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Preface

Environmental microbiology is a fascinating field of science which captured my imagination when I first got out my trowel 32 years ago to collect soil from beneath a suburban tree. While following the trail which ensued, I have sampled the drainage from coal mines, dug infiltration basins in the desert, dredged sediments from bays and rivers, and chopped holes through surface ice. I even have learned that sometimes the sample bottle gets filled in unusual ways, such as when the surf washed over my head or when I slipped sideways into a river. The latter event was observed by three of my students, who were courteous enough not to laugh too loudly. The *Manual of Environmental Microbiology* is an effort to combine the results and my excitement from my own discoveries with those of many colleagues. I feel honored to be a part of this collaborative effort. It is an idea which began handwritten on the back of an envelope in January 1991, and I still cherish that envelope.

The process of organizing, writing, and finally publishing this manual has been a joint volunteer effort by many hundreds of people, each of whom has contributed generously of their own experiences and expertise. Together, as a group of 136 scientists, we saw our efforts come to initial fruition with publication of the first edition of the *Manual* in the fall of 1996. By bringing to you this third edition, we now present a complete update of our field. Jay Garland, David Lipson, and Aaron Mills have been added as new volume editors for this edition, replacing Michael McInerney and Guy Knudsen, who now have become emeritus editors. Four of the section editors from the second edition, Robert Christian, Steven Newell, David Stahl, and Linda Thomashow, likewise have become emeritus editors. It is with pride that I welcome in their stead Meredith Hullar, Jonathan Lloyd, Seán O'Connell, and Ming Tien, who have joined the editorial board as new section editors for this edition. Many of the contributing authors from the first two editions have been called in other directions, and those departing contributors now are our alumni, each of whom knows that his or her efforts were appreciated by our readers. Two hundred seven scientists have contributed to this third edition of the *Manual*, including more than 100 new authors, and I welcome each of them. I offer special appreciation for the fact that nearly all of the former editors have remained among our group as authors.

The most difficult part of compiling such a volume is deciding which topics will receive center stage and which must be given a reduced representation. It also is important to recognize that the research of any given field flows and sometimes changes its path over time. The authors and editors of this manual are dedicated to helping you, our readers, keep a balanced perspective. We always will be certain that the basic foundations of environmental microbial methodology are thoroughly covered and also try to keep you up to date with the front of that flow. To keep that objective and meet our responsibility to our readers, we have updated all of those chapters that were carried over from the second edition, and many of them have been completely rewritten. You also will find that this edition contains numerous chapters on new topics.

In addition to thanking the authors and editors, without whose selfless volunteer efforts the American Society for Microbiology could not bring forth this manual, I offer particular thanks to several specific individuals. Jeff Holtmeier, the director of ASM Press, has lent us his utmost support. Ken April of ASM Press served as our production editor. Ken, most of all, has coordinated the entire process and kept us on course. Although his job is not easy, Ken somehow manages to seem calm and cheerful while surviving it all. The editors also thank the following individuals who assisted us by serving as ad hoc reviewers for this edition of the *Manual:* Anne Bernhard, Haluk Beyenal, Rima Franklin, Jim Fredrickson, Robert Genter, Tim Griffin, Jo Handelsman, Heribert Insam, Scott Kelley, George Kling, Mike Lehman, John Lindquist, Henry Mainwaring, Andrew Martin, Andrzej Paszczynski, Gary Sayler, Kathleen T. Scott, Claus Sternberg, Ben Van Mooy, and Darla Wise.

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INTRODUCTION TO ENVIRONMENTAL MICROBIOLOGY

I

VOLUME EDITOR
JAY L. GARLAND

Introduction to Environmental Microbiology

CHRISTON J. HURST

1

Environmental microbiology is the study of microorganisms which exist in natural or artificial environments. The origin of scientific research in this field rests in the observations of Antony van Leeuwenhoek that were published in 1677 (6). van Leeuwenhoek used a microscope of his own creation to discover what he termed "animalcula," or the "little animals" which lived and replicated in rainwater, well water, seawater, and water from snow melt. During the intervening centuries, the expansion of our knowledge regarding environmental microorganisms has been based on increasingly detailed observations and experimentation, in which we have been aided by advancements in microscopy and the development of biochemical and mathematical tools. However, many of the individual topics which we have since come to better recognize and characterize in environmental microbiology were initially described in van Leeuwenhoek's publication.

Many of van Leeuwenhoek's observations were based on the examination of environmental samples which he variously maintained in a wine glass, porcelain dish, and glass bottle in his house. In current terminology, we would say that he had created small artificial environments, or microcosms. While we often tend to think of artificial environments as taking the form of small laboratory systems, the term also encompasses both production-scale fermentations and anthropogenic structures emplaced in the natural world. Field-emplaced anthropogenic structures are often open to the environment and thus conceptually merge with the spectrum which represents natural environments.

It is an interesting coincidence that van Leeuwenhoek's first discovery of life was in water because that is almost certainly where the earth's life began. We have come to understand that the evolution of life on this planet would have depended on water because the bipolar nature of that molecule induces the three-dimensional configuration of our biomolecules. Also, we have continued to examine both the presence and metabolic activities of microorganisms found in surface waters and in the hyporheic world which underlies it. Since the studies of van Leeuwenhoek, we have discovered that microbial life is amazingly diverse (chapter 3). Microorganisms literally cover our planet. They live in surface environments even as extreme as the fumaroles of volcanoes and in sedimentary rocks within the dry valleys of Antarctica. Microbes can be found as deep as several kilometers both in glacial ice sheets and in bedrock. In fact, it seems that microorganisms can be found living in any site where the combination of physical conditions is such that water could occur in its liquid state. At deep-ocean thermal vents, where the temperature of the water can reach far above its normal boiling point, the high barometric pressure keeps water in its liquid state and microbial life abounds. The microbes living there provide the biological basis for the surrounding ocean floor life. When the ocean's surface layer freezes during polar winters, the dissolved salts become excluded in minute vacuoles and channels within the forming ice. The water contained within these minute spaces remains in a liquid state because of the high salt concentration. There, within these "brine channel" spaces, microbial life likewise abounds and provides the basis for some elaborate sea ice endofaunal food chains. Since many types of microorganisms have developed resistance to prolonged freezing and even to desiccation, the microbes seem to have much of our planet available to them. Amazingly, albeit perhaps not surprisingly, microbes even live on and within paintings and other artworks (1), where their digestive processes show that the microbes clearly have a taste for art, although they may not appreciate these objects in the same sense as we do. While we study these many wonders, we also further our recognition that microbes comprise the basis of our biological legacy. They represent the very origin of the biofilm on Earth, and we are but a part of that biofilm. Just as the microbes are the critics that seem to have the final say about our works of art, in the end they undoubtedly will have the final word regarding life on this planet.

In his paper, van Leeuwenhoek mentioned an observation that the types and relative concentrations of organisms present in his environmental samples changed and fluctuated with respect to time. We have continued his observation that microbial colonization of environments is a process which involves a succession of organisms, and we now know that this type of succession is brought about by waves of organisms which differ in their ecological requirements. The microorganisms chemically interact with their physical environment, and their most notable effect has been the creation of an oxidizing atmosphere on this planet. By way of these chemical interactions, microbes remain crucial to the biogeochemical cycling which supports the continuation of life on our planet, turning over the elements that represent the basic ingredients of life such as carbon, hydrogen, nitrogen, oxygen, phosphorus, and sulfur.

Over the course of millennia, we have learned to use naturally occurring microbial products for our benefit, including petroleum and some of the fungal metabolites which have antimicrobial properties. While expanding the extent to which we understand microbial processes, during the last few decades we have begun learning how to harness microbial biosynthetic and degradative activities. This harnessing, including the intentional manipulation of microbial activities, constitutes the basis of microbial biotechnology, whereby we direct the activity of microorganisms within both natural and artificial environments for a variety of purposes. As one example, we utilize microorganisms to help us achieve goals such as the production of materials which are beneficial to our existence, including numerous antibiotics, vitamins, fuels such as biogas and ethanol, and perhaps eventually usable electricity (4). Microorganisms also are used as tools to help us intentionally degrade both natural and anthropogenic materials in wastewater digestors, composters, landfills, natural terrestrial environments, and natural or artificial aquatic environments. Sometimes we use microorganisms as tools to achieve agricultural goals such as protecting plants from insect damage. Furthermore, microbial processes such as using microorganisms to leach metals from ores and to enhance the recovery of petroleum from wells have been used as a means of minimizing the application of hazardous chemicals in geochemical recovery operations. Just as we sometimes use our knowledge of beneficial microbial processes to optimize their usefulness, at other times we try to prevent natural microbial activities such as those which contribute to the biofouling, corrosion, and decay of objects exposed to the environment.

As scientists, we show a typical human penchant for trying to organize and categorize things. We extend this habit even to the microbes which surround us. Presently, we use a microbial classification scheme which divides the microorganisms into three overarching taxonomic groups. Two of these are considered to be cellular, meaning that they possess cell membranes. These are the Eukaryota, or Eucarya, and the Prokaryota, or Procarya (5). The Eukaryota contains those cellular organisms with membrane-bounded intracellural structures, and its members more traditionally are known as the algae, fungi, and protozoa. The Prokaryota contains those cellular organisms which lack membrane-bounded internal structures, and its members are more traditionally known as the bacteria plus those subdivided out into the archaea. The third overarching group consists of acellular microbes, the viruses and some of their biological relatives, which previously never were allowed shelter within any biological kingdom but could fit into the proposed domain *Akamara* (3). However, from the viewpoint of environmental microbiology, the most important aspect is our increasing understanding that, within ecosystems, these groups of microorganisms naturally organize among themselves as they go about their interactions both with one another and with the macroorganisms on this planet. Perhaps the greatest question remains whether the minimal functional unit of life truly is an individual member of a species or the entire ecosystem within which that individual exists. The interactions between the members of an ecosystem (chapter 2) occur and can be studied on many levels: spatially, biochemically, and even genetically (2). We have tended to divide our descriptions of these interactions into categories based on whether the relationships are neutral, positive, or negative with regard to the organisms involved. Neutral relationships are ones in which there is neither perceived harm nor advantage for the involved organisms, as when two coexisting populations ignore the presence of one another either because their numbers are sparse or because they occupy different ecological niches. Commensal relationships occur when one organism is perceived to benefit while the other is unaffected, as when microbes which colonize the external surfaces of animals or plants derive energy by metabolizing exudates produced by their hosts. There are also mutually advantageous relationships in which both populations benefit, examples of which are synergism and mutualism. Synergism is a voluntary interaction wherein both populations of organisms could exist on their own but do better when living together. Certain intestinal bacteria which are harbored within human hosts provide an example of synergism. In return for being sheltered, these bacteria produce vitamins that the host cannot manufacture on its own and would otherwise have to acquire from its diet. Mutualism, or symbiosis, occurs when neither organism seems capable of living on its own in that particular environment. An example of symbiosis is the interrelationship between certain chemoautotrophic bacteria and their hosts, the *Riftia* marine tube worms which inhabit ocean floor thermal vents. Through a highly coevolved relationship, those chemoautotrophic bacteria are housed within and provide nutrients for the tube worms. Lastly, there are relationships which are termed competitions, and these are harmful to at least one of the involved species. In the vast majority of instances, these interactions involve independently living organisms which suffer by competing in the open for scarce resources and arise because those organisms attempt to coexist within a common ecological niche when their combined numbers are too great to be supported by that niche. The parasitism represented by infectious diseases is a specialized form of competition, in which the scarce resource is produced within the body of the victim. The interactions between organisms are not fixed in nature, and a relationship which normally is benign may become detrimental, as is the case with some opportunistic pathogens which normally exist as benign members of the skin surface microflora but can cause disease if they gain access to the interior of an immunologically weakened host. This list of interactions ends with predation, in which one organism devours and digests another as food.

Not only have we tried to elucidate the natural fate of microorganisms in the environment, but also we have often attempted to eliminate from the environment some microorganisms which are pathogenic to either humans or the plants and animals on which we depend for our sustenance. Some of these pathogens are indigenous environmental organisms, while various others are of human origin or from animals and plants and are released into the environment through natural processes. The study of their persistence in environmental media, including survival and transport in soil, water, and air, provides clues that help us anticipate and control their populations.

Readers of this manual will find that the core sections are structured with regard to the type of environmental medium being discussed. The subject of water, the hydrosphere, has been divided into two sections, one containing chapters which address the fact that water often serves as a vehicle in the transmission of pathogenic microbes (section III) and the other containing chapters on general aquatic ecology (section IV). The terrestrial environments of the lithosphere have been divided into soil and plant zone interactions (section V) and the microbiology of deeper

subsurface environments and landfills (section VI). While microbes are not known to colonize the atmosphere, air serves as one of the vehicles by which both they and their often toxic by-products are transported (section VII). The subject area of microbially mediated chemical transformations bridges the hydrosphere and lithosphere due to the relatedness of the involved microbial metabolic processes, which often are performed by either the same or related genera of organisms. For this reason, the topics addressing biotransformation and biodegradation have largely been grouped into a common section (section VIII). Likewise, the basic principles of environmental microbiology (section I) and general analytical methodologies (section II) tend to be common across the range of environments that we study, and the last two subject areas have been placed in the front of the manual because of their primary importance.

I am not sure whether Antony van Leeuwenhoek could have foreseen where his discoveries have led: to the diversity of environmental microbiology subjects that we now study and the wealth of knowledge that we have accumulated. However, just as I have always enjoyed reading his account of environmental microorganisms, I feel that he would enjoy our efforts at once again summarizing all of environmental microbiology in a single document as represented by this manual. I thank the numerous microbiologists who have collaborated in creating this manual, many as contributors and some as editors. I also thank those giants in microbiology upon whose shoulders we have stood, for we could not have accomplished the creation of this manual without the advantage that those giants have afforded us.

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Neighborhoods and Community Involvement: No Microbe Is an Island

CHRISTON J. HURST

2

> No man is an *Iland*, intire of it selfe; every man is a peece of the *Continent*, a part of the *maine*; if a *Clod* bee washed away by the *Sea*, *Europe* is the lesse, as well as if a *Promontorie* were, as well as if a *Mannor* of thy *friends* or of *thine owne* were; any mans *death* diminishes *me*, because I am involved in *Mankinde*; And therefore never send to know for whom the *bell* tolls; It tolls for *thee*.
>
> John Donne, Meditation XVII, original publication in *Devotions upon Emergent Occasions*, 1624; quoted from reference 15

The most important thing which must be understood about microorganisms in their environments is that no microbe exists by itself. Extracting a parallel out of the above quotation from John Donne, we should consider that in the environment no microbe is an island existing independently. There are only a few, extremely rare instances when a single species exists naturally in pure culture. The interactions and interdependence between members of different species are a consequence of the evolution of those species into their occupation of specific niches. The second most important thing to recognize is that, as we look around nature's neighborhoods and observe the types of interactions that exist within the communities of macrobial organisms which live there, all of those types of interactions likewise exist in the microbial world. These interactions include such things as peaceful coexistences, competitions, hunting and hiding, and the use of chemical attractants, repellents, and toxicants. In fact, these approaches to surviving in community life would have existed within the microbial realm long before the first macroorganism evolved.

Microbes form the basis of our biological heritage as humans and indeed are the basis upon which all macroorganisms, including vascular plants and animals, evolved. Microbes form the understructure which supports what we perceive as being the macrobial realm. The fact that this assemblage of microbes and macrobes has evolved together binds it together. No single species selected out of this assemblage could survive for very long on its own in the wild without the biological activity provided by those species which occupy the connecting niches. This is a guiding concept in species ecology, and occasionally we will refer back to this concept as we progress through this chapter.

We also know that the life which exists on this planet evolved by using the hydrogen bonding bipolar nature of water molecules in their liquid state to serve as an organizing force. Through this force, biomolecules are arranged into their functional configurations by virtue of their having evolved hydrophilic and hydrophobic zones. Thus, the life on this planet requires water. The pervasiveness of this biology is such that it seems that on this planet active indigenous microbial life can be found in any environmental site where the laws of physics can allow water to exist in its liquid state (see chapter 46). The additional fact that many types of organisms have evolved mechanisms which allow them to survive periods of freezing or even desiccation means that much of this planet is available for supporting life. Microbes would have formed the initial basis of the food chains for all of this planet, serving as its primary producers. Vascular plants have taken over the role of primary production in those land surface areas of the planet where the air temperature is above the freezing point of water for at least part of the year. Vascular plants also have taken over the primary production role in many of the shallow aquatic areas where sunlight reaches in abundance. However, deep inside the earth the microbes still hold sway (19), and phytoplankton in the oceans is assumed to serve as the earth's primary source of fixed carbon. Microbes also perform the primary production role in such intriguing places as the communities of sea ice endofaunal organisms, where microfauna and meiofauna (small size category of macroorganisms) live in the brine channels which exist within the Arctic and Antarctic pack ice (23, 24) and in the unconsolidated layers of platelet ice which underlie the pack ice (26). Microbes apparently live within the water of Lake Vostok which exists in Antarctica beneath about 4 km of glacial ice and due to the absence of sunlight must serve as the primary producers for that lake (30). At the other end of the production line, microbes are the key to the recycling of available nutrients, including the important aspect of cellulose recycling within the gut of termites (41) and the recycling which occurs in deep-sea sediments (52).

Microbial communities function as interacting, coevolved assemblages (11, 17, 22, 25, 53). Of course, as we first begin to look at microbial communities and the interactions among microbial populations, we immediately are faced with three significant problems. First, an enormous number of different microorganisms exist in a wide array of highly dissimilar environments (48) and communities (20). These microorganisms include archaea and bacteria (see

chapter 35), fungi (see chapters 38, 51, and 52), algae and cyanobacteria (see chapters 30 and 37), protozoa (chapter 37), and viruses (chapter 36). Second is the enormously complex task of understanding and profiling the interactions among microbial populations. Such interactions not only are numerous and dissimilar but often are poorly characterized or totally undefined. Furthermore, there is difficulty in understanding the interactive role and major impact of invertebrate and vertebrate animals and vascular plant populations on microorganisms, a subject which is not within the usual scope of interest of microbiologists. For the rest of this chapter, I will try to limit our focus to just the microbes, although the more complicated reality is that microbial populations often are intimately linked with coevolved invertebrates (21, 27, 33, 54), some of which in turn consume microorganisms; vascular plants (35, 46) that in turn provide readily available organic nutrients; and vertebrate animals (3, 36) that may consume all of the above. At times it does seem that evolution works its way down strange pathways. Third, there exists a woeful lack of agreement about the various definitions that are appropriate for characterizing microbial populations, communities, environments, and interactions.

This chapter is not designed to be encompassing. The topic of microbial communities and interactions would easily fill an entire monograph. Rather, the text will serve as a prelude, presenting an introduction to some of the principal issues of environmental microbiology.

TERMINOLOGY: AS IF MERE WORDS COULD DEFINE HOME

> "When I use a word," Humpty Dumpty said, . . . "it means just what I choose it to mean—neither more nor less." "The question is," said Alice, "whether you *can* make words mean so many different things." "The question is," said Humpty Dumpty, "which is to be master—that's all."
>
> Lewis Carroll, original publication in *Through the Looking-Glass, and What Alice Found There*, 1872; quoted from reference 9

Environmental microbiology is blessed, or possibly cursed, with a multitude of terms that often are contradictory, sometimes misused, and frequently misunderstood even by the specialists. The ability to use language properly is an art and helps us to understand one another. But semantic sensitivity is frequently not the hallmark of the experimentalist, and this lack of sensitivity often is the basis for confusion and disagreement. The purpose of this section is to briefly define some of the more critical terms used in this chapter. As Lewis Carroll might like it stated, the words are defined even if the reader does not agree totally or even partially with the definitions used. Whether it is the word, the author, or the reader which is master will remain an open question (as Humpty Dumpty may have wished it to be).

Defining the ecology of a species is a complex issue, but the issue can be divided into different ecological aspects. The first aspect can be consideration of the terms "boundary" and "barrier." The descriptions provided here are brief, but the concepts of boundaries and barriers will be taken up a bit more extensively later in the chapter. The word "boundary," as used here, represents an abstract concept which describes the environmental limits beyond which the physiological requirements and capabilities of a given species do not allow its members to function (C. J. Hurst, "The Consequences of Crossing Barriers and Boundaries," presented at the 100th General Meeting of the American Society for Microbiology, 21 to 25 May 2000, Los Angeles, Calif.). This boundary encompasses the overlap of a combination of physically and chemically defined environmental characteristics and is absolute. The members of a species are obliged to live within their boundary because of the fact that their metabolism ceases to actively function beyond that boundary. While they may venture outside, any individual's existence in a live state for very long outside the boundary would require either artificial environmental support or the ability of that individual to enter temporary metabolic stasis. Often, the boundaries of different species overlap and this allows for biological interactions between the members of those species. If the boundaries of two species do not overlap, then it is unlikely that the members of those two species will biologically interact with one another. This concept of a boundary is not something marked out as if it were a physically defined territory on a map. However, we may be able to mark on a map spatial zones, territories, or physical locations which meet the requirements for being encompassed by the boundary of a species. Those spatial zones would represent potentially habitable sites for the members of that species. Most physical interaction between the members of species would occur within areas of spatial overlap. These potential habitats may be either contiguous or separated by barriers. "Barriers" are obstacles, and they can be classified by their nature as either physical, chemical, or biological (Table 1). Their nature as obstacles relates to the boundary of some particular species which is being considered. What constitutes a barrier to one species may not be a barrier to some other species. Barriers can restrict or prevent the interaction of two species even if those species have identical boundaries. This contributes to the reason why parallel evolution can produce two similar species which may occupy nearly identical niches but never encounter one another.

We now can move forward to the next ecological aspect, which is to consider that the ecology of a species has two traditionally defined components: its "niche" and its "habitat." Each of these components can be defined both in a potential perspective and in a more limited operational perspective. The major difference between these components is that, whereas a niche is biologically defined, a habitat is physically defined. The word "habitat" describes only the place where a species lives. The word "niche" describes how this species fits into that place, representing its interactions and accommodations with respect to the abiotic and biotic conditions found there. Thus, for any particular species, the description of its niche consists largely of the interactions between the members of that particular species and other species which occupy connecting niches. However, the definition of niche also includes changes in the environment effected by the species' biological activity. Among ecologists, the niche of a species represents what that species does; that is, its function in its natural habitat. The potential niche of an organism, expressed in terms of its total, genetically provided capacity for biological interactions, may be a marvelously broad thing and enables potential evolution of that species. In nature, however, competition and other interactions with and against different species narrow this potential niche to a smaller, more functionally restricted operational niche. The physically defined term "habitat" brings us to the point of identifying territories, places to which we could point and sometimes even identify

TABLE 1 Examples of barriers applying to different species[a]

- Physical
  - Thermal
  - Acoustic (usually ultrasonic)
  - Pressure (barometric, hydrostatic, osmotic)
  - Radiation (electronic, neutronic, photonic, protonic)
  - Impaction (includes gravitational settling)
  - Adhesion (adsorption, electrostatic, van der Waals)
  - Filtration (size exclusion)
  - Geographic features
  - Atmospheric factors (include meteorological aspects such as humidity, precipitation, and prevailing winds)
- Chemical
  - Ionic (includes pH and salinity)
  - Surfactant
  - Oxidant
  - Alkylant
  - Desiccant
  - Denaturant
- Biological
  - Immunological (includes specific as well as nonspecific)
  - Naturally induced (intrinsic response)
  - Naturally transferred (lacteal, transovarian, transplacental, etc.)
  - Artificially induced (includes cytokine injection and vaccination)
  - Artificially transferred (includes injection with antiserum and tissue transfers such as transfusion and grafting)
  - Biomolecular resistance (not immune related)
  - Lack of receptor molecules
  - Molecular attack mechanisms (includes nucleotide-based restrictions)
  - Antibiotic compounds (metabolic inhibitors, either intrinsic or artificially supplied)
  - Competitive (other species in ecological competition), including predation or parasitism against the subject species by members of some other species or, if the subject species is an infectious agent, competition against a vector which the subject species might need in order to achieve transmission between host organisms

[a]Information adapted from reference 29.

on a map. A habitat is a site, generally having some physical uniformity, which possesses those characteristics that appear to be of ecological importance for supporting residence by the members of a species. The potential habitat of a species can be very large but operationally may be more restricted due to deterring interactions such as competition against or predation by the members of other species. Some species end up being restricted to perhaps only a single and frequently unique habitat, whereas other species are cosmopolitan and successfully reside in a wide variety of seemingly dissimilar habitats. An individual habitat may be a specific layer in the depth profile of a characterizable soil type, the intestinal tract of members of a particular animal species, the outer surfaces of a plant root, or some zone within lake bottom sediments. Frequently, however, owing to the small size of microorganisms, the concern of the microbial ecologist is the microhabitat (49), inasmuch as individual propagules, filaments, or cells often are restricted to a site that is no larger than several millimeters or sometimes even micrometers. Thus, an organism may be found to exist only within a specific depth range in the water column of a particular lake and interact only with the other species found in that vicinity. This may be the case even though the boundary of that species might be broad enough that the organism could exist at other depths and in different bodies of water. Yet that one place then represents the operational habitat of that species, even if its potential habitat could be much larger. The interactions of this species with other organisms existing within that small vicinity are a defining part of this species' operational niche, even if its potential niche could be far larger. The reasons for restriction to that operational habitat and operational niche may be the existence of barriers, which may include a biological inability to successfully compete against, or may result from predation by, the members of other species found at other depths within that water column or found within other bodies of water.

Within a particular site, we find what we call a community. Perhaps we could consider the term "neighborhood" to define the site itself, and "community" then could define those which live therein. The community comprises all members of all species that occupy a particular site. These members of the community generally represent different populations. A "population" of a particular organism is an assemblage of individual organisms having common characteristics. In microbiology, the term "population" variously may be used to represent all individuals of a single species, subspecies, variety, race, or other subspecies designation. Within a population, there may be a large number of individual cells, or a population may be composed of a multicellular filament, such as is found among the fungi and algae. An appropriate term for an individual microbe belonging to a population is "propagule." However, the reader should be aware that many specialists use the term "community" to encompass only a particular category of organisms. For this reason, some would describe an environment as having separate communities of bacteria, fungi, algae, plants, or animals. The community of a soil in general is somewhat different from that of the subsoil or underlying aquifer, and the community in the water column is not the same as that inhabiting the underlying sediment. A term synonymous with "community" but not widely used in English is "biocoenosis." Although clear distinctions between communities and populations thus exist, the words are frequently and mistakenly used as synonyms.

"Consortium" is a term used more commonly for bacteria in associations than for assemblages of other organisms (18), but this concept does not have to be limited to just bacteria. It is a useful word when it refers to a collection of organisms that have some functional association with one another (see chapter 9) and when we talk about the properties which describe particular communities (see chapter 11). For example, one species may provide growth factors for a second species or serve to eliminate inhibitory compounds that affect a second species. To some degree, a consortium may represent a commensal or protocooperative interaction whose basis has not yet been well established. Unfortunately, however, mixtures of bacteria that have yet to be separated into pure cultures are frequently designated consortia; in these instances, the mixture may not represent a functional association but, rather, reflects the lack of isolation of individual components of the mixture. "Facilitation" is a term which describes situations when the activity of one species may facilitate a similar or different activity by another species.

UNDERSTANDING THE WAYS TO BEHAVE

The degree of species diversity within communities can vary enormously. Communities typically may have several, many, or innumerable species. Although microbiologists are prone to work with pure cultures, communities having only one species are rare in nature, except in those monospecific communities associated with disease processes or in environments so extreme that few species can survive or proliferate. There often is some benefit gained by one species from coexistence with other species. Although the benefits are unquestionable, the basis for those benefits and the mechanisms associated with the interactions in natural communities are rarely understood and rarely studied. The purpose of this section is to describe the kinds of interactions which have been observed between species.

We know that in order to become a member of a community, the propagules of a species not only must reach the environment where that community exists, but also must successfully cope with the detrimental interactions occurring there, including competition with the species which already exist in that site. Some sites seem not only readily accessible to many different types of microorganisms, but also supportive towards the survival and proliferation of those new arrivals. These receptive sites thus often end up with a high species diversity. Soils, surface waters, sediments, and other environments such as sites rich in decaying organic materials support a multitude of species of bacteria, fungi, protozoa, and sometimes algae. On the other hand, sites with high salinity, extreme pH, low nutrient levels, or high light intensity often are characterized by low species diversity, which may be reflective of a low receptivity.

An old truism states that microorganisms are potentially everywhere. That truism is little more than a platitude, and as a statement it takes on a degree of ecological inaccuracy when it is used as the basis for assuming that species or genera that are widely disseminated are also widely established. Potentially cosmopolitan species frequently are able to grow, in culture at least, very rapidly in the absence of other organisms so that a few propagules or a small biomass can multiply to reach a high cell density or large biomass in a matter of hours or days. Such largely unchecked proliferation rarely occurs in nature, however, and effective checks exist to prevent the unbridled multiplication of widely disseminated propagules. The operation of these various checks is the basis for the appropriate second half of the truism, namely, that the environment selects. Hence, the full and ecologically appropriate statement is that microorganisms are potentially everywhere, but the environment selects.

The impact of that selection is evidenced by the characteristic communities of individual habitats. Many of the bacteria, fungi, and protozoa present in surface waters are thus not characteristic of soils, and the types of heterotrophs (organisms which derive their operating energy from organic compounds found in their surrounding environment, as contrasted with autotrophic organisms, which derive their operating energy either from photosynthesis [photoautotrophic] or chemosynthesis [chemoautotrophic]) found in soils are often quite different from those that would be found on the surfaces of leaves. The forces of selection are often nonbiological, and an area that has a low pH, is exposed to high light intensity, has no available oxygen, or contains low concentrations of readily available carbon sources supports a community that is able to cope with these restraints or limitations. Such abiotic (not biological in origin) factors are often reasonably easy to demonstrate. However, more difficult to establish and perhaps more interesting from the biologist's perspective are the biotic (of biological origin) stresses that are operating in environments in which major abiotic stresses do not determine community composition.

Involvement and Homeostasis Are Evolutionary Consequences

The indigenous populations that make up the community are responsible for the biotic balance that is maintained. They regulate the population densities or biomass of the individual component species of the community, and they act to prevent the establishment of invading species. These various biotic interactions determine the stability of established microbial communities, and they reflect what is designated "homeostasis." From a biological perspective, homeostasis is the numerical and biochemical equilibrium resulting from the activities of the different species which comprise the community. From an ecological standpoint, homeostasis is the capacity of a community to maintain its stability and integrity in an environment subject to abiotic and biotic modifications. These homeostatic mechanisms are constantly operating, and although they are often modified somewhat, they generally are not altered appreciably unless an enormous perturbation occurs. But stable does not mean permanently unchanging. Both the levels of activity and the proportional numbers of the component species forming a community may fluctuate and even respond cyclically as a response to abiotic or biotic perturbations. That concept includes seasonally related changes. Characteristically, it is abiotic perturbations that may totally upset the community. Only in rare instances is a biotic influence, such as the mere introduction of propagules of a nonindigenous microorganism, sufficient to permanently alter the community to an appreciable extent. Indeed, in many instances, the new arrival is not even allowed to become established.

Ignoring the potency, as well as the limitations, of homeostatic mechanisms has led to a number of viewpoints that run counter to observations in nature. For example, individuals who want to introduce bacteria or fungi into soils, subsoils, or aquifers for bioremediation assume that the introduced species will become established and bring about the destruction of a contaminant of concern. In doing so, it is assumed that just because humans judge that the introduced microorganism has a beneficial trait (in this case, the capacity to grow on and thus destroy an organic pollutant), that organism indeed will become established and perform what is desired of it. While the humans performing the experiment may think that this important trait of the introduced microbe is desirable from their own perspective, the humans are not in charge of the situation. The introduced organism may not be accepted by the existing microbial community. Why might the microbe not be accepted? The capacity of the microbe to use a particular substrate for growth, although of paramount importance in culture, simply may not be sufficient for ecological success: the organism also must be able to cope with the various factors associated with homeostasis. An attribute that is a necessary requirement for growth is not a priori an attribute sufficient for establishment, and both necessary and sufficient traits must be present. Success requires that an introduced organism be able to compete effectively for limited resources other than the specific organic compound which laboratory studies

may have proven can serve as its carbon source. The introduced organism also must be able to cope with the stresses associated with predation and parasitism, which are of great importance in many environments. In contrast with the vendors of microorganisms, who always hope for success from their introductions, there are other individuals fearfully concerned that the environmental introduction of genetically engineered microorganisms could result in successful establishment. Many of the latter individuals must optimistically presume that all introductions will fail and that modest changes in the genotype of an existing organism will not result in its establishment. Again, it helps to emphasize that natural events often prove humans to have been wrong in their assumptions. It is necessary to consider not only the strengths but also the limitations of homeostatic mechanisms. While the operation of homeostatic mechanisms does eliminate most introductions, these mechanisms are not omnipotent, and some introduced organisms do succeed in becoming established. Borrowing an analogy from the world of macroorganisms, farmers know that sowing a new variety of plant, one that has major beneficial attributes, is not sufficient to obtain high yields of that variety. The introduced crop species frequently is unable to cope with competition by weeds, parasitism by insects and plant pathogens, poor soil structure, and other stresses. Few introduced species become established, regardless of whether the introduction has the capacity to do good or is potentially injurious. Yet in some instances, an alien species does become successfully established, as evidenced by the major upsets that have occurred because of invasion by plants and animals. Attempts have been made to predict the capacity of an introduced organism to become established (32, 47), and studies have been directed towards establishing the traits that might be used to predict the outcome of an introduction. Our inability to predict the success or failure of introductions is a reflection of incomplete knowledge of the various components of homeostasis.

Being Kind and Helpful

To the general public, microorganisms are frequently considered to be solely harmful. This is evident in the use of the word "germ" (commonly referring to a microorganism thought to cause disease but perhaps also definable as a microbe unwelcome in the place where it has been found). Admittedly, it is true that when we examine the world of microorganims we find competition, parasitism, and predation to be of great importance and that disease-producing microorganisms are widespread. And yet altruism also is a widespread attribute among all major categories of microorganisms. Despite the fact that detrimental interactions are inherent to biology at all levels, we should not overlook the good that many heterotrophic and autotrophic microbial populations do for their neighbors, including the natural and often protective microfauna associated with plants and animals. In truth, it is the neutral relationships which likely are the most common in biology. "Neutrality" describes those relationships in which there is neither perceived harm nor advantage for either of two species being considered relative to one another. Interestingly, though, there sometimes is a tenuous balance between neutrality and a harmful state. That balance may hinge even on something apparently so minor as ambient temperature (4).

Using and Abusing

Some apparent types of beneficence are commonly categorized, from the ecological viewpoint at least, by the terms "commensalism," "protocooperation," and "symbiosis." Although semantically sensitive readers might expect a clear distinction among those three terms, in fact a continuum exists so that the range of each of these types of beneficial interaction merges into the next. Indeed, it is likely that there is a continuous evolution so that organisms which at one time were commensals evolve to exist in protocooperative relationships and those that are involved in protocooperation will, with time, evolve into highly dependent and mutually beneficial symbioses.

"Commensalism" defines an association in which one species benefits a second but the first gets no apparent advantage in return. The second species which is provided with something essential for its replication is deemed the commensal. The mechanisms underlying commensal associations are numerous, but only a few have been characterized. Such interactions are evident between algae excreting photosynthetically fixed carbon and heterotrophic bacteria which as commensals consume that carbon (10). Commensal relationships apparently also exist between populations jointly contributing to many biochemical transformations and to degradative processes (see Section VIII). In these, the first population or species may convert a compound which otherwise metabolically is unavailable to a second population into some product that can be used as a nutrient source by the second species. Sometimes the second population is auxotrophic, meaning that its members cannot grow in the absence of the supplied compounds, and those required compounds are termed to be the second population's growth factors. An organism which excretes a growth factor may allow for the development of this auxotroph as a commensal. Studies of water and soils indicate that a large number of indigenous species are able to excrete one or more vitamins or amino acids which may be capable of serving as growth factors. Another type of commensalism is evident in environments containing organic or inorganic inhibitors, and a species that destroys an organic inhibitor or somehow detoxifies an inorganic ion serves an altruistic role for its sensitive neighbor.

The fact that terrestrial and aquatic communities contain a high percentage of microorganisms that are auxotrophic means that commensalism must be biologically favorable in those communities. An organism that relies on its neighbors for the synthesis of a carbon source, growth factors, or detoxifying enzymes does not need to expend energy of its own to either make or detoxify those substances. Thus, although the commensal is dependent upon the helpful neighbors found in that environment, given all other factors to be equal the commensal has a competitive energetic advantage over some other organism that might reject dependence by instead choosing to expend energy to make all of its own vital compounds or to detoxify by itself all harmful substances. In turn, a chain or network of commensal reliances can evolve. And if evolution thus energetically benefits a commensal, we can understand that it would also likely favor a development in which two species in contact with one another mutually develop a greater biochemical interreliance. This is a gradient along which facilitative relationships conceptually may lead into a kind of loose confederation called "mutualism" (3). Mutually, each of the two species provides some substance needed by the other, and thus by being helpful the two species cooperate and energetically as confederates they gain in competition against otherwise similar microbial populations whose individual member species instead develop their own full armament of enzymes and physiological mechanisms. In this

way, for as long as those two interreliant species decide to cooperatively coexist, they jointly would have an evolutionarily selective advantage over other partially or fully independent populations. This facilitative interaction conceptually can become the basis for a stronger interaction called "protocooperation," an association in which each of the two interactants needs and benefits the other. When functioning as a unit, a two-member protocooperative association has greater fitness than would the two species functioning independently. Still, the associate species involved in a protocooperation are not fixed in that relationship, in the sense that neither is it obligatory nor is it specific that only these two species can fulfill those roles for one another. Well-studied protocooperative relationships include those in which there is interspecies electron transfer (14), degradation of aromatic compounds (34), and the fermentation of polysaccharides (40). In a simple protocooperation, only a single benefit needs to be conferred from one population upon the second. However, if a simple protocooperation gives the interactants additional fitness, so too might greater degrees of integration. Each population may thus evolve to contribute more than one requisite to the second population. "Synergism" refers to a more closely interactive process in which two species clearly do better and appear to actively cooperate for the purpose of surviving within a particular environment. This interaction may involve jointly causing physical or biochemical changes within that environment which otherwise neither species could perform alone, or else it could be that these changes would occur much more slowly when the two species are not together. The synergants could, however, live in that environment independently of one another. The basis for the benefit-promoting synergism may be that the two interactants function better together because one provides something to the other, such as a carbon or energy source, a growth factor, O_2, or detoxifying enzymes. It often seems that synergism results from protocooperation. But, when the change to synergism occurs, the association becomes somewhat less flexible, and the identities of the two interactants become somewhat more restricted because each must provide the full complement of benefits required by the second. A further progression of synergism would seem to be the basis for the tight interactions that characterize symbiotic associations.

"Symbiosis" represents those instances when it seems essentially obligatory for different species, termed symbionts, to live together as one in a particular environment. Symbioses may involve two or more species of microorganisms, or else microbial species interacting with either plant or animal species. The most famous symbioses in microbiology are the lichens (42), each representing the association of a cyanobacterium species with a fungus species, and whose stability as symbionts has risen to the point that in the past some lichens were considered to be species. Endosymbiotic associations represent an even greater commitment to symbiosis in which one of the symbionts, termed an endosymbiont, lives within the body of the other, which is termed a host. Several endosymbioses involving plants (see chapter 52) have been intensively investigated. In some plant-microbe relationships the endosymbionts are segregated to nodule-like compartments, as is the case for the associations between legumes and members of the genera *Rhizobium* and *Bradyrhizobium* which generate fixed nitrogen, essentially creating a self-fertilizing plant. Agricultural studies of these, and the fungus-root endosymbioses termed mycorrhiza (46), have provided huge benefits to human society. Luminescent organs in deep ocean fish are another notable example of microbial endosymbionts housed within specialized tissue compartments created by the host. There also are endosymbiotic relationships wherein bacteria reside within protozoans, and it has been speculated that this type of endosymbiosis led to the evolution of intracellular organelles.

The Hunters and the Hunted

Many types of microorganisms feed upon other microorganisms. Often, the same microbes which did the feeding then serve as prey and hosts for other microorganisms and macroorganisms in elaborate food chains. There is a vast body of literature on laboratory studies of some of these general kinds of relationships, but far less is known about the ecological role played in nature by those species that parasitize or prey upon microorganisms. This shortfall in knowledge surely affects us, because it is almost certain that ultimately some of the predator-prey relationships must affect either human health or farm production. At least in part, this shortfall in terms of understanding how microbial predator-prey relationships affect natural systems probably exists because it is easier to carry out laboratory studies with only two organisms living in culture media than to establish the very significant but far more complex interactions which naturally occur in highly heterogeneous microbial communities. Sadly, however, our understanding of the importance of specific predator-prey systems also may be biased because all too often it depends on the interest of the researcher and the ease of working with some organisms as compared to others. Thus, the more visible and stepwise simpler topics and trophic chains tend to be better examined. The complex and more difficult but potentially far more important topics then get ignored. In turn, the relative abundance of literature may lead the nonecologist to conclude that a particular group of parasites or predators is indeed very important in nature and that all other groups are unimportant. The truth, then, as to which players and roles are the most significant is not yet revealed.

The viruses are subcellular microbial predators and they are obligate intracellular parasites (29). Among the cellular microbial predators are protozoa, myxobacteria, acrasiomycetes, and a number of chlorophyll-containing flagellates that are often but not always classified as algae. Of these various groups of cellular organisms, there is incontrovertible evidence for the importance of at least the protozoa as contributing factors in determining the composition, structure, and perhaps even the the function of microbial communities (5, 6, 7, 31, 44). Wastewaters, many surface waters, and the rhizosphere of actively growing plants often contain large numbers of protozoa, and a high percentage of the individual protozoa are present in a trophic stage that is associated with active feeding on bacteria. A single protozoan cell, upon division, consumes 10^3 to 10^4 bacteria. Moreover, by use of eukaryotic inhibitors, it has been shown that inhibition of protozoan growth is associated with the maintenance of large numbers of potential prey individuals and often the existence within a community of populations that otherwise would have been suppressed. Thus, eliminating protozoa can alter homeostasis.

Nevertheless, despite their abundance and activity, protozoa generally do not eliminate their prey. It may appear that a predator which is able to markedly reduce the size of a large population is somehow unable to effectively destroy a small population. Instead, however, it seems to be the case that the protozoa and most other predators are prudent. The elimination of prey by an obligate predator would result in

the subsequent elimination of the predator species itself. This prudence in feeding appears to be density dependent rather than due to altruism and often has been interpreted in terms of the balance between energy needed for hunting and energy gained by the hunt. Thus, when a predator population is getting more energy by feeding on the prey, as would occur at high prey densities, than it needs to hunt for those prey, it will continue to graze. On the other hand, at low prey densities, the predator must use considerable energy to move to the few surviving prey cells or to bring those survivors to itself for consumption, and it thus will not continue to feed on that species (1). The options then available to the predatory protozoan would be to begin feeding upon another species, die of starvation, or encyst and hope for a happier future when its prey species would again be more abundant. This is not to say that the predator cannot eliminate a single prey species, which it does if the density of alternative bacterial prey is sufficient to sustain feeding (38). Thus, the effects of protozoa may be viewed in terms of reducing the size of the bacterial community and also, under circumstances when the bacterial community is large, possibly eliminating individual species. Still, soils contain higher bacterial densities, at least by total counts, than are predicted on the basis of the capacity of the indigenous protozoa to reduce the bacterial densities. For example, the total count of bacteria in soil may be in the vicinity of $10^9/cm^3$, but in solution the protozoa may reduce the bacterial density to approximately $10^6/cm^3$. Therefore, it is likely that there is a refuge in soil that possibly results from the inability of protozoa to penetrate pores large enough for bacteria but too small for the predator or from the inability of protozoa to feed on bacteria that are adsorbed to particulate surfaces. Adsorption onto particulates would effectively convert the bacteria into objects too large to be engulfed by the protozoa. It also is possible that susceptibility to grazing by protozoa is checked by other factors, perhaps including evolutionary pressure favoring potential prey organisms which can effectively conceal themselves by changing their "signature" of secreted compounds.

Less defined and still subject to extensive discussion are the roles of virus and *Bdellovibrio* species in nature. The viruses are acellular; bdellovibrios are cellular and in appearance are much like small bacteria. The bacteria, algae, fungi, protozoa, and archaea, plus all species of plants and animals, are affected by viruses, which are obligate intracellular parasites. Bdellovibrios can replicate in very complex defined media, but presumably in nature they exist as obligate intracellular parasites of bacteria. The ecology of their host, or prey, is integral in the ecology of both virus (2, 8) and *Bdellovibrio* species. Indeed, as intracellular parasites, the viruses and bdellovibrios rely on a host organism for their development and even their very existence in nature. The interactions which comprise the ecology of viruses have been examined and described by Hurst and Lindquist (29). Much less is understood about the ecology of *Bdellovibrio* (37).

WHY THEY ARE WHERE THEY ARE WHEN THEY ARE THERE

We know that in fact all of the life on earth represents a single interconnected biofilm. But the participant species involved in even similar events and interactions are not static with respect to space or time. The purpose of this section is to help the reader understand why the observed things may be happening in any given place and time.

Boundaries and Barriers

One of the concepts which is important in consideration of the ecology of the members of a species is that in nature each species has a boundary. As indicated earlier in this chapter, boundaries are operationally defined, and the physiological limitations of a species do not allow its members to actively function in the conditions found beyond that species' boundary. For example, we know that almost no species of fish can survive for very long when out of water. Thus, the interface between the atmosphere and the surface of the water represents a boundary for those species of fish. Equally, most terrestrial species cannot survive unaided for very long when submerged in water, so the water's surface likewise serves as a boundary aspect for those terrestrial species. The ecological boundaries of many amphibians and reptiles cross the water's surface, attested to by the fact that those organisms have both aquatic and terrestrial capabilities. The concept of a boundary includes a complicated mixture of factors, sometimes very highly species specific. However, we often can identify both biotic and abiotic components for some of those factors; i.e., oxygen requirements (biotic) may restrict the altitude or depth (abiotic) at which the members of a species can function. A species cannot move its boundary except through evolution. For most species, this evolution must be biological in nature. Humans, however, can move their boundary by cultural evolution. For example, although we lack effective body insulation by fur, the development of clothing and weather-resistant shelters allows us to live even in polar environments, places whose physical conditions normally would make them beyond humans' boundary. The development of the aqualung, an early portable self-contained underwater breathing apparatus (scuba), and modern rebreathing technology represents a cultural evolution which allows humans to exist underwater at least temporarily. Within the confines of its boundary, a species will find at least one habitat in which its members successfully can reside (otherwise, it would become extinct). While a particular species cannot cross and survive beyond its own boundary, its habitat may be sufficiently diverse that, while moving within that habitat, the members of that species may cross the boundaries of many other species. This overlap of boundaries allows biological interactions between the members of different species.

The parts of a species' habitat may be contiguous or may be separated by barriers (Table 1). These barriers can be physically defined and are determinable by physical measurements. They also may be tangible. For example, a species' oxygen requirements may turn geographical features into barriers. Barriers are not fixed with regard to time and space. Rather, they can appear, disappear, and move with time (e.g., as mountain ranges rise and fall, glaciers advance and retreat, continents shift toward either more polar or more equatorial zones, and competing species evolve or become extinct). Species tend to evolve survival mechanisms which allow their members to successfully move as barriers move and even evolve mechanisms which allow them to cross those barriers. Indeed, some species have evolved to a situation in which cyclical migrations between parts of their habitat are necessary for continuance of the species. For example, seasonal migrations occur among many species of bats, birds, butterflies, caribou, salmon, and whales. Even maple trees migrate within the northern hemisphere, albeit on a longer time scale, advancing and retreating with the glacial cycle (43). In the cases of monarch butterflies and maple trees, no individual member

of the species completes the full migration. The capability of a species' members to migrate also facilitates colonization of new areas. Cyclical migrations could be viewed as highly evolved forms of dispersal. And, as the macroorganisms migrate, so too must there be a corresponding migration of those microbe species whose lives are either dependent upon or interdependent with the migrating macrobes.

Staying at Home versus Venturing to New Places

What does the importance of microorganisms as major pollutants have to do with dispersal? Dispersal of the host population can reduce the general incidence of disease. For example, sometimes wealthier humans can flee to other, presumably more healthful areas. In those preferred destinations, costly medical capabilities such as prophylaxis and chemotherapy have some impact upon either reducing the incidence of or ameliorating the misery resulting from microbially induced diseases. Sadly, these luxurious options do not exist in remote or poor areas of developing countries. For people in much of the world, the major means of controlling or preventing these infectious diseases is by interfering with microbial dispersal. Dispersal thus not only is important for populations of humans, animals, and plants as they try to survive the onslaught by pathogenic microbes but also is critical for viruses and other types of infectious or otherwise dependent microorganisms in their own efforts to maintain their existence by keeping up with their dispersed hosts. Dispersal also is a key factor for free-living archaea, bacteria, fungi, protozoa, and algae, which must seek new home territories in order to avoid competition against their parental populations for available space and nutrients.

Some of the scientific interest in microbial dispersal comes from basic research. There have been major breakthroughs in the use of genetic and biochemical techniques to understand chemotaxis, which is movement in response to a chemical stimulus (39). In addition, a number of very useful mathematical models have been developed in aerobiology and for use in predicting microbial dissemination through aquifers and soils (28). Interest in microbial dispersal also comes from concern with bacteria and viruses that cause diseases of importance to humans (12) and with fungi that, as a consequence of their aerial movement, are contributors to declines in food and feed production. The disposal on land of agricultural and urban wastes containing pathogens has also resulted in considerable research and monitoring of microbial movement.

Potential new habitats regularly become available for microbial colonization. For example, habitats appear as seedlings emerge from seeds and with the extension of growing root or shoot tissues, during wounding, which creates access to the interior tissues of multicellular beings (including damage to fruits), with the first appearance of a newborn infant, and at the site of bruised tissues. All of these habitats contain sterile sites which could be inhabited by a variety of microorganisms that might find the sites through successful dispersion. Similarly, possibilities occur when nutrients arrive at new locations in water bodies because of vertical and horizontal mixing of water, and new environments are created both by soil erosion and by the building of dams and ponds. The first half of the earlier statement that microorganisms are everywhere, or at least potentially everywhere, is clearly a platitude when one considers that the issue is not whether a microorganism will appear but whether members of a particular species will have reached a site in which they can grow. However, the organism also must have evolved the capability of staying at that site long enough to grow. Staying around to grow often involves adhesion to the existing surfaces (13, 51) and the formation of complex biofilms (see chapter 45). I would suggest acknowledgment that humans are just another part of the biofilm which has evolved and spread itself over and into the nooks and crannies of this planet. Finally, after adhering, the organism would have to successfully compete for nutrients and defend itself against any organisms already found in that site.

Sometimes microbial communities completely consume the supply of limiting nutritional elements or other limiting resources available in the specific sites where they reside. If there is no further input or regeneration of a limiting nutrient or resource, then the species that make up those communities will die away at those sites. Should this happen in all of the ecosystems within which some particular rare species resides, and if the members of those populations have not evolved adequate survival mechanisms, extinction will occur. Avoiding extinction thus can require that a species have evolved a means of escape either in time (via a persistence mechanism) or in space (via a dispersal mechanism). Escape in time can occur by the establishment of starvation existence, wherein an individual shuts down unneeded metabolic activity and hopes for the arrival of nutrients before it dies from starvation. Starvation modes are common among aquatic bacterial species. Escape in time also can occur by the combined development of metabolic inactivity and protection of an environmentally resistant structure. This combined approach is represented by the spores formed by fungi and by some species of bacteria, the cysts and oocysts formed by many types of protozoa, and the nucleocapsid structure formed by viruses. Escape in space dictates dispersal, leaving in search of a more favorable environment.

Traveling by Air, Sea, or Land

Microorganisms have developed many means for dispersal, and each species must have one or more methods for accomplishing its migration. A few microbial groups have evolved specialized structures to launch their cells into the air or send them swirling into the water. These organisms depend upon those vehicles to deliver their progeny to a new home. There are parasitic species which possess mechanisms which cause changes in the behavior of their animal or plant hosts that result in dispersal of the parasite (29). Still other microorganisms utilize very efficient vectors, such as mosquitoes and biting flies, which almost unerringly deliver the progeny microbes to the correct address (29). Some means of dispersal have a higher risk of failure than others, and species with more efficient dispersal mechanisms do not require as great a level of production and shedding of new propagules because each of their released propagules has a greater likelihood of success at encountering a hospitable site.

Active dispersal, in which the physiology of the microorganism controls its transport (automobility), has been the subject of considerable research, probably because it is more comforting to the microbiologist to have his or her pet control its own fate. Active dispersal, either by motility or by growth of filaments, may be somewhat random rather than resulting from a specifically directed movement (taxis) or growth in a specific direction (tropism). Still, the motility of protozoa and the growth of either bacterial chains or the filaments of fungi in soil and algae in water are sufficient to result in those species successfully encountering and colonizing new environments (28). As a consequence, considerable research has been done on the effects of chemical

stimuli. In the short term, active microbial motility generally is restricted to limited distances because of the energy requirement for movement either toward or away from a chemical stimulus. In the long term, the result can be global dispersals and migrations.

In contrast with active dispersal, there also exists passive dispersal, during which the microbe is transported by a carrier. Passive dispersal is something which we normally might think of as occurring over short distances (45), but it can result in relatively quick dissemination of an organism to locations meters, kilometers, or hundreds of kilometers away from its original reservoir. Aerial dispersal is frequently of considerable importance to fungi (see chapter 73). For organisms dispersed through the atmosphere to be successful, they must produce enormous numbers of propagules to survive the random chance that one or several might alight in an environment in which that species can stay around and grow. A propagule that is transported through the air must have mechanisms to overcome three major hazards: radiation, desiccation, and extremes of temperature. It is not clear in all instances what physiological adaptations are responsible for successful resistance against such environmental hazards. However, the presence of thick walls and dark pigments in many fungi transported through the air and of carotenoid pigments in many aerially dispersed bacteria suggests that these features are important adaptations for this mode of transport. The extent of migration of some of these organisms is truly impressive. For example, in a single year *Helminthosporium maydis*, a pathogen of corn, may spread over areas of thousands of square kilometers, doing enormous financial damage to the intensively planted corn crops of North America. Similarly, spores of the fungal genus *Hemileia* apparently have spread from Angola to Brazil via trade winds which cross the Atlantic Ocean, a distance of more than 1,000 km.

Passive dispersal in water (see reference 7 and section III) and soils (see chapter 50) has also been the subject of considerable inquiry, in part because of public health problems. For example, algae associated with red tides move for some distances and then suddenly create blooms (population explosions visible to the eye because of the pigmentation of these organisms [see chapter 30]), often only to be decimated by viruses (see chapter 36). Still, the toxic products from those blooms can bioaccumulate in the food chain and may have major consequences for aquatic fauna and people who consume seafood animals from the area of the algal bloom. The interest in passive dispersal through soil is often a result of concern with the vertical migration of bacteria or viruses that cause human disease and the entry of those microorganisms into aquifers underlying soils into which the microorganisms were inadvertently or deliberately introduced (see chapter 72). However, appreciable research on the vertical movement important to microbial colonization of roots has also been conducted.

Bacteria, viruses, or fungi that are transmitted by living vectors typically have efficient dispersal mechanisms and require fewer propagules for the species to be maintained. This mode of transmission is considered outside the scope of the *Manual of Environmental Microbiology*, but the general concept of vector transmission is described elsewhere by Hurst and Lindquist (29).

Prepared for the Climate? And What about the Location?

Climate? Even geography? Yes, climate is an important consideration for microbes, and there is a microbial geography. Restricted distributions on a macroscale as well as on a microscale characterize all groups of organisms, and the microbes are no exception. The literature dealing with the geography of microbial groups is often unknown to laboratory scientists, but an investigation of microbial communities in natural environments quickly shows marked and sometimes extreme localization of microbial groups. For example, geographical distributions are evident among aquatic and terrestrial algae; free-living and pathogenic fungi; protozoa in marine water, freshwater, or soil; and bacteria in countless habitats. Many genera, and often even species, seem cosmopolitan, but their widespread distribution does not mask the restricted nature of their occurrence within particular regions, sites, or microenvironments (16, 50, 55).

A key element for any particular species of alga, archaeon, bacterium, fungus, or protozoan to successfully exist in an environment is the ability to endure all of the abiotic stresses characteristic of that environment. These stresses include essentially the same list of things which can serve as either physical or chemical barriers: factors such as unsuitable pH or temperature (what is just fine for one species may be too high or too low for another species), occasional drying or freezing in some environments, intense solar radiation, high pressures deep in the ocean, or salinity in certain terrestrial or aquatic ecosystems. The combination of these factors often produces amazing outcomes. Some algae can be found broadly distributed over the surfaces of lakes and oceans but exist in patches at each site. Some of these patches are no more than a few centimeters across, whereas other patches extend over areas of more than 300 km^2. Well-known to phycologists are the limited distributions of diatoms. Some diatom species are present only in subtropical or tropical waters, whereas others characteristically are found only in the Arctic or Antarctic and even there often are stratified by depth (24). Other algae have snowfields as their habitats. Bacteria such as *Beijerinckia* species similarly have a restricted distribution, being commonly but not solely present in soils of the tropics or subtropics. Often, the biogeography of a microorganism that is transmitted by a living vector or that is an obligate parasite is determined largely or exclusively by the biogeography of its vector or host. This is true of both the protozoan genera *Plasmodium* and *Trypanosoma*, for example, and the explanations for their distributions thus are quite simple. This also is true for vector-borne viruses, which can exist only within the same geographical areas where both their host and the vectoring species can be found (29). In the case of insect-vectored protozoans and likewise insect-vectored viruses, success requires a community interaction of the residential predatory parasitic species (remember that despite the terminology often used by protozoologists, both the protozoans and the viruses are parasitic) which lives inside the vector, a predatory but nonresidential parasitic vectoring species (the mosquito), and the free-living host species which simultaneously serves as prey for both of the predators. Not quite as simple to explain are rare distribution patterns of pathogenic microorganisms that do not have a known vector and whose distribution does not seem obligately associated with a particular known host organism. A notable example is *Coccidioides immitis*, which has a unique distribution in the Western Hemisphere (55). This organism causes disease in humans, but although that host is largely global, the fungus is restricted to certain localized geographical and climatic sites. This fungus is characteristically found in the soils of certain semiarid regions that typically are

exposed to high temperatures, receive little rainfall, and frequently have high salinities. Although C. *immitis* is transmitted by wind, wind movement alone cannot account for its biogeography. Even if the fungus is cold intolerant, that possible sensitivity would not explain why it is not present in warm, humid areas to which it may be carried by the wind. Having failed to find a climatic reason for this geographical restriction of the habitat of C. *immitis*, we may in time find that the answer lies in an examination of biotic factors. It is possible that this narrow operational habitat of C. *immitis* is related to the need for a supporting community composition and interactive community structure, which then determines the sites where the organism is successful and thus is likely to be found. Conversely, then, an unfavorable community composition or structure may prohibit the organism from residing in other potential habitats.

Natural geographic and climatic restrictions are called "zonations." Zonation is evident even at a microenvironmental level. Marked horizontal and vertical differences in either the distributions or the occurrences of algae, bacteria, and fungi are evident in waters, sediments, and soils. Such microscale zonations almost certainly reflect biologically important differences in the physical and chemical characteristics of the environment. However, in only a few cases have the causes of this highly localized microenvironmental distribution been established by either physical or chemical analyses. Among the factors either known or postulated to be important in microscale biogeography are nutrient concentration and type, temperature, pH, oxygen, grazing by zooplankton, mechanical barriers, and inhibitory substances (some of which may be biotic, while others certainly are abiotic).

The fitness traits of the initial colonists in environments that are largely free of microorganisms or that have been drastically disturbed are often reasonably simple to predict. Such environments are not unknown, and they exist, for example, on the previously uncolonized surfaces of roots growing through soil, on plant materials that become bruised, and in waters that receive sudden influxes of organic or inorganic materials. The fitness traits associated with successful colonization, and with an organism's hoped establishment, frequently represent the basis for a capacity to use the organic nutrients present at the new site. For example, with root-dwelling microbes, those nutrients may be either compounds excreted by the emerging root segments or constituents of the tissues that become bruised and thus accessible for microbial utilization. Frequently, the initial colonist preempts the site so that a propagule that arrives later but has the same enzymatic capacity will be unable to multiply. In these instances of preemptive colonization, the key fitness trait is frequently associated with dispersal (getting there first).

Finding common traits among organisms can indicate a similar ancestry, albeit horizontal gene transfer can scramble phenotypic traits and complicate our efforts at understanding how phenotypic traits and physiology relate to ancestry. Still, the very fact that species can be distinguished is evidence that each has some uniqueness with respect to its combination of biochemical, physiological, and morphological traits. Some of these distinguishing traits help explain the presence of a species in one environment but its absence in another and the relative abundance and activity of the various inhabitants which exist in a given environment. This combination of a species' traits provides the basis for natural selection, accounts for the geography of a species, and explains the role (i.e., the niche) of a species in its environment. The sum of the traits of all species present within an ecosystem represents an identifying signature for that ecosystem.

How Is the Food?

An obvious need that must be satisfied for an organism to become established is the presence of all nutrients that it requires. Often, in aquatic environments, phosphorus is a limiting nutrient for photoautotrophic organisms. Many natural terrestrial ecosystems contain most and sometimes all inorganic nutrients in concentrations sufficient to maintain a reasonably large community. Heterotrophic organisms often seem more finicky than autotrophs. Thus, when considering the needs for heterotrophic organisms and any single specific site, we often find that overall the supply of energy resources is the limiting factor and that the kinds of carbon sources available affect the selection of organisms which can maintain a presence in that site. In most environments containing readily or slowly available organic molecules, a variety of dissimilar propagules able to use those carbon sources arrive, yet only a few become established. In this instance, as in so many cases in environmental microbiology when causation is being sought, the presence of a suitable energy source is a necessary requirement but in and of itself not sufficient for microbial establishment.

How can we understand what sufficiency requires? We gain this knowledge by understanding the basis for natural selection, or selection in nature. From an environmental viewpoint, the basis upon which selection operates is the set of fitness traits that underlies ecological success and sometimes the achievement of community dominance by the members of a species. These traits are the specific biochemical, physiological, or morphological characteristics that determine a species' boundary, barriers, habitat, and niche. Nature tries many different approaches to solving a particular problem. Evolution is based upon the fact that those solutions which work are retained and hopefully will again prove adequate at some future point. The issue for scientists is to identify those attributes of organisms that are necessary (the organism could not do without them) and sufficient (with those, the organism will have a chance at competitive success) to enable the organism to survive and occasionally multiply in particular environments. After we first show the importance of tolerance to the abiotic factors that are detrimental to one or another group of organisms and secondly understand the need for a supply of inorganic nutrients and a carbon or energy source, then what? The methodologies associated with enrichment or selective culture techniques are ideal for examining natural selection under artificial conditions. But we must understand the associated limitations of these methodologies and of artificial conditions. The technical limitations focus on the fact that the use of such methods to examine the community of organisms present in an environmental sample typically results in finding only the one organism that grows fastest under those artificial conditions. This would represent selection of those isolated based upon what is termed an *r* characteristic. The *r* growth strategy is one in which organisms invest in quick reproduction. These are the microbial equivalents of the plants known as dandelions. In contrast, the competitive conditions in nature are such that many inhabitants of natural communities grow slowly. For these inhabitants, the focus is upon long-term survival of the individual, and we term this pattern to be the *k* growth strategy. These are the microbial equivalents of the sequoia trees. But, don't think that the members of some species cannot shift from an *r* to a

k strategy if it befits them! This kind of a switch happens when some intestinal bacteria try surviving in an aquatic environment. Even more confounding to our efforts is the reality that most of the organisms existing in nature do not appear to grow at all under conditions imposed by laboratory media. The reason why they do not grow in the artificial environment which we supply simply may be that we have not adequately duplicated the natural setting into which those organisms evolved. Hopefully, newer biochemical tools will help us in sorting out many of the remaining answers, allowing us to achieve success in areas where present cultural techniques are not sufficient tools.

Settling in with Relatives (In-Laws and Outlaws) and Meeting the Neighbors

Abundant literature on the biochemistry, physiology, and morphology of microorganisms has been derived from studies of pure (axenic) cultures of organisms conducted under artificial conditions, usually in liquid media, and in the absence of any other species. However, it is far from certain which of those biochemical, physiological, or morphological properties either truly are of ecological significance or facilitate the establishment of a particular microbe in a particular site. Many facile extrapolations have been made from in vitro to in vivo conditions, but rarely have these extrapolations been verified as being ecologically relevant. We can surmise that if an organism identified in culture is unable to survive and grow when exposed to artificially simulated environmental stresses, it is unlikely to be an inhabitant of an environment in which those stresses occur naturally. Some environmental stresses are easy to establish and duplicate in the laboratory, and it is simple to show by process of elimination which of these may be important in determining the absence of a specific organism in a specific habitat. Conversely, understanding the presence of a particular organism in a particular place and time can be very difficult. Many species are transported to environments in which they are able to tolerate all the abiotic stresses, but they still do not become established. What lies behind this failure of establishment? We may surmise that it stems from the homeostatic mechanisms operating in the community in which the new arrival alights and that these mechanisms are extremely effective at eliminating many of the arrivals. Yet the exact reasons for eliminating or allowing the continued presence of the new potential inhabitants are rarely understood.

We do know that with time, the initial colonizing species present at a site can become displaced as more recently arrived organisms become abundant within the community. These displacements represent natural successions. The initially dominant species often seem to become of less significance to the community as a succession proceeds, and some of the initially dominant species may be eliminated totally. Those displaced species succeed overall by having dispersed propagules to colonize other, more recently available sites before their parental population loses its foothold on the present site. Yet, despite these successional eliminations of specific species, often the overall trend of succession with time is toward an increasing species diversity. As succession proceeds, the identities of the fitness traits associated with allowing any single species to maintain a place within the community become increasingly less certain. However, we do know that among the factors that either contribute to or determine selection during succession are the availability of nutrients that are synthesized by the temporally preceding species, the alterations in concentrations of inorganic nutrients and the formation of toxic products by those earlier arrivals, successful competition for limiting resources (especially the supply of organic carbon), and the inevitable appearance of organisms that parasitize either the pioneering species or the subsequent colonists. The last factor tells us that if a species chooses to remain in the community, it must successfully dodge or repel parasitic organisms such as viruses and grazing organisms such as protozoa and invertebrates.

The displacement of organisms which occurs during the process of colonization and succession may ultimately lead to a mature or climax community. This is the assemblage of organisms most characteristic of any habitat. Often the communities of chief concern to environmental microbiologists are the climax communities, because they tend to be the most prevalent. As might be imagined, the organisms making up a climax community interact in a variety of ways and it is a dynamic structure. Not all of the interactions between the members are peaceful, and many are outright antagonistic. The climax community tends to reproduce itself, and yet it is the net functions fulfilled by the community which may be more constant than are the relative numbers of each of the component species. At this mature stage, the nature of the interactions between species can be very complex and difficult to unravel, and it may seem impossible to discern the specific fitness traits that underlie any particular organism's position in the community hierarchy at any given point in time. Despite the fact that the relative proportions of the component species may vary, as a general rule the component members of a climax community are not eliminated and many rare species can be found there.

In some instances, a community simply seems dominated by a species that has preemptively colonized the site, and its role is associated with its presence at the site before other organisms arrived. In other instances, however, a variety of organisms endowed with appropriate physiological capacities have reached the site, and they thus may end up competing for dominance. Undoubtedly, competition is one of the major interactions in climax communities. As microorganisms usually grow readily when growth is freely allowed, one or more metabolically required factors in the environment must become limiting. These limiting factors may contribute to the bases for competition. For communities dominated by heterotrophic bacteria and fungi, the limiting factor is frequently the supply of available carbon. Many environments, such as soils and sediments, contain large amounts of organic matter, but much of that organic matter is not in a readily available (biologically utilizable) form. Thus, the heterotrophic organisms that are successful are often those that are able to make use of the less readily available organic materials. In communities containing chemoautotrophs, the limiting factor is frequently the supply of the inorganic compound or ion that serves as their energy source. In surface waters, the limiting factor for the photoautotrophic algae or cyanobacteria is often the concentration of either phosphorus or nitrogen. Fluctuations in both the net activity and community composition also may cycle with time due to factors such as climatic variables and seasonality.

By way of summation, what determines the outcome of competition and obtaining a place in the community? It is tempting to suggest that the successful competitor gains its dominance based upon having an *r* strategy, meaning that the organism simply outgrows all others. However, the fact that many of the dominant organisms in natural environments

adopt instead the *k* strategy and do not grow quickly suggests that it is imprudent to extrapolate to natural environments from growth rates obtained by studies performed with pure cultures under noncompetitive circumstances. Clearly, more is involved than simply growth rate per se because of other stresses which exist in nature. Likewise, we must consider the need for an organism to be transported to sites where there is an available supply of limiting nutrients and the need to avoid predation after it has arrived. Sometimes, dominance does result simply from being the first arrival, but even those first arrivals may lose dominance and become displaced during community successions. Perhaps it is simply "peacefully fitting in" which describes the recipe for success. Alternatively, it may be subterfuge. Both thievery and enslavement of other species may be involved. The success of new arrivals and their inclusion in the community also may depend in part upon the biochemical weaponry which those species bring with them, since microbes seem to have invented the concepts of laying siege and attack.

WHAT DOES IT ALL MEAN?

Perhaps the key aspect of understanding ecology is development of a comprehension that the individual species are connected, i.e., that the ecology of one species coordinates with those of other species. This coordination usually includes both macrobes and microbes. All of these species then function together as a community. An ecologist has been euphemistically defined as an individual whose feet are firmly planted in midair, possibly because of the enormous time which must be spent pondering it all. Perhaps, like Buddhist monks, we seek some "true understanding of nature" as we regard both interrelations among the activities of different species and their interactions with the physical environment which surrounds all of them. Much of ecology, including environmental microbiology, is concerned with gaining insight through the process of basic science. Indeed, basic science can help us to find the keys which then allow us to open some of the seemingly locked doors beyond which lies a better understanding of nature. However, it is also clear that environmental microbiology is partly an applied science. It has much to offer to our knowledge regarding the maintenance and restoration of environmental quality, prevention of the transmission of diseases of animals and plants, and approaches to improving human health.

The guardianship of environmental quality relies upon understanding of the role of microorganisms in preventing pollution, destroying noxious organic materials before their concentration becomes objectionable, or destroying toxic chemicals before they have an impact on humans, animals, or plants. Indeed, using information which they have gained through their studies, environmental microbiologists now are engaged in bioremediation technologies, designing ways to enhance the capacity of microbes to bring about the destruction of pollutants.

The epidemiology of communicable diseases is, to a significant degree, an extension of evaluations of microbial dispersal. The spread of viruses, bacteria, fungi, and protozoa thus has a tremendous impact on the protection of plant, animal, and human health. Plant pathologists have long recognized the significance of information on the ecology not only of the disease-producing fungi, bacteria, and viruses but also of other microorganisms, many competitive and some even protective, that reside in the same habitats. Many plant diseases are not effectively controlled by chemical agents or by sanitation procedures, and it is the activity of those other, nonpathogenic members of microbial communities that must be used to form the basis for effective control of particular diseases. Much of environmental microbiology research is interdisciplinary. For example, aerobiologists and soil microbiologists frequently interact with plant pathologists. Preventive animal husbandry and human medicine have acquired valuable information from environmental microbiology. Notably, in the field of medical treatment, the development of antibiotics and other forms of chemotherapy has provided us with some powerful tools, many of which have been based upon knowledge of upsets and restorations in microbial communities.

Thus, knowledge of microbial community structure and community function has a key role in improving our lives. Furthermore, this knowledge will aid us in understanding ways of maintaining the environment and its microbial communities. As John Donne correctly stated, "No man is an *Iland*." Carrying his understanding a step further, humans and all macroorganisms are connected to the microbial community and have evolved from it, and our future depends upon that community.

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Prokaryotic Diversity: Form, Ecophysiology, and Habitat

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3

RECOGNITION OF PROKARYOTE DIVERSITY AND THE LIMITS OF OUR COMPREHENSION

Our perceptions that microbes as a group possess extremely diverse characteristics and live and function in quite diverse habitats (many of which seem "extreme" to those acquainted only with macrobiotas) had their origins in the penetrating observations and studies of two particular individuals—the Russian S. Winogradsky and the Hollander M. Beijerinck. As van Niel noted (146), those who studied with Beijerinck and his successors in Holland and elsewhere (the "Delft school") were among the major contributors to the breadth and depth of our comprehension of prokaryote diversity, the crucial functional roles these microbes play in the biosphere, and the recognition that in the absence of these diverse microbial activities other forms of life on this planet would promptly cease to exist. The success of enrichment culture and isolation approaches (22, 47, 63, 112, 147, 153), coupled with new analytical techniques (2, 4, 51, 54, 57, 98, 119), reveals how little we understand about so many of the presumptive prokaryotes present in nearly every habitat we explore. We continue to be inspired by the extent of microbial diversity as contemporary approaches to light, electron, and other forms of microscopy have evolved and as tools of a molecular biological nature (e.g., analysis of DNA and RNA sequences, use of RNA-targeted oligonucleotide probes, stable-isotope probing, metagenomics, and community proteomics) have been brought to bear in the examination of mixed microbial populations of many habitats (4, 73, 102, 104, 107, 117, 118, 121).

Frequent reports now cite the cultivation and characterization of organisms that had been refractory to study in the laboratory or the presence of one with apparently novel features detected in a habitat that had seemingly already been well explored or the fact that the microbial content of some novel habitat is worthy of inspection. Still, we may never be able to describe completely either the enormous diversity in features of the many prokaryotes currently recognized, the wide range of habitats in which they are known to occur, or the microbial composition of the populations present in those habitats. There is no little irony in the fact that as we learn more and more about ever more members of the *Bacteria* and *Archaea*, we come to realize that we comprehend less and less, both qualitatively and quantitatively, of the diverse features of different prokaryotes and of environmental factors affecting, and reflecting, their growth, activity, metabolic capabilities, and persistence. Twenty-five years ago, some might have surmised that perhaps as much as 40% of the prokaryotic world was recognized and understood; today it is probably overly optimistic to suggest that the figure is more than 5%, if even that. And just what we consider, or how we define, prokaryotic "species" (for details, see chapter 13) has been perplexing for decades and will likely remain enigmatic.

Readers are reminded that in this assessment of prokaryotic diversity and the range of habitats in which these organisms are known to multiply (or at least survive), the phrase "as known at present" needs to be kept uppermost in mind. This limitation challenges those interested in enlarging our understanding of microbes' contribution to life on the planet. This chapter is intended to be a descriptive outline of the *Bacteria*, the *Archaea*, and some new chemical boundaries of their habitats in the more usual environmental sense (e.g., association with soils, waters, and some extreme environments) and also includes a consideration of microbes associated with macrobes.

SOME FACTORS AFFECTING LIMITATIONS IN OUR UNDERSTANDING

Many factors are responsible for our inability to fully describe the prokaryotic world. Often, major advances in the development and application of new technologies may be required to permit significant increases in our comprehension. In addition, new findings are not always integrated with an understanding of the microhabitat dimensions and features that are so essential for understanding the context of microbial habitation in a given place. If coupled to detailed measurement of the corresponding abiotic parameters in a given environment, new microbial characterization methods carry the real promise of discerning which microbial cells are the basis for maintaining the biologically tractable chemical and physical features of the habitat. Some of the hurdles that we must overcome are noted below.

Rarely does the size or shape alone of a prokaryote permit one to identify it and equate its properties with those of an organism already described. This limitation is no less serious if one encounters a novel or "new" organism, and even determining whether an object is in fact an organism or is instead some inanimate material can be difficult. Another important consideration is that for a single cell or

even a few cells to be detected in a typical field of view by light microscopy, an amount on the order of 10^6 cells per ml needs to be present. Clearly, it would be easy to overlook the presence of many less abundant organisms. Also, because of the physiological diversity of microorganisms and our lack of knowledge of their physiology it is difficult to duplicate in the laboratory the conditions that facilitate the growth of all but a small subset of the *Bacteria* or *Archaea*. In addition, in many instances cells are attached to surfaces or present in biofilms the removal from which may damage the cells. New methods that rely upon extraction of DNA from the sample and subsequent molecular characterization have made advances in this area; however, for many habitats it is not readily possible either to enumerate the prokaryote population present or to isolate and then describe thoroughly the types present. Pure cultures are rarely found in habitats other than the laboratory. Even though we continue to explore the metabolic and physiologic properties of pure (as well as some simple, mixed) cultures in controlled laboratory environments and to use these insights to make reasoned suggestions about functional attributes that might occur in natural habitats (23), we obviously lack information about the capabilities of those organisms not amenable to cultivation. The advent of large-fragment DNA libraries (also called metagenomes) constructed of DNA extracted from whole communities has permitted the simultaneous determination of key functional traits and the associated 16S rRNA classification of community members without the need to culture cells and without the bias introduced by PCR (16, 121). These and other molecular methods are covered extensively in this volume in chapter 12. Of significance are the interactions of different prokaryotes with each other, or with other biota (for examples, see references 1, 17, and 32), with the result that the behaviors of members of mixed populations differ from those predicted on the basis of the study of component pure cultures. These are but a few reasons why a particular organism's presence in a habitat does not necessarily provide evidence concerning the precise in situ activities of that organism and its contributions to habitat functions (60). New investigations that target mixed cultures and complex communities collected from the environment address the lament that too many "microbial ecology" studies deal with single species and move our science progressively towards a system level understanding of microbes in their environment.

Another complication is that many bacteria exhibit an enormous degree of metabolic flexibility (e.g., nutrients consumed, metabolic end products, and mode of energy conservation); these traits are often regulated by numerous and complex environmental factors that are difficult to ascertain with accuracy for the microscale environments occupied. In contrast, not all prokaryotes possess such a broad spectrum of traits; instead, many possess a specialized metabolism or physiology. The detection of such organisms in a habitat may allow a more confident prediction of their roles in the habitat than is possible for the more metabolically flexible ones. Similarly, the dominance of metabolically specialized microbes in a given habitat may provide a gauge of the stability of the habitat.

The enrichment culture approach has resulted in many significant contributions to our understanding of the connections between particular microbes and cause-effect relationships in many habitats. Many who have carefully employed this method to determine (i) whether organisms with already known attributes were present in samples from a habitat being examined or (ii) whether a novel transformation of compound C to compound D, or some other alteration of a habitat feature, could in fact be mediated by a microbe have recognized the need to quantify the numbers of the particular organisms present as an important aspect of establishing causal relationships (147).

Molecular probe technologies are now well accepted for describing the microbial composition of the habitat of interest, yet some caveats are associated with this methodology (2, 95). In general, the probes are designed on the basis of our knowledge of molecular signatures of well-described and understood organisms, that is, organisms that have been studied in pure cultures. As long as the habitats being examined using these probe methodologies contain recognized organisms with previously described molecular signatures, then there is a reasonable likelihood that the cells will be detected. It must be remembered that because of the inherent metabolic and/or physiologic flexibility of many prokaryotes, even if probe studies reveal the presence of (for example) sulfate-reducing bacteria in a habitat, this information alone does not establish whether it is sulfate, sulfite, thiosulfate, some other sulfur-containing electron acceptor, or even nitrate that is being anaerobically respired by the population or whether it is lactate or some other electron donor that is serving as the energy source. By coupling molecular studies with water chemistry analysis of the sample, investigators may be able to understand the actual processes that are being used by the cells. Another issue that arises when one depends upon molecular probes, or any nucleic acid-dependent characterization, is the degree to which representative nucleic acid can be cleanly extracted from the samples. Still, the potential and utility of molecular probe approaches, when used in combination with those of classic enrichment culture for habitat analysis, are likely to lead to greater comprehension. The combination of culture-based and molecular strategies has thoughtfully been suggested as a wholly new means for identifying new microbial physiologies, as was demonstrated for the recently discovered anaerobic ammonia oxidizers (139).

But perhaps the most significant factors responsible for our limited understanding of bacterial diversity relate to the relatively small numbers of analysts who have devoted themselves to these tasks and to the fact that often their revelations lead to a type of "reductionism," namely, the exploration and elucidation of the properties of the organism(s) newly isolated. Because the discovery of novel properties of such isolates continues to be both stimulating and rewarding, individuals with interests in surveys and elucidation of microbial diversity have had their attentions refocused. The best explanation for the enormous numbers of newly described prokaryotes (for an example, see reference 111) possessing newly recognized biochemical, morphological, and physiological properties is probably a real increase over the past several decades in the numbers of investigators who have pursued this type of scholarship. Even so, the total number of practitioners of this art and science is not large, and their goals, significant as they are, often have not been widely appreciated or understood, nor has the research been well funded. The continued exploration of microbial diversity may best be accomplished through integrated and balanced use of culture-based approaches and new autecology investigations in which cells are studied in the context of their microniche, a method made possible through the use of sophisticated new equipment.

Many thoughtful students of prokaryotic diversity have long recognized that only a fraction of the presumed prokaryotes seen in habitat samples or appearing in enrichment

cultures have ever been brought into pure culture (or into stable mixed culture) so that traits could be evaluated and the organisms could be identified (151). Seemingly insignificant modifications of the culture medium or incubation conditions often resulted in pronounced changes in the populations that developed. Even when similar modifications were made in isolation media in attempts to further cultivate such organisms, the efforts were often unsuccessful. Clearly, the organisms' physiological needs remained unmet. Possible explanations are legion. An oft-noted situation is that the populations developing in an enrichment medium with or without agar present as a solidifying agent (an example of the accumulative versus the separative enrichment culture approach) are recognizably different. Many investigators note that some organisms that do appear in accumulative (liquid) enrichments are unable to grow in an otherwise presumably identical agar-solidified medium (55, 112). Nevertheless, progress towards growing cells that were unyielding to many cultivation regimes is occurring. Attempts to physically isolate target microbes from other microbes include optical "tweezers," cell-sorting techniques, traditional dilution-to-extinction approaches, filtration, and density-gradient centrifugation (78). Subsequent to the physical isolation of the cells, creative tools have been devised to encourage cell growth (74) including diffusion chambers (63) or miniaturization techniques using cell encapsulation in gel microdroplets (153) or new cell arrays (22). High throughput of samples is a key to success and to reducing the labor required in order to test the conditions needed to grow heretofore-uncultivatable cells. Still, those practicing the art of isolation and cultivation frequently counsel patience as the key to obtaining cultures of obstinate cells (57, 74).

The historical discord between what can be seen and what can be cultivated (140) has reached a new level. Analytical approaches that are founded upon extraction of nucleic acids from populations followed by amplification and cloning of genes for rRNA allow us now to ponder the incongruency between what can be detected and what can be cultivated. In many instances, rRNA with sequences unlike those already characterized from cultivated organisms is found, providing the initial clue of the presence of a novel, undescribed organism(s) in the habitat sampled. Now, methods like stable-isotope probing (102, 115), metagenomics (16, 51, 121), and community proteomics (117) are providing striking information on community members and their relationships. Recently, such knowledge gained from molecular studies of an extremely acidic system with low microbial diversity permitted the design of cultivation conditions such that one microbe, first known only by its molecular signature from an environmental sample, was successfully grown in culture (142). Still, these powerful new tools for determining the molecular nature of microbes or communities are not ends in themselves. Their careful use requires the accompanying measurement of physical and chemical parameters of the specific environment from which the cells are derived. If these contextual data are collected, then the molecular biology data that are becoming so accessible will yield new insight into the microbial ecology and controls on microbial diversity.

MORPHOLOGIES AND SIZES OF PROKARYOTIC CELLS

We consider prokaryotic cells to have shapes described simply as spheres, rods, vibrioids, spirals, or pleomorphic, and we now recognize cells that are nearly square, rectangular, triangular, or even star shaped. Many of these different morphological types are characterized as well by the presence of constrictions, protuberances, lobes, or other geometrically irregular aspects of their surfaces. Cells may exist as single entities or as units forming chains, clumps, or filaments. Table 1 provides a selected list of the diverse morphological traits of representative organisms.

This morphological diversity contributes to the difficulty encountered in distinguishing cells from inanimate material. Of additional significance is the fact that the morphology of a given organism can undergo change depending on the extracellular environment or the stage of growth. In addition, cells subjected to drying for purposes of staining sometimes appear different from the same cells in the living, unstained state. The range of sizes for different prokaryotic cells is quite large. One unicellular organism may be barely large enough to be resolved by and seen in a light microscope, while others can be just large enough to be seen with the unaided human eye (7). The reclassification of one "protist" as, instead, yet another large prokaryote (47) generates wonder about the actual upper size limits for prokaryotes and how frequently we may revise the estimate (128). At the other end of the size spectrum, some cell types are known to respond to starvation by becoming so-called ultramicrobacteria (5). The newly cultivated *Nanoarchaeum* (56) appears to be one of the smallest microbes. While the question of minimal permissible size of a microorganism has been the subject of contention (20, 64, 71, 144), it seems that a lower size boundary must still be large enough to contain a ribosome (92). Table 2 provides a selected listing of the cell sizes currently recorded for prokaryotes.

PHYSIOLOGICAL DIVERSITY AMONG THE PROKARYOTES

In the biological world, there are no known parallels to the abilities of the *Bacteria* and *Archaea* to utilize an enormous array of energy sources to support growth and metabolism. The recognition that beneath the widely disparate nutritional and environmental needs for the growth and sustenance of different microbes there was an underlying unity in their physiological attributes was a major conceptual contribution (69) that had a marked practical influence on the development, nature, and extent of our understanding of the significance of prokaryotic diversity (70).

This encompassing view was that energy was conserved and made available for life processes as a result of cellularly mediated, coupled oxidation-reduction reactions and that as a group the *Bacteria* exploited in this way nearly every conceivable source of energy available. Then novel, this concept of an extraordinary versatility, along with the wide range of environments in which different prokaryotes were shown to grow or remain metabolically active, led to the notion that prokaryotes are unique in their ability to inhabit and thrive in environmental extremes (i.e., those not conducive to growth of macrobes) (31, 94). One outgrowth of these considerations was the recognition that every naturally occurring organic compound is subject to attack (utilization as a growth-supporting nutrient or as a cometabolic substrate) by one or another microbe and mineralized as a result of participation in the biogeochemical cycles. We have come to realize that not all compounds synthesized by animals and plants are subject to biodegradation at identical rates (the slow attack on lignocellulosic material is one example) and that the initial degradative steps of

TABLE 1 Examples of diversity in morphology of selected prokaryotes

Morphology or other cell anatomical feature	Representative genera
Coccus	
Single	*Acidianus*, *Megasphaera*
In chains	*Lactococcus*, *Streptococcus*
In other groupings	*Pediococcus*, *Sarcina*
Coccus-rod, lobed	*Sulfolobus*
Coccus ↔ rod[a]	*Arthrobacter*
Circular (or nearly so)	*Cyclobacterium*
Cyst (or microcyst) formed[a]	*Azotobacter*, *Sporocytophaga*
Endospore formed[a]	*Bacillus*, *Clostridium*, *Acetonema*, *Sporosarcina*, *Thermoactinomyces*
Exospore formed[a]	*Methylocystis*
Irregular	*Nocardia*, *Mycobacterium*, *Streptomyces*[a]
Myxospore formed[a]	*Myxococcus*, *Stigmatella*
Rectangle	*Methanopyrus*
Rod	
Long, single	*Bacillus*
Short, single	*Pseudomonas*
Often in chains	*Bacillus*, *Lactobacillus*
Spiral, small	*Bdellovibrio*, *Desulfovibrio*, *Rhodospirillum*, *Methanospirillum*
Sheathed	*Thermotoga*, *Sphaerotilus*
Square	Not yet named
Stalked[a]	*Asticcacaulis*, *Caulobacter*
Triangle	*Pyrodictium*
Vibroid	*Bdellovibrio*, *Marinomonas*, *Vibrio*
Wall-less	*Mycoplasma*, *Thermoplasma*

[a]Cell undergoes morphogenesis.

some biosynthesized materials are carried out not by *Bacteria* or *Archaea* but instead by other microbes. The initial contributions of Beijerinck and Winogradsky (134) that established the utilization of inorganic ions and molecules for energy conservation coupled to growth have since been extended in terms of both the scope of the *Bacteria* and *Archaea* involved and the range of inorganic entities able to serve in this capacity (3, 61, 90, 125, 143).

TABLE 2 Diversity in prokaryote cell size

Size (μm)[a]	Representative organism(s)
< 0.2	Some "picoplankton"
0.3–0.5	*Veillonella*
0.4	*Nanoarchaeum*
2–3	*Megasphaera*
5–25	*Thiovulum*
0.6–1.2 × 2.5–5.8	*Bacillus*
1.1–1.5 × 2–6	*Escherichia*
5–6 × 8–12	*Chromatium*
2.5–4 × 40–100	*Thiospirillum*
1–100 × 500–200	*Beggiatoa*
80 × 600	*Epulopiscium*
100–750	*Thiomargarita*

[a]Approximate diameter (× length where appropriate).

It is customary (8, 10, 35, 108) to categorize and contrast the several ways prokaryotes employ the coupled oxidation-reduction reactions listed below. Table 3 provides examples of some additional aspects of these processes.

- Aerobic respiration: molecular oxygen serves as the oxidant in a redox reaction and appears in reduced form as water, one end product of this metabolism.
- Anaerobic respiration: in environmental conditions where molecular oxygen is absent or in limited supply, an inorganic ion such as nitrate, sulfate, or carbonate serves as the terminal oxidant and becomes reduced to dinitrogen (or ammonia), sulfide, or methane, respectively. It is now recognized that a variety of other ions such as oxidized (ferric) iron or organic molecules such as fumarate, trimethylamine oxide, dimethyl sulfoxide, or the sulfonic acids can also serve as terminal oxidants for a variety of anaerobically respiring prokaryotes.
- Fermentation: an organic compound, most often a metabolic intermediate, that results from oxidation of the organic compound serving as the energy source, serves as the terminal oxidant, and a more reduced organic molecule is a metabolic end product(s).
- Phototrophy: radiant energy is absorbed by chlorophyll-, bacteriochlorophyll-, or "accessory pigment"-containing pigment complexes, resulting in an excitation of electrons present in the complex and leading to an oxidation and charge separation. When water is employed as the ultimate reductant for the sequential reactions, the

processes are characterized by evolution of molecular oxygen ("oxygenic photosynthesis"); when either inorganic or organic compounds replace water, molecular oxygen is not formed, and the processes are termed, instead, "anoxygenic photosynthesis."

We may summarize, then, one aspect of the prokaryotes' physiological diversity by noting that some can persist and multiply only in habitats that are in regular contact with the earth's atmosphere and the molecular oxygen it contains, employing aerobic respiration (i.e., are strictly or obligatorily aerobic). Other prokaryotes function only in the absence of air (58), employing either fermentation or anaerobic respiration, and thus pursue other modes of energy acquisition and conservation; we term such organisms strictly or obligatorily anaerobic (but might also describe them as strictly fermentative or as living strictly by anaerobic respiration). Another group may possess, for example, the ability either to live by aerobic or anaerobic respiration; the adjective "facultative" is added to describe such an organism's respiration. The same term is used in describing the ability of an organism to live either by aerobic respiration or by fermentation (e.g., facultatively fermentative) and in the description of a bacterium able to live either by anoxygenic phototrophy or by aerobic respiration. Evolution has resulted in a continuum of traits rather than a set of neatly packaged ones. The inadequacy of words such as "facultative" to describe the physiologies of a bacterium able to live by either fermentation, aerobic respiration, or anoxygenic phototrophy, for example, becomes readily apparent. Despite our inability to succinctly categorize microbial metabolic strategies, we recognize that life requires an accessible and energetically sufficient oxidation-reduction gradient from which to gain energy and that this requirement is a signature feature of an environment if it is to sustain life. Conceptually, this theme has been proposed as a potential marker of extraterrestrial life (39) even if microbes display a variety of strategies for accessing such energy.

In a related sense, our understanding of the roles of prokaryotes in biosphere functions may be constrained. For example, we may not even know that a nutritional lifestyle based on acetogenesis remained to be discovered in a distinct morphological prokaryotic group (spirochetes [76]), that phosphite oxidation could be coupled to dissimilatory sulfate reduction by anaerobes (123), or that N_2-dependent growth was a trait of metal-metabolizing bacteria (13). Perhaps for such reasons it has become customary to categorize microbial physiological traits in ways other than those having to do strictly with oxidation and reduction reactions.

One such way refers to the source(s) of carbon assimilated for biosynthesis: "autotrophy" describes the ability of an organism to utilize carbon dioxide as the principal source of carbon (save perhaps the need for vitamins or an amino acid[s]), while "heterotrophy" or "organotrophy" describes the use of carbon atoms of organic molecules as the principal carbon source. When categorization is focused on the source of energy to be conserved, an organism doing so at the expense of reduced inorganic ions, or molecules such as

TABLE 3 Some nutritional aspects of physiological diversity

Electron donor(s) utilized (examples)	Electron acceptor, reduced end product(s)[a]
In aerobic respiration	
Organic molecules: carbohydrates, amino acids, purines, pyrimidines, lipids, fatty acids, alcohols, hydrocarbons (both aliphatic and aromatic), sulfonic and aromatic acids	Molecular oxygen, **water**
Inorganic molecules or ions: carbon monoxide, molecular hydrogen, metallic sulfides, ammonium, nitrite, ferrous and manganous salts, elemental sulfur	
In anaerobic respiration	
Organic molecules: much as given above; possible exceptions are some sulfonic and aromatic acids	Nitrate, nitrite; **nitrite, nitrogen gas, ammonium**
Inorganic molecules or ions: much as given above	Sulfate, sulfite, elemental sulfur; **sulfite, sulfide**
	Fumarate, **succinate;** dimethyl sulfoxide, **dimethyl-sulfide;** ferric salts, **ferrous salts;** trimethylamine oxide, **trimethylamine**
But methanogens typically utilize primarily hydrogen gas, formate, or acetate	Carbonate, **methane**
In fermentation	
Organic molecules: carbohydrates, purines, pyrimidines	Organic molecules, protons; **alcohols, fatty acids, ketones, hydrogen gas**
In phototrophy	
Organic molecules: alcohols, fatty acids, organic acids (e.g., malate, succinate, benzoates)	Carbonate, **cellular components**
Inorganic compounds or ions: hydrogen gas, ferric, sulfide, elemental sulfur, thiosulfate	
But, for cyanobacteria, water	

[a]Reduced end products are in boldface type.

TABLE 4 Some terms used in relation to bacterial growth or metabolic activity

Acidophiles: organisms with growth or activity optima at pH values of ca. 1–5
Aerobes: organisms that use molecular oxygen in redox reactions coupled to energy conservation
Obligate: cells that cannot grow or that remain inactive in the absence of molecular oxygen
Facultative (or euryoxic): cells that grow or are active in the absence of molecular oxygen
Alkaliphiles: organisms with growth or activity optima at pH values of ca. >8
Anaerobes: organisms that are unable to use (i.e., consume) molecular oxygen
Obligate: those that cannot grow or that remain inactive in the presence of molecular oxygen
Oxyduric: those not killed by (i.e., tolerant of) molecular oxygen
Oxylabile: those killed by the presence of molecular oxygen
Aerotolerant: those able to grow or remain active in the presence of molecular oxygen even though they do not use it
Microaerophiles: organisms that require molecular oxygen for growth or activity but can tolerate its presence only when present at low levels (often ca. 10% of atmospheric levels)
Mixotrophs: organisms that are both autotrophic and heterotrophic, usually simultaneously, in order to conserve energy and assimilate nutrients for growth or activity
Phototrophs: organisms that use radiant energy (light) as a source of energy for growth or activity
Obligate: those that cannot grow or that are inactive in the absence of light
Facultative: those able to grow or remain active by gaining energy in the absence of light
Symbiosis: two or more dissimilar organisms that interact and live together
Syntrophy: the relationship between proton-reducing organisms and other organisms that consume hydrogen gas
Obligate: the relationship when the proton-reducing organism is unable to grow or remain active in the absence of the hydrogen-consuming one

hydrogen gas, is regarded as a "lithotroph." The term "organotroph" describes an organism utilizing organic molecules; note the use of the identical word to describe both the carbon and the energy sources. Table 4 lists examples of descriptive terms often used in categorizing what are indeed different lifestyles of prokaryotes.

Prokaryote physiological diversity extends beyond relationships to molecular oxygen or how energy is derived. Optimal growth under conditions of low pH (acidophiles) or high pH (alkaliphiles) is characteristic of many different prokaryotes. Similar diversity exists in temperature optima for growth: cold-loving organisms (psychrophiles) contrast with those that are unable to grow at temperatures less than ca. 80°C, some of which (hyperthermophiles) have been shown to grow at temperatures in excess of 110°C (135). The current high temperature record holder grows at 120°C and can briefly survive at 130°C (66). Among other traits in which prokaryotes show remarkable variation from organism to organism are the abilities to tolerate (34) or need inorganic salts (as for the strictly halophilic subgroup of the *Archaea*) or to grow only in environments with low nutrient levels (oligotrophs) or high nutrient levels (copiotrophs). Yet another trait noted for certain organisms is a requirement for pressure in excess of that at the earth's surface (barophiles [110] or piezophiles [152]). Table 5 summarizes ranges of selected traits.

As noted above, the scope of nutritional diversity among the prokaryotes is not only impressive but also important, as it affects the ability of these microbes to colonize and to thrive in nearly every imaginable habitat. Some bacteria are able to oxidize compounds containing no carbon-to-carbon bonds (C-1 compounds) and to assimilate the oxidized moiety for synthesis of molecules characteristic of all cells. While many of these organisms are also able to oxidize and assimilate methanol carbon, they are generally incapable of assimilating the carbon of other simple or complex organic molecules as the sole carbon and energy source for growth or maintenance. In contrast to these "methanotrophs,"

TABLE 5 Environmental extremes in which prokaryotes are thought to multiply

Characteristic	Value
Pressure	1 to ca. 1,000 atm[a]
Temp	−1.2 to 120°C
Ionizing radiation	Up to 15,000 Gy[b] instantaneous dose
Depth	2,000 m (terrestrial subsurface) to ca. 10,000 m (ocean floor)
Salt conc.	Up to ca. 4–5 M
Acidity or alkalinity	pH of <1 to 11–12
Available water	a_w (water activity) as low as 0.6

[a] 1 atm = 101.29 kPa.
[b] 1 Gy (gray) = the absorption of 1 J of energy by 1 kg of matter.

another group, the "methylotrophs," differs in two ways. Methylotrophs cannot utilize methane for growth but can grow or remain metabolically active at the expense of methanol, formate, and methylated amines; more strikingly, however, they can use acetate, other organic acids, and amino acids. Most of the well-studied methanotrophs are specialized in terms of nutrients utilizable for growth or activity, while the methylotrophs are much less so.

Another example of a nutritional specialization is shown by at least one *Bacillus* species which can be sustained readily with urate, a purine, or compounds such as allantoin and allantoate, both of which are intermediates in the degradative pathway for urate oxidation and assimilation. However, in contrast to the scores of other *Bacillus* species, *Bacillus fastidiosus* cannot survive by using sugars, polysaccharides, amino acids, or simple proteins. In similar fashion, some *Cytophaga* species, unable to be sustained with amino acids as carbon and energy sources when first isolated from a natural habitat, are strictly dependent upon polymers such as cellulose or chitin as a source of carbon and energy for growth. Other cytophagas, by contrast, cannot use cellulose or chitin for growth, but they can grow on complex media containing peptides, amino acids, yeast extract, or simple sugars. These specificities contrast to those of some pseudomonads and their relatives which are able to use scores of organic compounds, including simple organic acids, the range of amino acids, and benzenoid and polycyclic compounds, to support cell division.

As a group, autotrophs also may vary the reductant that they use with respect to their nutritional needs. Some oxidize hydrogen gas or may use a compound such as hydrogen sulfide from which to capture energy for growth, or they may be able to grow as organotrophs by use of organic compounds. However, not all chemolithotrophs are so versatile; some "sulfur-oxidizing" chemolithotrophs use any of several reduced sulfur compounds to acquire energy, while others display much more selectivity and specificity. For another group of autotrophs (e.g., *Nitrosomonas* spp.), ammonia serves as an energy source, while nitrite does not, yet *Nitrobacter* spp. oxidize nitrite to form nitrate but are unable to gain energy from ammonia. Reduced forms of iron are suitable electron donors in energy-conserving processes for other autotrophs.

At least two other aspects of physiological diversity are significant for the discussions that follow. In one case, even though a particular organic molecule may not function as a sole source of carbon and energy for growth of an organism, the compound may be metabolized (i.e., either oxidized or reduced in whole or in part, and all or part of the molecule may be assimilated into cellular components) as long as the organism is utilizing a different molecule as a carbon and energy source. This phenomenon, first termed "cooxidation" and now most often referred to as cometabolism, has been shown to be of more general significance and distribution in prokaryotes and is essential for many bioremediation processes (83). A second trait is syntrophy (85). In anoxic environments, the degradation of reduced organic compounds (e.g., simple alcohols, fatty acids, and certain aromatic acids), resulting in the accumulation of acetate, carbon dioxide, hydrogen gas, and formate (among other end products), is energetically unfavorable. However, if the concentrations of these products are kept sufficiently low, the overall energetics for growth become more favorable. Methanogens and sulfate-reducing bacteria (sulfidogens), commonly present in anoxic habitats, consume hydrogen gas and thus lower its partial pressure in the microhabitat. Thus, one member of a coculture functions to permit the biodegradation of compounds that would otherwise be refractory to attack by pure cultures. The term "interspecies hydrogen transfer" has also been used to describe such interactions. Although not yet fully physiologically characterized, the anaerobic methane-oxidizing community that occurs in marine sediments where methane and sulfate are present as the electron donor and acceptor, respectively, appears to represent a recent example of such a microbial consortium (17, 102).

One final observation related to the physiological diversity of microbes is that many of these cells display a range of levels at which they may be metabolically active. Although cells grown in the laboratory are often pushed to grow optimally, under environmental conditions cells can enter a state of maintenance level activity or dormancy enabling their survival for periods of time far longer than the generation times or metabolic rates deduced for them in laboratory cultures. Although dormancy is a trait often attributed to cysts or endospores, it is well recognized that dormancy is a significant phenomenon in the persistence of an enormous variety of cells (65, 96). In fact, it seems that the metabolic activity of cells in numerous environmental settings can be classified according to whether the cells are actively growing, sustained at maintenance levels of activity, or clearly dormant (114).

DIVERSITY: INSIGHTS FROM MACROMOLECULAR ANALYSES

Because prokaryotes have been on this planet for perhaps four-fifths of its age, there has been ample time for repeated mutations, their accumulation and natural selection, and an introduction and accumulation of widely varied traits in these organisms. For years, the lack of a substantial traditional fossil record impeded critical considerations of evolutionary change in the microbial world. It was not until Zuckerkandl and Pauling's (155) proposal of macromolecular traits as indicators of evolutionary changes that we could ponder events of the past. Data from protein (30), rRNA (48, 130), and high-throughput DNA processing that generates complete genome sequences (e.g., the U.S. Department of Energy's Joint Genome Institute [http://www.jgi.doe.gov/]) are now used widely for this purpose and for making inferences about phylogenetic relationships. The evolution of prokaryote diversity has become a subject of intense interest, warranted because more than one-third of the phylum level prokaryotic lineages are known only by the phylotypes or environmental clone sequences (i.e., DNA sequences in a database) that reside within them and not by cultured representatives (57).

A widely accepted phylogenetic tree (Fig. 1) that classifies life in three major categories, *Archaea*, *Bacteria*, and *Eucarya*, is based on the inferences that *Archaea* and *Eucarya* diverged from ancestors of the *Bacteria*, first as a single lineage and only later diverging and becoming separately recognizable entities. The timing of this divergence, as well as the estimates for the divergence point of the eukaryotes and prokaryotes, has been considered often (30).

The potential for lateral gene transfer in natural populations remains a matter of concern in using sequence information for phylogenetic considerations and in reconciling the concept of species in microorganisms (15, 42, 45, 48, 80, 93). As the number of known microbial genomic sequences increases, there is mounting evidence that horizontal (or lateral) gene transfer has occurred frequently in

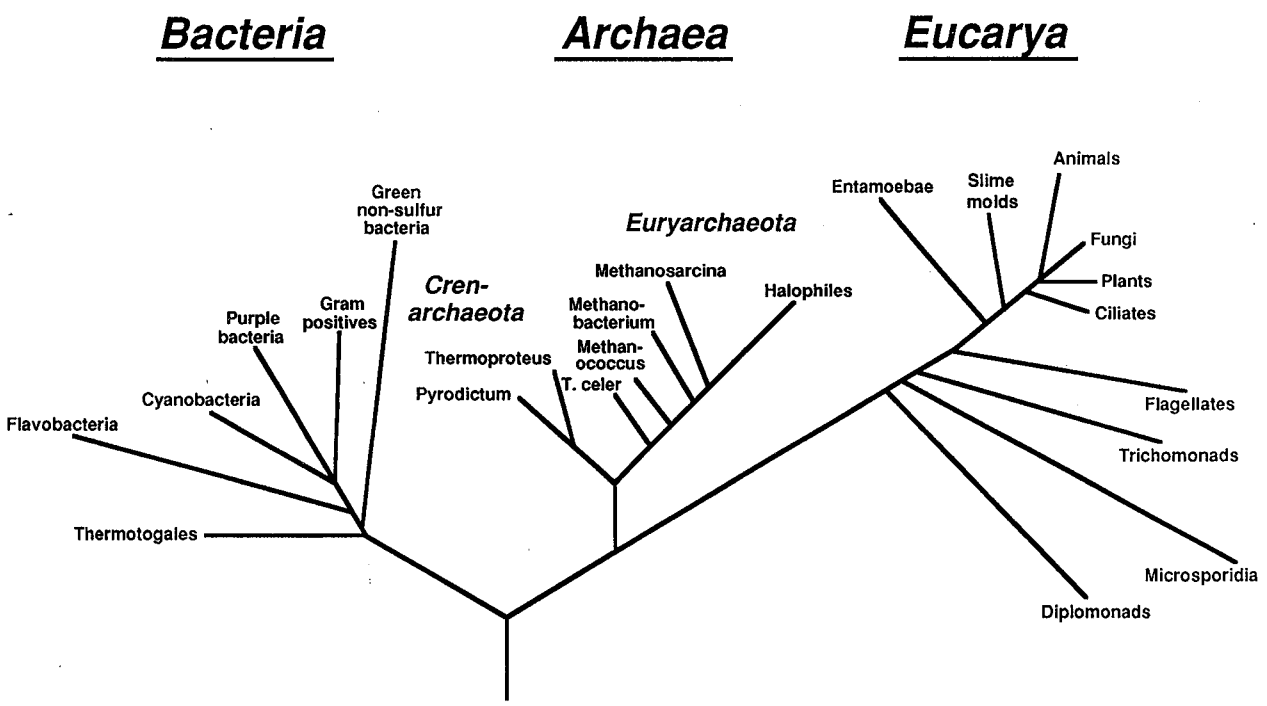

FIGURE 1 A phylogenetic tree based on evaluation of 16S rRNA sequences. The three major lineages of life (*Archaea*, *Bacteria*, and *Eucarya*) are shown. T. celer, *Thermococcus celer*. Reprinted from *The FASEB Journal* (99) with permission.

the course of evolution. Accordingly, the oft-accepted consensus of cellular evolution as outlined in the following paragraphs seems vulnerable to considerable revision as the details of genomic sequences become clearer. Our view of microbial evolution will be clarified by additional database surveys of gene variation and the development of models that describe how gene transfer affects evolving microbial populations (15) and ultimately, with the inclusion of experimental data that test ecologically important selection criteria (45).

In the line leading to the *Bacteria*, an initial bifurcation leads to thermophilic cells now represented by genera such as *Aquifex*, *Fervidobacterium*, and *Thermotoga* (119). It is a matter of curiosity and concern that *Aquifex*, regularly placed at the bottom of the tree, is an aerobic, respiring chemolithotroph. This represents an anomaly, as the planet presumably was devoid of molecular oxygen at the time of this evolutionary event (44, 145).

Most current *Bacteria* trees show that the metabolically versatile, green nonsulfur phototrophs were among the next groups to diverge (119). Perhaps at about the same time a group that possessed cell walls devoid of peptidoglycan emerged: the *Planctomycetales*, including genera such as *Gemmata*, *Isosphaera*, *Pirellula*, and *Planctomyces* (44, 145).

The remaining well-studied and recognized bacterial groupings designated the flavobacteria, cyanobacteria, gram-positive bacteria (of which there appear to be at least two major subgroups), and purple bacteria or proteobacteria (a group itself considered to warrant five subdivisions: alpha, beta, gamma, delta, and epsilon) appear to have emerged later as a single radiation which then underwent divergence.

Even before this refined evidence about likely events in bacterial evolution, it was suggested that thermophilic anaerobes were candidates as the most ancient of living beings. Arguments over whether autotrophs preceded heterotrophs or vice versa abounded but were based more on conjecture than on persuasive evidence. Among the candidates for the earliest of organisms were the methanogens, members of the *Archaea*, appearing later on the evolutionary scene.

Despite microscopic similarities, *Archaea* are as unlike the *Bacteria* as they are unlike the *Eucarya*. Their possession of unusual ether-linked lipids and the lack of peptidoglycan in their cell walls are among their distinguishing characteristics. The group is now divided into the subcategories of methanogens, thermo- and hyperthermophiles, and strict halophiles. The limits of our understanding of the *Archaea* are demonstrated by a series of surprising discoveries of new types of these organisms: in cold, marine environments (26, 127), as contributors to anaerobic ammonia oxidation in the oceans (36, 72), as yet uncultivated anaerobic methane oxidizers (86), as extreme acidophiles that entirely lack cell walls (46), and even as contributors to disease (77).

Although the *Eucarya* are usually cited as characteristically containing a membrane-bound nucleus, they are also usually considered to possess subcellular organelles termed chloroplasts or mitochondria. It may be significant for evolutionary considerations that in the *Eucarya*, three groups at the base of the tree—the diplomonads, the microsporidians, and the trichomonads—are devoid of mitochondria.

One of the many surprises arising from such approaches to the study of phylogeny, and of special interest for considerations of prokaryotic diversity, has been the demonstration that some biochemical and physiological traits appear in quite distantly related organisms. For example, sulfate reduction occurs both in bacteria and in archaea; the same is true for autotrophic assimilation of carbon dioxide as well as the trait of hyperthermophily.

ASPECTS OF BEHAVIORAL DIVERSITY AMONG PROKARYOTES

Direct observation of either mixed microbial populations or pure cultures reveals differences in behavior. Some organisms swim through liquids with ease and rapidity, others move more slowly; some dart about randomly as if in a frenzy, while others move for longer distances and appear to be swimming smoothly; in some, no swimming motility is ever discerned. Typically, motility reflects the presence and function of either a single flagellum or multiple flagella and the arrangement of these on the cell surface(s). Many of the *Bacteria* termed spirochetes swim much better through viscous liquids than they do in typical water samples or laboratory media; this may be a reflection of the position of their flagella (axial filaments, or endoflagella) between the cell wall and an external sheath of the cell. This location clearly does not permit the flagellum to rotate in the extracellular milieu and propel the cell in a manner similar to that of, for example, the well-studied enteric bacteria. Usually the behavior of swimming flagellated bacteria has been described as alternating "runs" and "tumbles." The observations made with the enteric bacteria may be of limited application; however, *Azospirillum* spp. (154), pseudomonads, and rhizobia are among organisms observed not only to behave differently but also to have different intracellular regulatory mechanisms involved.

A group of bacteria with rather diverse physiological properties move in a different manner, not by swimming through liquids but rather by migrating over solid surfaces (e.g., agar, cellulose, chitin, and other cells). This motility, for which locomotor organelles have not yet been visualized and for which the mechanism for motility remains unknown, has often been termed "gliding." The implication of type IV pili in one form of myxobacterial motility and in the motility termed "twitching" (characteristic of some acinetobacters and pseudomonads) is a welcome development in visualization, although the mechanistic basis (e.g., the "motor") for these functions of pili remains unclear. An earlier explanation for the gliding motility of some cyanobacteria, namely, polysaccharide extrusion through a "wall" pore complex, has recently been reassessed and supported. Cells can be seen to move (glide) on a solid surface, and the colony can spread a considerable distance (often more than 1 cm) beyond the point of cell deposition (inoculation). Such colonies usually are characterized as much thinner (very little vertical height) than those typical of enteric bacteria or pseudomonads, for example, growing on an identical nutrient-poor medium. Nutrient concentration usually exerts a significant effect on the ability of cells to glide and hence to form spreading colonies; the ability of a cell to glide is ordinarily not expressed on a nutrient-rich medium such as nutrient agar. Among the *Bacteria* best known for gliding ability are those of genera such as *Beggiatoa*, *Cytophaga*, *Flexibacter*, and *Myxococcus*; however, many others share this capability. And at least one nonlliding cyanobacterium (a *Synechococcus* sp.) swims through liquids without flagella; nor have any locomotor organelles yet been discerned for this organism (109).

It has already been suggested (and previously noted) that cells alter their physiological phenotypic properties; such occurrences are myriad, and alterations in anatomical features and motility phenomena also develop. One such example is the motility of some flagellated *Bacteria* on solid surfaces referred to as "swarming." Certain *Serratia*, *Proteus*, and *Vibrio* species, among others, synthesize additional, specialized flagella as a result of contact with a solid surface, and these additional flagella enable the organism to spread. Surface features, among them the concentration of agar used in solidifying the medium, affect the cell's synthesis of these appendages for motility.

The chemotactic response of cells to concentration gradients of attractants (e.g., nutrients) or repellents by employing temporal sensing mechanisms has been extensively studied as one category of behavioral responses. Although phototrophic eukaryotic cells have long been known to respond by phototaxis to light gradients, no unambiguous instance of this phenomenon has yet been established for prokaryotic phototrophs (116, 122). Studies of the in situ behavior of prokaryotic populations have established phenomena such as diel migration (40) which undoubtedly reflect both chemo- and phototactic (and possibly other types of) behavior of members of these communities (18).

Some cells undergo either cellular or colonial morphogenesis in connection with entrance into a dormant or nongrowing ("resting") state. The formation of the remarkably resistant endospore within the cells of genera such as *Acetonema*, *Bacillus*, *Clostridium*, *Desulfotomaculum*, *Sporomusa*, *Sporosarcina*, and *Thermoactinomyces* generally is regarded as a response to nutrient-poor conditions, although this may not always be the case. Endospore production in *Epulopiscium*-like cells appears to occur at night possibly as a way of ensuring dispersal to new hosts of this intestinal symbiont of surgeonfish (33). Still, starvation seems to be an important precursor for myxospores and microcysts formed by myxobacteria and cytophagas, in which the entire cell shortens, thickens, and becomes spherical (or nearly so). This is probably the explanation for exospore formation by some methanotrophs. Other examples of changes in cell morphology resulting in specialized functions are the formation of heterocysts in some cyanobacteria and the formation of akinetes in others and stalk formation in prosthecate bacteria, as typified by *Caulobacter* spp.

Numerous investigations have verified biofilm development as a critical aspect of microbial behavior (50). Cells that are present in biofilms experience higher proximal cell densities and are subject to higher concentrations of metabolic by-products or metabolites than cells that exist in a planktonic phase (106). Cell-to-cell communication (i.e., quorum sensing) has become a key aspect in the investigation of biofilm development (24) and the term "sociomicrobiology" has been coined to represent the study of any group behavior of microbes (106). The recent discovery of so-called "nanowires" which are conductive pili used by metal-reducing microbes, possibly as an electron shunt (120), represents yet another new area of research that branches from the general field of cell attachment processes.

ENLARGING PERSPECTIVES OF THE DIVERSITY OF HABITATS EXPLOITED BY PROKARYOTES

> Everything is everywhere; but the milieu selects . . . in nature and in the laboratory.
>
> L. G. M. Baas Becking, *Geobiologie ov in eidintot de milieukunde* (9)

Why is it that prokaryotes have been known to occur in so many different habitats and that our comprehension of "new places" in which they are found is increasing?

Certainly, as may be obvious from the degree of physiological diversity outlined above, prokaryotes have the potential to exploit (81) habitats judged to be extreme in comparison with those that support the existence of animals and plants.

If we accept the dictum that prokaryotes are found virtually everywhere (although perhaps not in the totally cosmopolitan scope of the past [82, 132, 133]), then all of the habitats and the microbes therein cannot be enumerated, let alone described, in this chapter. Nonetheless, given the dynamic and exciting state of the study of microbial diversity, selected recent descriptions, reflecting either novel revelations or reconsiderations of earlier findings, are noted briefly below.

As ever-increasing numbers of unique habitats are examined by use of macromolecular sequence, stable-isotope research, and new cultivation strategies, it is ever more evident that prokaryotic diversity has been regularly underestimated by classical isolation and cultivation approaches. It is true not only in seemingly extreme habitats such as hot springs (11, 148), where this was demonstrated early, but also for habitats that are cold (25, 38, 43, 97)—as well as for those that are anoxic (e.g., the rumen [118] and the deep ocean [75]), where we encounter indications of once-unexpected population diversity and microbial impact (76, 150). Estimates derived from microbial inventories and determinations of the habitable space within the Earth's subsurface (21) suggest that among the various places where life can exist, the deep earth is the location of the greatest biomass (149). Within this subterranean realm are newly recognized habitats where cells are apparently sustained by radiolysis of water (79) or fossil hydrocarbons (52), coexist with methane hydrates (59), are able to tolerate radioactive waste (37), or are energetically driven by molecular hydrogen (19, 87, 89, 136). Although the activities of these cells are often deemed to be at or near the threshold required for cell survival (28, 68, 100), observations from some deep habitats suggest that higher levels of activity can occur at considerable sediment depths (ca. hundreds of meters) (105, 124).

The remarkable advancements in molecular characterization strategies notwithstanding, direct observational approaches to habitat analysis continue to play a large role in expanding our comprehension of the impact of prokaryotes in different habitats. The presence of purple sulfur bacteria in the form of macroscopically visible "berries" in salt marsh ponds (129) and the dense accumulation (as "plates") of another phototrophic bacterium in a meromictic saline lake (103) remind us of our limited understanding of the roles that such prokaryotes play in these and other habitats. The discovery of a green sulfur bacterium captured near a deep-sea hydrothermal vent and found to be capable of anoxygenic photosynthesis by capturing light emitted by geothermal radiation expands permissible phototrophic habitats to include those disconnected from solar input (14).

Another example of a transformation long wondered about but only recently demonstrated is that of the oxidation of ammonium under anaerobic respiratory (denitrifying) conditions (88); even more exciting is the indication that the prokaryote involved is a previously unknown member of the *Planctomycetales* (138) and the prospect that new aspects of bacterial physiology may exist in this poorly studied group. It is worth noting that continued microscopic examination played an important part in establishing cause and effect for this microbe. Although no pure cultures of these cells have been sustained, significant advancements have been made in the ability to cultivate and study them (62).

The unaided eye, too, remains a significant part of the exploration of the microbial world. Although dense mats of members of the genus *Thioploca* oxidize sulfide by reducing nitrate (35) near the coasts of Chile and Peru, these organisms were present in amounts far smaller than expected in a similar situation in Namibian shelf sediments. Instead, giant (up to 750 μm in diameter) cells of the previously unknown prokaryote given the name *Thiomargarita namibiensis* (128) were readily visible and extensively studied. Our perception of just how large a prokaryote might be was once again challenged.

A reminder that so many habitats on the planet have very limited molecular oxygen content is made evident by the emerging significance of bacterial respiration of, for example, arsenic (101) and selenium (137) and the evidence that hydrocarbons such as hexadecane can be metabolized via methanogenesis (6).

Studies of *Buchnera* spp., the prokaryotic intracellular symbionts of aphids (12), are but one reminder that not all organisms of environmental significance are free-living and that some may exist in mutualistic states. The phenomenon of bacterial luminescence, so long associated solely with the marine habitat, is associated with terrestrial biotas as well (91). Study of one squid-vibrio interaction (84) makes clear that the microbe-macrobe interaction that may result in a mutualistic (as opposed to parasitic) outcome has effects on the "normal" development of both the microbe and macrobe.

While research has determined that microhabitats are abundant and significant, there is an extreme need to reconcile the multiple impacts of microbes on large-scale Earth processes. And this last aspect of microbial diversity, how microbes from numerous distinctive habitats impact the earth through the cycling of key elements and so have the ability to scale their activities to a global dimension, has yielded some of the most startling revelations in the past few years. The long-predicted and recently verified "anammox" process may contribute 30 to 50% of the N_2 that is generated in the oceans (27). Methanogens, as they contribute to the enormous cache of methane in marine sediments, have been implicated as the governor of the globally important "gas hydrate capacitor" (29). And, of course, microbes also insinuate their way into and may exacerbate human impacts on the environment. To the now familiar explanations of such processes as microbial methylation of mercury or acid mine drainage there are new ways in which we may unknowingly work in concert with these single cells. The excess release of sulfates into the atmosphere and the subsequent deposition into some freshwater locations has caused a change in the terminal electron accepting process from carbon dioxide reduction to sulfate reduction (41). The occurrence of arsenic in groundwater in Bangladesh is now believed to be part of the biogeochemical cycling of the element (113) and an increase in the respiration of microbes in the rhizosphere of trees is thought to offset the capacity of plants to sequester carbon dioxide from the atmosphere (53). The continued systematic probing of microbial diversity and explanation of where microbes live and how they live will lead to a better understanding of the path that we are traveling together (126).

The advent of new molecular technologies heralds a time of seductively immense data sets that catalog the genomes of individuals and groups of microbes. Accordingly, microbial diversity studies that describe the communities from a range of habitats will be profuse. Yet it will be essential to acquire the corresponding environmental data

along with the molecular data to enable the testing of hypotheses related to cells and their capabilities. With complete and accessible data collection, there is a good chance that even complex systems with multiple species may yield information that is comprehensible, leads to more accurate predictions, and may be used by the greater scientific community (67). In this regard, integrated teams of experimentalists and modelers working iteratively to develop a better understanding of the controls on microbial diversity should be a high priority for future research. Some excellent examples already exist (49, 131, 141).

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GENERAL METHODOLOGY

II

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Overview: General Microbiology

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4

If the only tool you have is a hammer, you tend to see every problem as a nail.

Abraham Maslow

A human being should be able to change a diaper, plan an invasion, butcher a hog, conn a ship, design a building, write a sonnet, balance accounts, build a wall, set a bone, comfort the dying, take orders, give orders, cooperate, act alone, solve equations, analyze a new problem, pitch manure, program a computer, cook a tasty meal, fight efficiently, die gallantly. Specialization is for insects.

Robert A. Heinlein (quoted from *Time Enough for Love*)

The last decades of the 20th century saw both a proliferation of new tools for studying microorganisms and an increased awareness of the global importance of microbial activities. The challenge for environmental microbiology in the new millennium is to develop a predictive understanding of microbial communities in order to develop effective strategies for important global issues such as greenhouse gas mitigation, clean water generation, pollution remediation, and soil maintenance (2). Predicting the behavior of complex ecological communities, difficult even when individual elements can be clearly identified such as is the case in plant and animal systems, is further complicated by the large numbers of total individuals and different types of microbes present. A gram of soil, for example, typically contains over 10 billion individual prokaryotic cells belonging to 4,000 different "species" (3) or more, depending on the type of analysis which is used (1). A tendency toward specialization, in terms of both the types of organisms studied and the techniques employed, is natural when faced with this great diversity of organisms and the increasingly broad range of available analytical tools. However, successful solutions to our global challenges in environmental microbiology will require multiple tools and perspectives to link physiological and genetic changes at the individual level to shifts in metabolism and composition of whole communities. The goal of this general methodology section is to foster the development of generalists who will not be limited, or biased, by their knowledge of available techniques.

The following chapters are meant as intellectual portals for different methodologies, leading to more detailed information contained in both the references and subsequent sections within this manual. Rather than specific protocols and complete inventories of methods, the conceptual basis and broad classification of different approaches are emphasized. The overall section organization and brief summaries of specific chapters are presented below.

The placement of the microscopy chapter at the beginning of the section is consistent with its role in the origins of environmental microbiology (see chapter 50). Technological advances in microscope lenses, automated image capturing, computerized analyses, and novel ways to examine samples with minimal disturbance in three dimensions allow for a less biased view of microbial communities. Techniques such as confocal laser scanning microscopy (CLSM) coupled with fluorescence of discrete molecules and/or real-time gene expression allow the modern environmental microbiologist to better infer relationships between the structure of a community and its function. This gap of understanding about which species are in a community and what they are doing, termed the Heisenberg uncertainty principle as it relates to microbial ecology (2), continues to be the bane of diversity studies.

The effort to bridge the chasm of understanding between identification of species and the role that they play in the natural world has historically relied upon the attainment of a pure culture, a single species that can be described from various biochemical, physiological, and molecular approaches. Pure cultures are necessary and an ultimate goal in microbiology; however, it is estimated that 1% or fewer of Earth's microorganisms are currently culturable. This is due to difficulty in reproducing in the laboratory the complex environmental parameters that support species, including interactions between populations as well as chemical growth factors (many that science has yet to discover) produced by other species. A major challenge for environmental microbiology is in understanding the degree of "imperfection" in nature, or, in other words, how much synergism is a necessary element of function within microbial communities. This is alluded to in the two chapters describing cultivation of bacteria and fungi (chapter 6) and algae and protists (chapter 7). Additionally, the role that viruses play in mediating interactions between species needs to be better understood, and their cultivation is discussed in chapter 8.

Wolfaardt et al. (chapter 9) describe the tradition in environmental microbiology of addressing Koch's postulates for ascribing function or effect to a single species and for the

use of enrichment culturing to ultimately isolate guilds performing the same function. However, they rightly suggest that the above two approaches cannot be directly applied to the interpretation of a microbial community in situ, due to the loss of interactions between populations. In the chapter on cultivation of communities, a community culture is described as one that by design keeps the structure and function of a set of interacting populations intact; this allows for the community to be resilient and adaptive, and if measured at the scale of a biofilm, for example, to be directly observed by using tools available through CLSM. One major difference between enrichment and community cultures is that the former are chemically defined at the beginning and change with growth of the populations while the latter are able to be manipulated experimentally throughout the time of culturing.

The limitation of individual-based approaches which led to community cultivation techniques was also the rationale for developing community-level methods for characterizing microbial communities. These methods all bypass the cultivation and isolation approach and instead directly assess community properties on the basis of direct extraction and analysis of unique cell constituents (i.e., cell membrane constituents, nucleic acids) or simultaneous analysis of multiple physiological properties. The multivariate profile of cell constituents or physiological properties is used as a surrogate for species abundance matrices to assess spatial or temporal changes in community state without the bias of isolation. More specific information on the function and structure of the community can be developed from these assays as well, as summarized below.

Phospholipid ester-linked fatty acids (PLFA) analysis (chapter 10) generates information on the physiological state and composition of communities based on the extraction and identification of cell membrane lipids. Overall lipid phosphate provides an estimate of total viable biomass, while distinctive PLFA "biomarkers" have been defined for a variety of different microbial groups and physiological status. Pattern analysis of PLFA markers, even if not directly linked to different bacterial types, can be used to assess dynamics in microbial community structure. Stable isotopic analysis of PLFA signatures can provide information on carbon flow within microbial communities.

Community-level physiological profiling (CLPP) involves rapid assessment of multiple sole carbon sources to characterize and classify heterotrophic microbial communities (chapter 11). The multivariate profile of carbon source use is a proven tool for assessing community dynamics in response to spatial, temporal, or experimental gradients. While this approach originally used microtiter plates from Biolog, Inc., in which respiration of 95 separate sole carbon sources is detected by the reduction of a redox-sensitive dye, studies have shown that selective enrichment in the wells provides a biased, limited view of most microbial communities. More functionally relevant and less biased CLPP approaches involving more direct measurement of respiration (i.e., CO_2 production and O_2 consumption) at lower substrate concentrations and shorter incubation times have been recently developed. These newer techniques hold promise for rapid functional assessments of microbial communities, either for generating estimates of functional richness or for examining the effects of physiochemical factors (e.g., nutrient limitation, pH effects, pollutants) on activity.

Because of the rapid expansion of molecular biology tools since the last edition of this manual, two chapters (chapters 12 and 13) are necessary to discuss the use of DNA- and RNA-based methods for assessing community structure and phylogeny. It is possible to select from a host of techniques to examine species richness, evenness, and gene diversity and expression (chapter 12) as well as more recent phylogenetic approaches for determining the relatedness of species using 16S rDNA and other gene sequences and whole-genome approaches (chapter 13). In the former chapter, approaches for obtaining high-quality nucleic acids from environmental matrices are discussed, as well as methods for community fingerprinting and techniques for determining functions of species by DNA and RNA probing. Discrimination between spatial and temporal factors affecting communities is highlighted, and deduction of ecological functioning by determining phylogeny is discussed. In the latter chapter, phylogenetic approaches to hypothesize relatedness among species are introduced. The use of genes other than 16S rDNA is expanded upon, as well as the role of secondary structure in RNA-based phylogenetic analyses. Furthermore, the use of amino acid sequences for comparison of some species groups and whole-genome approaches for others are outlined.

Advancement in molecular techniques has also enabled the manipulation of the genomes of specific organisms to provide an assayable response to environmental stimuli. The resulting bioreporters, and their fusion to miniature electronics to form biosensors, are discussed in chapter 14. An increasing number of genetically engineered organisms containing either luminescent (i.e., *lux* cassette) or fluorescent (i.e., green fluorescent protein) genes have been produced. The light response is a sensitive measure of the presence and distribution of labeled organisms, or, if the genes are fused to specific promoter regions of interest, specific microbial activities which can be of interest in themselves or as indicators of the presence of certain substrates. These approaches offer increasing promise for in situ assessment of activity, especially when linked to the advanced imaging techniques described in chapter 5 or optics-based microprobes.

The remaining three chapters of the section move away from specific methods, addressing broader issues related to the effective implementation and analysis of research tools. Chapter 15 addresses the incorporation of microbiology into the long-term ecological research at an increasing number of dedicated study sites. Long-term research, necessary to understand a range of ecosystem dynamics (e.g., natural changes, rare events, disturbances), involves manipulative and observational approaches at habitat types spanning desert and aquatic sites from polar to tropical regions. In addition to the more traditional incorporation of microbial process measurements considered important to ecosystem processes, recent application of advanced techniques for evaluating community composition provides the potential for developing predictive links between microbial community composition, microbial activities, and ecosystem processes.

Integrating modern microbial ecology into the long-term research sites has synergistic benefits beyond the application of new tools; the strong focus on research themes and ecological questions of global importance promotes the scientific rigor and relevance of microbial ecology research. While the emerging set of analytical approaches provides an improved tool set for assessing microbial community dynamics, the methods are too often applied without critical examination of the underlying theory. For example, the dogma that diversity is beneficial is largely untested, yet is a common goal of studies employing the community-level

methods defined above. Linking microbial ecology to the ecological community through the long-term study sites will help strengthen the linkage between microbiologists and the leading edge of current ecological theory.

The last two chapters address issues related to the reliability of data generated through research activities, either from the perspective of the confidence in the processes by which the data are collected (i.e., quality assurance [chapter 16]) or in the conclusions drawn from the data (i.e., statistical analysis [chapter 17]). Given the expense, in both equipment and manpower, required for many of the techniques outlined in this section, researchers need to diligently protect the quality of resulting data.

Chapter 16 emphasizes the general approaches necessary for environmental microbiology laboratories to provide a product or service (i.e., data) that meets defined standards of quality. These approaches focus on both the general system by which the laboratory operates (i.e., quality assurance) and the specific measurement processes (i.e., quality control). Quality assurance elements, including management requirements, training, and assessment, are addressed in relation to both large and small laboratories. A variety of quality control methods are discussed, with emphasis on the importance of the investigator in planning and selecting appropriate approaches.

The final chapter provides an overview of the challenging task of effectively linking conceptual models of the system under study to the appropriate statistical models used for measurement and testing. Careful integration of research objectives with the appropriate study design, sampling approaches, and statistical tests is stressed. The underlying theory of descriptive, associative, and comparative and confirmative approaches is discussed with specific reference to examples related to measurement of microbial community structure, abundance, and activity. The environmental microbiologist is encouraged to view the use of statistical tools with the same rigor as the application of the other research methods discussed in this section.

Pasteur is credited with saying that the role of the infinitely small in nature (microorganisms) is infinitely large. Our ability to see microbial cells, identify them, detect their activities, and link them to communities and processes on the visible scale is increasingly limited by our creativity and not our technology. The following section should serve as an overall guide to these technological advances in environmental microbiology.

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