# 19

# Taking the Measure of Microbial Systems

## microbiologynow

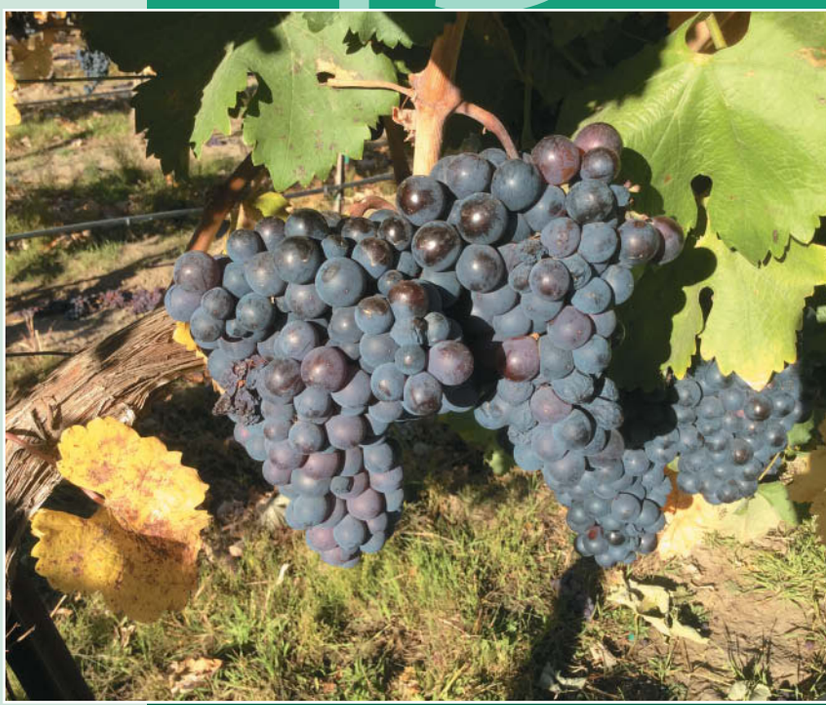
### The Vineyard Microbiome Revealed by Next-Generation Sequencing Technology

It has been recognized for millennia that the quality of wine produced from the same variety of grape can vary dramatically depending on where the grapevines are grown. The French refer to the influence of location on wine characteristics as *terroir* (from the French *terre*, meaning "land"). The influence of locale on wine quality is the primary basis for defining the appellation boundaries for vineyards within major growing regions. The Burgundy region in France, for example, has dozens of such appellations. It has long been considered that *terroir* is a function of climate and the physical and chemical characteristics of the local soil. However, a recent study using next-generation sequencing of 16S rRNA genes to determine the diversity and relative abundance of different microorganisms on grape plants and in soils from major wine-growing areas in the United States and France has revealed that *terroir* may result from a combination of microbial and abiotic factors.

Distinct microbial communities are imprinted on the grapes from different winegrowing regions, even for the same grape variety. Next-generation sequencing technology can unravel these microbial communities and define their species composition and abundance. Modern sequencing efforts have shown that the soil microbial community can vary significantly between appellations and that soil is the major source of the microbiota found on grapevine leaves and fruit. This suggests that *terroir* is influenced not only by soil physics and chemistry and the prevailing climate, but also by the local soil microbial community. Apart from a direct contribution soil microbes might make in wine fermentation by influencing the flavor, color, and quality of the final product, differences in the soil microbial community may also affect the physiology of the plant, for example, by controlling the production of specific plant compounds that alter grape chemistry.

These initial applications of next-generation sequencing to the study of *terroir* highlight the growing importance of modern microbial ecology in agriculture. As our understanding of plant–soil–microbial interactions develops, a soil microbial census could well become a valuable tool for improving soil quality and crop performance by ensuring that each crop plant has the best possible microbial associates.

**Source:** Zarraonaindia, I., et al. 2015. The soil microbiome influences grapevine-associated microbiota. *MBio:* 6 e02527-14.



We now begin a new unit devoted to microorganisms in their natural habitats. We learned in Chapter 1 that *microbial communities* consist of cell populations living in association with other populations in nature. The science of **microbial ecology** is focused on how microbial populations assemble to form communities and how these communities interact with each other and their environments.

The major components of microbial ecology are *biodiversity* and *microbial activity*. To study biodiversity, microbial ecologists must identify and quantify microorganisms in their habitats. Knowing how to do this is often helpful for isolating organisms of interest as well, which is another goal of microbial ecology. To study microbial activity, microbial ecologists must measure the metabolic processes that microorganisms carry out in their habitats. In this chapter we consider modern methods for assessing microbial diversity and activity. Chapter 20 will outline the basic principles of microbial ecology and examine the types of environments that microorganisms inhabit. Chapters 21–24 will complete our coverage of microbial ecology by exploring nutrient cycles, applied microbiology, and the role microbes play in symbiotic associations with other life forms, including humans.

We begin with the microbial ecologist's toolbox, which includes a collection of powerful tools for dissecting the structure and function of microbial communities in relation to their natural habitats.

# I • Culture-Dependent Analyses of Microbial Communities

The vast majority of microorganisms, more than 99% of all species by most estimates, have never been grown in laboratory cultures. Recognition of this fact, based on molecular diversity surveys (Sections 19.4–19.8) of various microbial habitats, has stimulated the development of new methods for isolating microbes from nature in order to establish pure cultures. Even though a host of sophisticated methods are available for studying microbes in their native environments, culturing a microorganism remains the only way to fully characterize its properties and predict its impact on its environment.

In the first part of this chapter we cover the enrichment approach, a time-honored and useful method for isolating microorganisms from nature but one with limitations. Enrichment is based on culturing in a selective growth medium, and thus the tools and methods used in this approach are considered *culture-dependent* analyses. As we will see, considerable progress has been made in culturing the more elusive microorganisms in natural populations by using robotics and associated microfabrication technology to establish large numbers of enrichment cultures that can be monitored simultaneously. In the second and third parts of this chapter we consider *culture-independent* analyses, techniques that can tell us much about the structure and function of microbial communities in the absence of actual laboratory cultures. In the final part of this chapter, we consider methods for measuring microbial activities in nature and linking them to specific organisms. Collectively, these methods allow the microbial ecologist to ask both "who's there" and "what are they doing."

## 19.1 Enrichment Culture Microbiology

For an **enrichment culture**, a medium and a set of incubation conditions are established that are *selective* for the desired organism and *counterselective* for undesired organisms. Effective enrichment cultures duplicate as closely as possible the resources and conditions of a particular ecological niche. Hundreds of different enrichment strategies have been devised, and Tables 19.1 and 19.2 summarize some simple and direct ones.

### Inocula

Successful enrichment requires an appropriate inoculum containing the organism of interest. Thus, the making of an enrichment culture begins with collecting a sample from the appropriate habitat to serve as the inoculum (Tables 19.1 and 19.2). Enrichment cultures are established by placing the inoculum into selective media and incubating under specific conditions. In this way, many common microbes can be isolated. For example, the great Dutch microbiologist Martinus Beijerinck, who conceptualized the enrichment culture technique (↩ Section 1.11), used enrichment cultures to isolate the nitrogen-fixing bacterium *Azotobacter* (Figure 19.1). Because *Azotobacter* is a rapidly growing bacterium capable of $N_2$ fixation in air (↩ Sections 14.6 and 15.12), enrichment using media devoid of fixed nitrogen, such as ammonia or nitrate, and

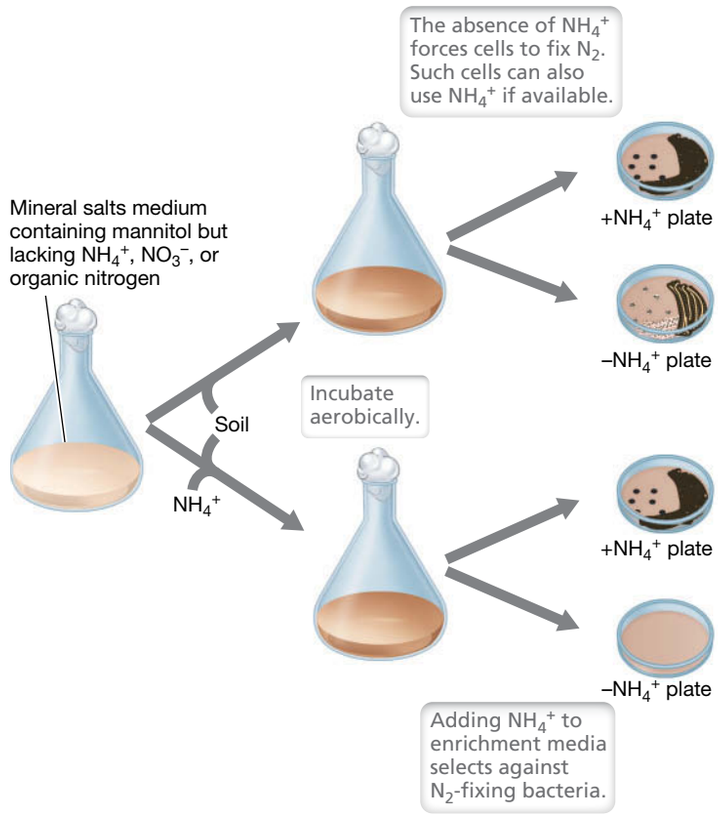



**Figure 19.1 The isolation of *Azotobacter*.** Selection for aerobic nitrogen-fixing bacteria usually results in the isolation of *Azotobacter* or its relatives. The selective basis of the enrichment is the absence of fixed nitrogen ($NH_4^+$ in this case) in the culture medium in the upper flask. Thus the medium *selects* from the microbial community those species that can fix $N_2$ aerobically, of which *Azotobacter* is one of the most rapidly growing. See Section 1.11 and Figure 1.33 for more on the historical importance of *Azotobacter*.

**TABLE 19.1 Some enrichment culture methods for phototrophic and chemolithotrophic bacteria**

| Light-phototrophic bacteria: main C source, $CO_2$ | | |
|---|---|---|
| ***Incubation condition*** | ***Organisms enriched*** | ***Inoculum*** |
| ***Incubation in air*** | | |
| $N_2$ as nitrogen source | Cyanobacteria | Pond or lake water; sulfide-rich muds; stagnant water; raw sewage; moist, decomposing leaf litter; moist soil exposed to light |
| $NO_3^-$ as nitrogen source, 55°C | Thermophilic cyanobacteria | Hot spring microbial mat |
| ***Anoxic incubation*** | | |
| $H_2$ or organic acids; $N_2$ as sole nitrogen source | Purple nonsulfur bacteria, heliobacteria | Same as above plus hypolimnetic lake water (↔ Section 20.8); pasteurized soil (heliobacteria); microbial mats for thermophilic species |
| $H_2S$ as electron donor | Purple and green sulfur bacteria | |
| $Fe^{2+}$, $NO_2^-$ as electron donor | Purple bacteria | |

| Dark-chemolithotrophic bacteria: main C source, $CO_2$ (medium must lack organic C) | | | |
|---|---|---|---|
| ***Electron donor*** | ***Electron acceptor*** | ***Organisms enriched*** | ***Inoculum*** |
| ***Incubation in air: aerobic respiration*** | | | |
| $NH_4^+$ | $O_2$ | Ammonia-oxidizing *Bacteria* (*Nitrosomonas*) or *Archaea* (*Nitrosopumilus*) | Soil, mud, sewage effluent, seawater |
| $NO_2^-$ | $O_2$ | Nitrite-oxidizing bacteria (*Nitrobacter, Nitrospira*) | |
| $H_2$ | $O_2$ | Hydrogen bacteria (various genera) | |
| $H_2S$, $S^0$, $S_2O_3^{2-}$ | $O_2$ | *Thiobacillus* spp. | |
| $Fe^{2+}$, low pH | $O_2$ | *Acidithiobacillus ferrooxidans* | |
| ***Anoxic incubation*** | | | |
| $S^0$, $S_2O_3^{2-}$ | $NO_3^-$ | *Thiobacillus denitrificans* | Mud, lake sediments, soil |
| $H_2$ | $NO_3^-$ | *Paracoccus denitrificans* | |
| $Fe^{2+}$, neutral pH | $NO_3^-$ | *Acidovorax* and various other gram-negative autotrophic bacteria | |

incubation in air selects strongly for this bacterium and its close relatives. Non-nitrogen-fixing bacteria and anaerobic nitrogen-fixing bacteria are counterselected in this technique.

## Enrichment Culture Outcomes

For success with enrichment cultures, attention to both the culture medium and the incubation conditions is important. That is, the *resources* (nutrients) and *conditions* (temperature, pH, osmotic considerations, aerobic or anaerobic, and the like) must closely mimic those of the habitat to offer the best chance of obtaining the organism of interest (↔ Table 20.1).

Some enrichment cultures yield nothing. This may be because the organism capable of growing under the enrichment conditions specified is absent from the habitat. Alternatively, even though the organism of interest exists in the habitat sampled, the resources and conditions employed in the enrichment may simply be incompatible with its growth. Thus enrichment cultures can yield a firm *positive* conclusion (that is, that an organism with certain capacities exists in a particular environment because it was enriched) but never a firm *negative* conclusion (that such an organism is not present because the enrichment failed). Moreover, the isolation of the desired organism from an enrichment culture says nothing about the ecological importance or abundance of the organism in its habitat. A positive enrichment proves only that the organism was present in the sample, and in practice, this can result from as few as a single viable cell.

## The Winogradsky Column

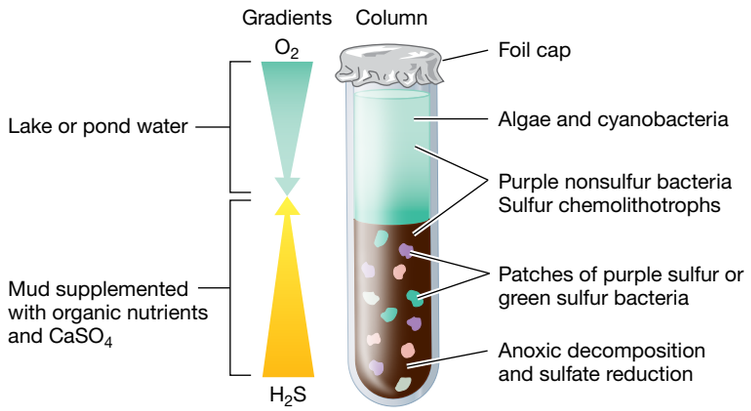
The **Winogradsky column** is an artificial microbial ecosystem and a long-term source of various bacteria for enrichment cultures. Winogradsky columns have been used to isolate phototrophic purple and green bacteria, sulfate-reducing bacteria, and many other anaerobes. Named for the famous Russian microbiologist Sergei Winogradsky (↔ Section 1.11), the column was first used by Winogradsky in the late nineteenth century in his classic studies of soil microorganisms.

A Winogradsky column is prepared by filling a glass cylinder about half full with organically rich, preferably sulfidic mud into which carbon substrates have been mixed. The substrates determine which organisms are enriched. Fermentative substrates, such as glucose, that can lead to acidic conditions and excessive gas formation (which can create gas pockets that disrupt the enrichment and let in air) are avoided. The mud is supplemented with small amounts of calcium carbonate ($CaCO_3$) as a buffer and gypsum ($CaSO_4$) as a source of sulfate. The mud is packed tightly in the cylinder, taking care to avoid trapping air, and then covered with lake, pond, or ditch water (or seawater if it is a marine column). The top of the cylinder is covered to prevent evaporation, and the container is placed near a window that receives diffuse sunlight for a period of months.

**TABLE 19.2 Some enrichment culture methods for chemoorganotrophic and strictly anaerobic bacteria[a]**

| *Electron donor (and nitrogen source)* | *Electron acceptor* | *Typical organisms enriched* | *Inoculum* |
|---|---|---|---|
| ***Incubation in air: aerobic respiration*** | | | |
| Lactate + $NH_4^+$ | $O_2$ | *Pseudomonas fluorescens* | Soil, mud; lake sediments; decaying vegetation; pasteurize inoculum (80°C for 15 min) for all *Bacillus* enrichments |
| Benzoate + $NH_4^+$ | $O_2$ | *Pseudomonas fluorescens* | |
| Starch + $NH_4^+$ | $O_2$ | *Bacillus polymyxa,* other *Bacillus* spp. | |
| Ethanol (4%) + 1% yeast extract, pH 6.0 | $O_2$ | *Acetobacter, Gluconobacter* | |
| Urea (5%) + 1% yeast extract | $O_2$ | *Sporosarcina ureae* | |
| Hydrocarbons (e.g., mineral oil, gasoline, toluene) + $NH_4^+$ | $O_2$ | *Mycobacterium, Nocardia, Pseudomonas* | |
| Cellulose + $NH_4^+$ | $O_2$ | *Cytophaga, Sporocytophaga* | |
| Mannitol or benzoate, $N_2$ as N source | $O_2$ | *Azotobacter* | |
| $CH_4$ + $NO_3^-$ | $O_2$ | *Methylobacter, Methylomicrobium* | Lake sediments, thermocline (Section 20.8) of stratified lake |
| ***Anoxic incubation: anaerobic respiration*** | | | |
| Organic acids | $NO_3^-$ | *Pseudomonas* (denitrifying species) | Soil, mud; lake sediments |
| Yeast extract | $NO_3^-$ | *Bacillus* (denitrifying species) | |
| Organic acids | $SO_4^{2-}$ | *Desulfovibrio, Desulfotomaculum* | |
| Acetate, propionate, butyrate | $SO_4^{2-}$ | Fatty acid-oxidizing sulfate reducers | As above; or sewage digester sludge; rumen contents; marine sediments |
| Acetate, ethanol | $S^0$ | *Desulfuromonas* | |
| Acetate | $Fe^{3+}$ | *Geobacter, Geospirillum* | |
| Acetate | $ClO_3^-$ | Various chlorate-reducing bacteria | |
| $H_2$ | $CO_2$ | Methanogens (chemolithotrophic species only), homoacetogens | Mud, sediments, sewage sludge |
| $CH_3OH$ | $CO_2$ | *Methanosarcina barkeri* | |
| $CH_3NH_2$ or $CH_3OH$ | $NO_3^-$ | *Hyphomicrobium* | |
| Hydrocarbons | $SO_4^{2-}$ or $NO_3^-$ | Anoxic hydrocarbon-degrading bacteria | Freshwater or marine sediments |
| Acetate + $H_2$ + $NH_4^+$ | Tetrachloroethene (PCE) | *Dehalococcoides* spp. | PCE-polluted groundwater |
| ***Anoxic incubation: fermentation*** | | | |
| Glutamate or histidine | No exogenous electron acceptors added | *Clostridium tetanomorphum* or other proteolytic *Clostridium* species | Mud, lake sediments; rotting plant or animal material; dairy products (lactic and propionic acid bacteria); rumen or intestinal contents (enteric bacteria); sewage sludge; soil; pasteurize inoculum for *Clostridium* enrichments |
| Starch + $NH_4^+$ | None | *Clostridium* spp. | |
| Starch + $N_2$ as N source | None | *Clostridium pasteurianum* | |
| Lactate + yeast extract | None | *Veillonella* spp. | |
| Glucose or lactose + $NH_4^+$ | None | *Escherichia, Enterobacter,* other fermentative organisms | |
| Glucose + yeast extract (pH 5) | None | Lactic acid bacteria (*Lactobacillus*) | |
| Lactate + yeast extract | None | Propionic acid bacteria | |
| Succinate + NaCl | None | *Propionigenium* | |
| Oxalate | None | *Oxalobacter* | |
| Acetylene | None | *Pelobacter* and other acetylene fermenters | |

[a]All media must contain an assortment of mineral salts including N, P, S, $Mg^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Ca^{2+}$, and other trace elements (Sections 3.1–3.2). Certain organisms may have requirements for vitamins or other growth factors. This table is meant as an overview of enrichment methods and does not speak to the effect incubation temperature might have in isolating thermophilic (high temperature), hyperthermophilic (very high temperature), and psychrophilic (low temperature) species, or the effect that extremes of pH or salinity might have, assuming an appropriate inoculum was available. Some enrichment substrates are naturally more specific than others. For example, glucose is quite nonspecific as an enrichment substrate compared with benzoate or methanol.

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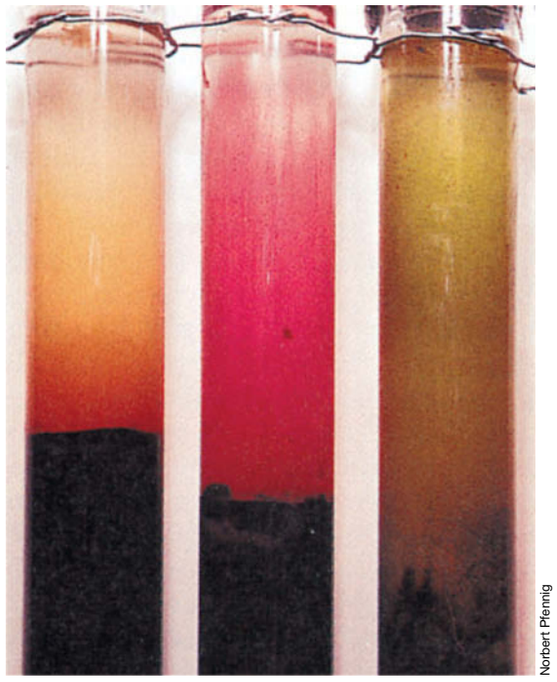


Norbert Pfennig

*(b)*

**Figure 19.2 The Winogradsky column.** *(a)* Schematic view of a typical column used to enrich phototrophic bacteria. The column is incubated in a location that receives subdued sunlight. Anoxic decomposition leading to $SO_4^{2-}$ reduction creates the gradient of $H_2S$. *(b)* Photo of Winogradsky columns that have remained anoxic up to the top; each column had a bloom of a different phototrophic bacterium. Left to right: *Thiospirillum jenense, Chromatium okenii*, both of which are purple sulfur bacteria, and *Chlorobium limicola* (green sulfur bacterium).

In a typical Winogradsky column, a diverse community of microbes develops (**Figure 19.2*a***). Algae and cyanobacteria develop quickly in the upper portions of the water column; by producing $O_2$ these organisms help to keep this zone of the column oxic much as they do in the upper zones of a lake. Fermentative processes in the mud lead to the production of organic acids, alcohols, and $H_2$, suitable substrates for sulfate-reducing bacteria (Section 14.14). Hydrogen sulfide ($H_2S$) from the sulfate reducers triggers the development of purple and green sulfur bacteria (anoxygenic phototrophs, Sections 14.3 and 15.4–15.8) that use sulfide as a photosynthetic electron donor. These organisms typically grow in patches in the mud on the sides of the column but may bloom in the water itself if oxygenic phototrophs are scarce (Figure 19.2*b*). The pigmented cells of the anoxygenic phototrophs can be sampled with a pipette for microscopy, isolation, and characterization (Table 19.1).

Winogradsky columns have been used to enrich both aerobic and anaerobic *Bacteria* and *Archaea*. Besides supplying a ready source of inocula for enrichment cultures, columns can also be supplemented with a specific compound to enrich an organism in the inoculum that can degrade it. Once a crude enrichment has been established in the column, culture media can be inoculated for the isolation of pure cultures, as discussed in Section 19.2.

## Enrichment Bias

Although the enrichment culture technique is quite useful and still widely practiced, there exists a bias, and sometimes a very severe bias, in the outcome of enrichments. This bias is typically most profound in liquid enrichment cultures where the most rapidly growing organism(s) for the chosen set of conditions dominate. However, using molecular techniques to be described later, we now know that the most rapidly growing organisms in laboratory cultures are often only minor components of the microbial community rather than the most abundant and ecologically relevant organisms carrying out the process of interest. This could be for several reasons including the fact that the levels of resources available in laboratory cultures are typically much higher than those in nature, and the conditions in the natural habitat, including both the types and proportions of different organisms present as well as the physical and chemical conditions, are nearly impossible to reproduce and maintain for long periods in laboratory cultures.

This problem of **enrichment bias** can be demonstrated by comparing the results obtained in dilution cultures (Section 19.2) with classical liquid enrichment. Dilution of an inoculum followed by liquid enrichment or plating often yields different organisms than liquid enrichments established with the same but undiluted inocula. It is thought that dilution of the inoculum eliminates quantitatively insignificant but rapidly growing "weed" species, allowing development of organisms that are more abundant in the community but slower growing. Dilution of the inoculum is thus a common practice in enrichment culture microbiology today. As discussed below, the problem of overgrowth by "weed" species can also be circumvented by physical isolation of the desired organism before introducing it into a growth medium. This can be accomplished by dilution and a variety of classical isolation procedures that we turn to in the next section. However,

more recently, sophisticated methods have been developed to physically isolate single cells of interest (or a single type of cells) and place them in a growth medium that is free of undesired cells. We consider these techniques in Section 19.3.

**MINIQUIZ**

- Describe the enrichment strategy behind Beijerinck's isolation of *Azotobacter*.
- Why is sulfate ($SO_4^{2-}$) added to a Winogradsky column?
- What is enrichment bias? How does dilution reduce enrichment bias?

## 19.2 Classical Procedures for Isolating Microbes

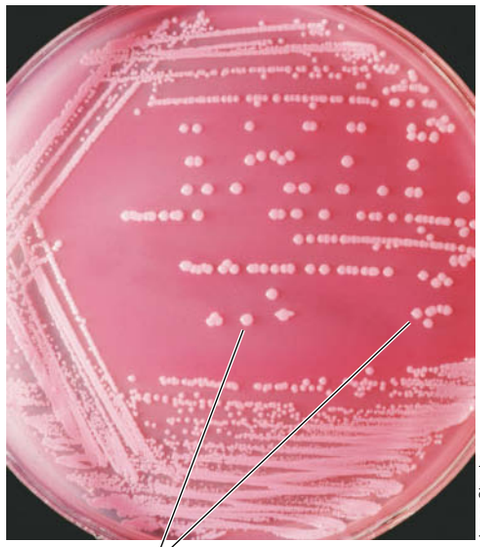
Once a positive enrichment culture has been obtained, the next step is typically to attempt to get the enriched organism in *pure culture*—one containing a single kind of microorganism. Pure cultures are valuable because genomes can be quickly dissected and experiments done under controlled laboratory conditions to clearly define the physiology of the isolate. Pure cultures have been studied since the days of Robert Koch (Section 1.10) and we considered some of these methods earlier (Section 5.5).

### Agar Dilution Tubes and the Most-Probable-Number Technique

Common isolation procedures include the streak plate, agar dilution, and liquid dilution. For organisms that form colonies on agar plates, the streak plate is quick, easy, and the method of choice (**Figure 19.3**); if a well-isolated colony is selected and restreaked several successive times, a pure culture is usually obtained. With proper incubation facilities (for example, anoxic jars or anoxic chambers for anaerobes, Section 5.14), it is possible to purify both aerobes and anaerobes on agar plates by the streak plate method.

In the agar dilution tube method, a mixed culture is diluted in tubes of molten agar medium, resulting in colonies embedded in the agar. This method is useful for purifying anaerobic organisms such as phototrophic sulfur bacteria and sulfate-reducing bacteria from samples taken from Winogradsky columns or other sources. A culture is purified by successive dilutions of cell suspensions in tubes of molten agar medium (Figure 19.3, Figure 15.23*g*). Repeating this procedure using a colony from the highest-dilution tube as inoculum for a new set of dilutions usually yields pure cultures. A related procedure called the *roll tube method* uses tubes containing a thin layer of agar on their inner surface. The agar can then be streaked for isolated colonies. Because the tubes can be flushed with an oxygen-free gas during streaking, the roll tube method is primarily used for the isolation of anaerobic microbes.

Another purification procedure is the serial dilution of an inoculum in a liquid medium until the final tube in the series shows no growth. When a 10-fold serial dilution is used, for example, the last tube showing growth should have originated from ten or fewer cells. Besides being a method for obtaining pure cultures, serial dilution techniques are widely used to estimate viable cell numbers in the **most-probable-number (MPN) technique**

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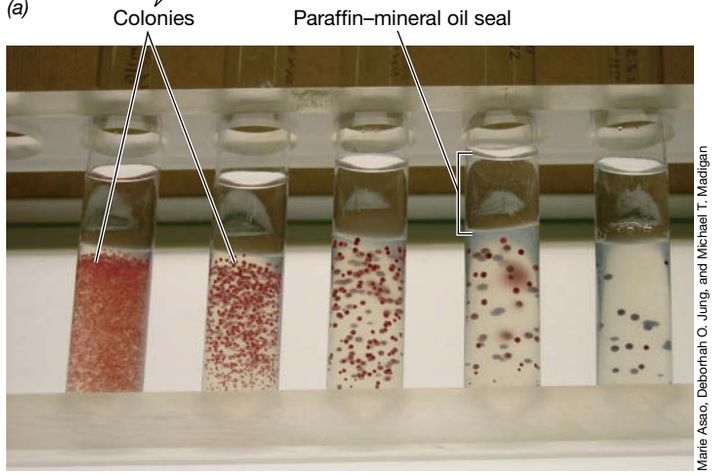



(b)

**Figure 19.3 Pure culture methods.** *(a)* Organisms that form distinct colonies on plates are usually easy to purify. *(b)* Colonies of phototrophic purple bacteria in agar dilution tubes; the molten agar was cooled to approximately 45°C before inoculation. A dilution series was established from left to right, eventually yielding well-isolated colonies. The tubes were sealed with a 1:1 mixture of sterile paraffin and mineral oil to maintain anaerobiosis.

(**Figure 19.4**). MPN methods have been used for estimating the numbers of microorganisms in foods, wastewater, and other samples in which cell numbers need to be assessed routinely. An MPN count of a natural sample can be done using highly selective media and incubation conditions to target one or a small group of organisms or a particular pathogen. Alternatively, a count can be done using complex media to get a general estimate of viable cell numbers (but see Section 5.7 for a caveat that applies to such estimates). Use of several replicate tubes at each dilution improves accuracy of the final MPN obtained.

### Criteria for Culture Purity

Regardless of the methods used to purify a culture, once a putative pure culture has been obtained, it is essential to verify its purity. This is typically done through a combination of (1) microscopy, (2) observation of colony characteristics on plates or in dilution

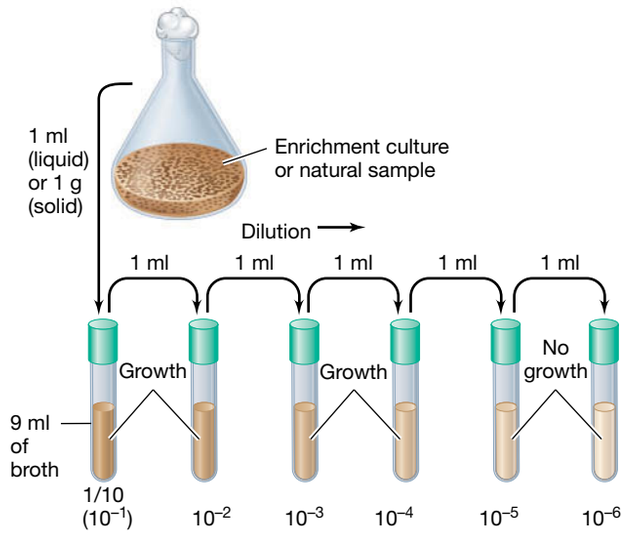



**Figure 19.4 Procedure for a most-probable-number (MPN) analysis.** Growth in the $10^{-4}$ but not the $10^{-5}$ dilution means that cell numbers were at least $10^4$ cells/ml in the sample used for inoculation. Because particle-attached microorganisms can skew numbers significantly, gentle methods to disassociate microorganisms from particles are often used prior to dilution. In addition, each dilution tube is mixed thoroughly before removing a sample for the next dilution.

tubes, and (3) tests of the culture for growth in other media. In the latter, it is important to test the culture for growth in media and under growth conditions in which the desired organism is predicted to grow poorly or not at all but in which contaminants will grow vigorously. In the final analysis, the microscopic observation of a single morphological type of cell that displays uniform staining characteristics (for example, in a Gram stain) coupled with uniform colony characteristics and the absence of contamination in growth tests with various culture media is strong evidence that a culture is a pure (*axenic*) culture.

Certain molecular methods described in this chapter for characterizing natural microbial communities can also be applied to the verification of culture purity. However, these techniques are complementary and do not substitute for the more fundamental observations of culture characteristics and cellular morphology.

**MINIQUIZ**

- What is a pure culture and why is obtaining one useful in microbial ecology?
- How does the agar dilution method differ from streaking to obtain isolated colonies?

## 19.3 Selective Single-Cell Isolation: Laser Tweezers, Flow Cytometry, Microfluidics, and High-Throughput Methods

The problem of enrichment bias has fueled the development of new methods for culturing microbes from nature. These advancements have emerged from the understanding that every microbe has a *fundamental niche* and a *realized niche*. The **fundamental niche** refers to the range of environments in which a species will be sustained when it is not resource-limited, such as may result from competition with other species. By contrast, the **realized niche** refers to the range of natural environments supporting a species when it is confronted with factors such as resource limitation, predation, and competition from other species.

Establishing laboratory conditions that fall within the fundamental niche may be sufficient to support an organism once it is in pure culture but may fail to selectively enrich the same organism from a natural sample. Because the realized niche of most microorganisms is unknown, there has been an increasing emphasis on developing methods that physically isolate single cells into separate compartments free from competition with other microbes. These include both manual and robotic methods that function to sort individual cells from an environmental sample, and we consider these methods now.

### Laser Tweezers and Flow Cytometry

**Laser tweezers** consist of an inverted light microscope equipped with a strongly focused infrared laser and a micromanipulation device. Trapping a single cell is possible because the laser beam creates a force that pushes down on a microbial cell (or other small object) and holds it in place (**Figure 19.5***a*). Then when the laser beam is moved, the trapped cell moves along with it. If a mixed sample is in a capillary tube, a single cell can be optically trapped

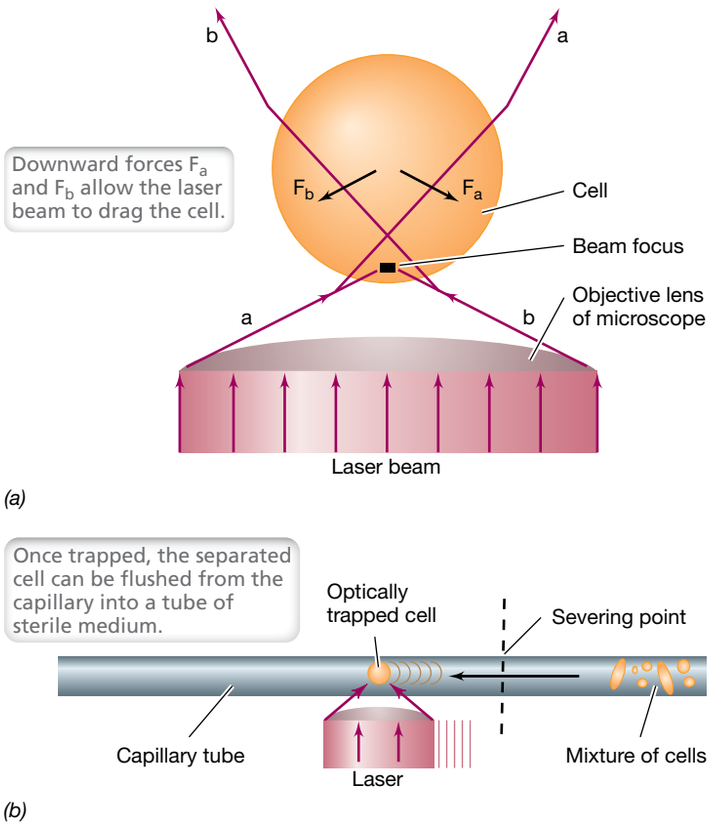



**Figure 19.5 The laser tweezers for the isolation of single cells.** *(a)* Mechanism by which individual cells can be isolated. *(b)* Once a cell has been isolated in a capillary tube, it can be tested for subsequent growth in pure culture.

and moved away from contaminating organisms (Figure 19.5*b*). The cell can then be isolated by breaking the tube at a point between the cell and the contaminants and flushing the cell into a small tube of sterile medium. Laser tweezers, when coupled with staining techniques that identify particular organisms (Sections 19.4 and 19.5), can be used to select organisms of interest from a mixture for purification and further laboratory study.

**Flow cytometry** is a technique for counting and examining a mixture of cells by suspending them in a stream of fluid and passing them through an electronic detector that sorts them according to defined criteria; for example, by cell size, shape, or fluorescent properties. This ability makes cell sorting useful not only for isolating single cells but also for enriching a particular cell type from a mixture. Cell sorters can deposit individual cells into wells of a microtiter plate where each well contains the same growth medium or a slightly different growth medium. Because the growth requirements of some organisms include organic compounds and metabolites produced by other organisms that share their environment, addition of filter-sterilized source water (for aquatic organisms) or soil water extract (for soil organisms) can be used to supplement the media tested. Each well in the microtiter plate can then be monitored for growth or some other property either manually or using robotic methods (high-throughput culture, see next subsection). We explore the mechanism and uses of flow cytometry in more detail in Section 19.12 (see Figures 19.36 and 19.37).

## High-Throughput Culture and Microfluidic Devices

Continuing innovations in single-cell isolation methodology have spawned **high-throughput culturing methods** and related methods for use on an even smaller scale. High-throughput methods require dilution (or cell sorting) of a sample to yield a single cell in each well of a microtiter plate (**Figure 19.6**). From there, each well is robotically monitored over time for cell growth or a specific target gene. High-throughput methods allow the experimenter to test many alternative growth conditions simultaneously in an attempt to replicate the realized niche or, alternatively, to allow the organism to occupy its fundamental niche by relieving it from competition. Microtiter wells that are positive for growth or a target gene of interest identify the acceptable resources and conditions for growth of a particular microbe and supply valuable clues for the design of laboratory culture media to obtain its growth in pure culture.

High-throughput cultivation has shown increasing success in isolating unique bacteria. For example, high-throughput methods were used for the isolation of one of the most abundant bacteria on Earth, the small marine planktonic bacterium *Pelagibacter ubique* (Figure 19.6). This bacterium thrives on the very dilute pool of dissolved organic matter present in the open oceans and eluded classical enrichment methods for years. But with high-throughput technology, this ecologically important bacterium was brought into laboratory culture where its biology could be studied in more detail.

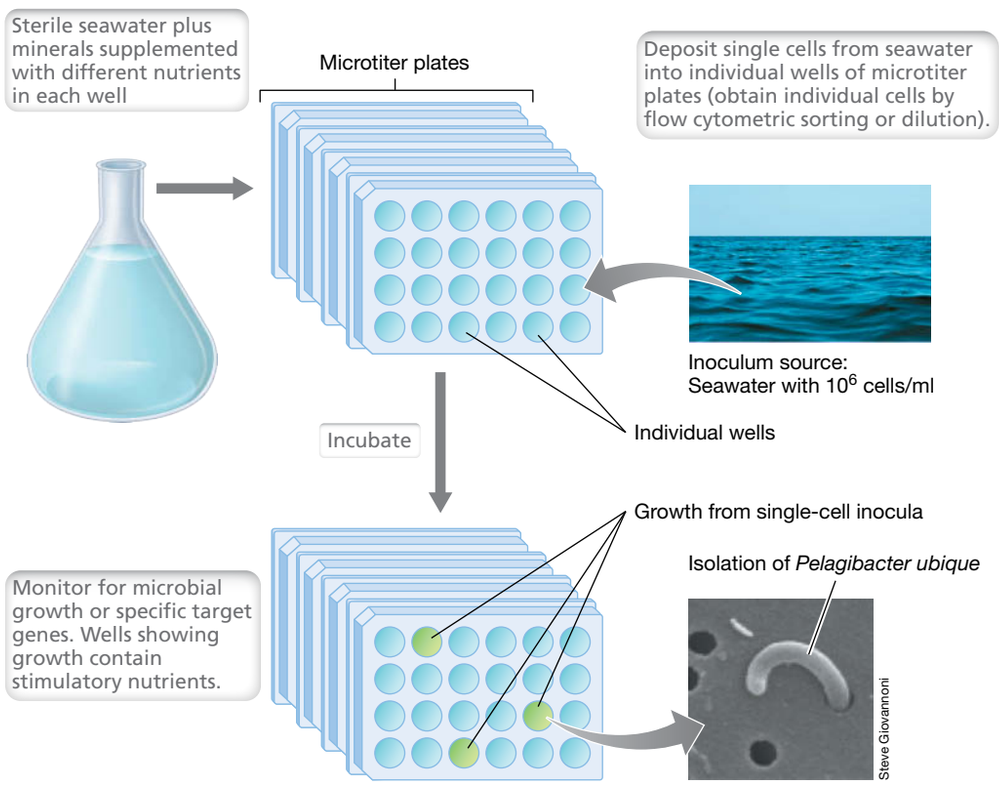



**Figure 19.6 Methodological pipeline for high-throughput cultivation of previously uncultured microorganisms.** The method shown here was used to isolate the marine bacterium *Pelagibacter ubique*. Following the addition of filter-sterilized seawater and low nutrient concentrations to the individual wells, pure cultures of *Pelagibacter* and other novel marine *Bacteria* were obtained. *Pelagibacter* is the most abundant bacterium in the open oceans (Section 20.11).

**Microfluidic devices** carry the high-throughput concept even further by using microfabrication technology to combine channels and wells for fluid transfer and collection on a miniaturized platform. One such device is less than 10 centimeters long yet holds 3200 nanoliter-sized wells, each well functioning as a small culture vessel (**Figure 19.7**). An environmental sample is introduced into the microfluidic device such that each well receives a single cell. Different medium formulations can be tested, and the media supplemented with a small amount of filter sterilized water or soil extract collected from the sampled niche (these additions may stimulate growth by providing trace nutrients missing in the culture medium).

Both growth and target genes can be assessed in each well of the microfluidic device; growth is assessed by direct microscopic examination of a well under the microscope. A variation on this technique employs a microchamber device modified such that each of the tiny chambers is separated from the external environment by a membrane that traps the microbes but allows soluble nutrients to diffuse in and out.

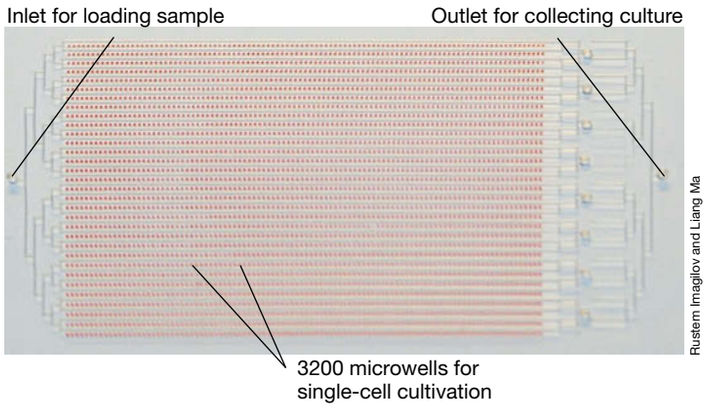



**Figure 19.7 Microfluidic platform for cultivation.** An environmental inoculum is suspended in a cultivation medium and loaded onto this microfluidic device, enabling confinement of as many as 3200 single cells in nanoliter wells to promote the growth of microcolonies. Following different periods of incubation, cultured populations are collected at the outlet and further grown under conditions demonstrated to support growth on the microfluidic device. The device is about 7 cm wide.

Following the introduction of a single cell into each chamber, the device is placed back into the environment from which the inoculum was obtained. Then, after incubation for a month or more, microbes that initiate growth only when incubated under the conditions and resources present in their habitats can often be isolated and subsequently propagated in the laboratory.

Although technology is rapidly advancing the art of isolating new microbes, patience is still needed in any cultivation effort, as the discovery of slow-growing or dormant organisms may require months of incubation. Also, many microbes in nature are likely adapted to extremely low nutrient concentrations and may be inhibited by levels of nutrients used to grow organisms commonly studied in the laboratory. Both high-throughput and microfluidic methods overcome these problems by their ability to separate individual cells from other cells that may release inhibitory materials and by surveying a nearly limitless variety of nutrient conditions. Currently, these methods offer the best opportunity for culturing the most interesting (and likely ecologically relevant) microorganisms from nature.

**MINIQUIZ**

- How might you isolate a morphologically unique bacterium present in an enrichment culture in relatively low numbers?
- What is meant by "high-throughput" in culturing microorganisms? How has it benefited microbiology?

# II • Culture-Independent Microscopic Analyses of Microbial Communities

Microbial ecologists quantify cells in a microbial habitat to estimate relative abundances of different species. Cell stains are necessary to obtain these types of data, and we detail these methods here. Organisms in natural environments can also be detected by assaying their genes. Genes encoding either ribosomal RNA (rRNA, Section 13.7) or enzymes that support a specific physiology are the usual targets in these studies. *Environmental genomics* is a method for assessing the entire gene complement of a habitat, revealing both the biodiversity and metabolic capabilities of the microbial community at the same time, and we consider this exploding field of microbial ecology in Section 19.8.

## 19.4 General Staining Methods

Several general staining methods are suitable for quantifying microorganisms in natural samples. Although these methods do not reveal the physiology or phylogeny of the cells, they are nonetheless reliable and widely used by microbial ecologists for measuring total cell numbers. One method also allows cell viability to be assessed.

### Fluorescent Staining with Dyes That Bind Nucleic Acids

Fluorescent dyes can be used to stain microorganisms from virtually any microbial habitat. **DAPI** (4′, 6-diamidino-2-phenylindole) is a popular stain for this purpose, as is the dye **acridine orange**. There is also increasing use of *SYBR Green I*, a dye that confers very bright fluorescence to all microorganisms, including viruses. These stains bind to DNA and are strongly fluorescent when exposed to ultraviolet (UV) radiation (DAPI absorption maximum, 400 nm; acridine orange absorption maximum, 500 nm; SYBR Green I absorption maximum, 497 nm), making the microbial cells in the sample readily visible and easy to enumerate. Cells stained with DAPI fluoresce blue, cells stained with acridine orange fluoresce orange or greenish-orange, and cells stained with SYBR Green I fluoresce green (Figure 19.8).

Dyes that stain DNA are widely used for the enumeration of microorganisms in environmental, food, and clinical samples. Depending on the sample, background staining is occasionally a problem with fluorescent stains, but because these dyes specifically stain nucleic acids, they are for the most part nonreactive with inert matter. Thus, for many samples, from soil as well as aquatic sources, they can give a reasonable estimate of the cell numbers present. Staining with the brightly fluorescent SYBR Green I also provides excellent enumeration of aquatic virus populations (Section 20.11). For dilute aquatic samples, cells can be stained following collection on a membrane surface by filtration.

DNA staining is a nonspecific process; *all* microorganisms in a sample are stained. Although this may at first seem desirable, it is not necessarily so. For example, DAPI and acridine orange fail to differentiate between living and dead cells or between different species of microorganisms, so they cannot be used to assess cell viability or to track specific microorganisms in an environment.

### Viability Staining

Viability staining differentiates live cells from dead ones. Hence, viability stains yield both abundance and viability data at the same time. The basis of differentiating between live and dead cells lies with whether a cell's cytoplasmic membrane is intact. Two dyes that fluoresce green and red are added to a sample; the green-fluorescing dye penetrates all cells, viable or not, whereas the red

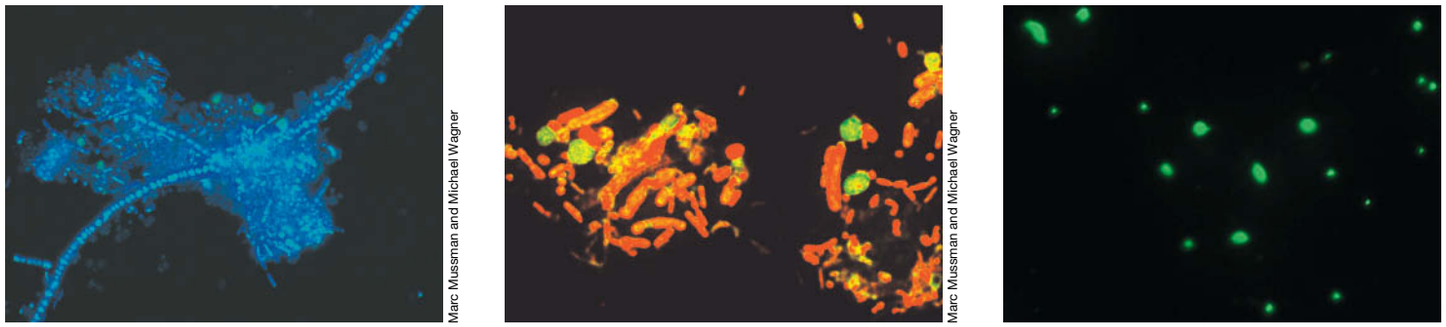



**Figure 19.8 Nonspecific fluorescent stains.** *(a)* DAPI and *(b)* acridine orange staining showing microbial communities inhabiting activated sludge in a municipal wastewater treatment plant. With acridine orange, cells containing low RNA levels stain green. *(c)* SYBR Green-stained sample of Puget Sound (Washington, USA) surface water showing green-fluorescing bacterial cells. The large cells near the center of the field are 0.8–1.0 μm in diameter.

dye, which contains the chemical propidium iodide, penetrates only those cells whose cytoplasmic membrane is no longer intact and that are therefore dead. Thus, when viewed microscopically, green cells are scored as alive and red cells as dead, yielding an instant assessment of both abundance and viability (**Figure 19.9**).

Although useful for research that uses laboratory cultures, the live/dead staining method is not suitable for use in the direct microscopic examination of samples from many natural habitats because of problems with nonspecific staining of background materials. However, procedures have been developed to overcome this problem in analyses of aquatic environments; a water sample is filtered and the filters are stained with the live/dead stain and examined microscopically. Thus in aquatic microbiology, live/dead staining is often used to measure the viability of cell populations in the water column of lakes or oceans, or in the flowing waters of streams, rivers, and other aquatic environments.

## Fluorescent Proteins as Cell Tags and Reporter Genes

Bacterial cells can be altered by genetic engineering to make them autofluorescent. As discussed earlier, a gene encoding the **green fluorescent protein (GFP)** can be inserted into the genome of virtually any cultured bacterium (↔ Sections 7.1 and 12.5). When the gene encoding the GFP (*gfp*) is expressed, cells fluoresce green when observed with ultraviolet microscopy (**Figure 19.10**). Although GFP is not useful for the study of natural populations of microorganisms (because these cells lack the GFP gene), GFP-tagged cells can be introduced into an environment, such as plant roots, and then tracked over time by microscopy. Using this method, microbial ecologists can study competition between the native microbiota and a GFP-tagged introduced strain and can assess the effect of perturbations of an environment on the survivability of the introduced strain.

The gene *gfp* and those encoding other **fluorescent proteins** have also been used extensively in laboratory cultures of various bacteria and in controlled environments as *reporter genes*. When the gene is fused with an operon under the control of a specific regulatory protein, transcription can be studied by using fluorescence as the indicator (a "reporter") of activity. That is, when genes containing the fused fluorescent protein gene are transcribed and translated, both the protein of interest and the fluorescent protein are made, and cells fluoresce the characteristic color (↔ Figures 7.2 and 12.16). For example, expression of *gfp* was used to demonstrate that colonization of alfalfa roots by *Sinorhizobium meliloti* (legume–root nodule symbiosis, ↔ Section 23.3) is promoted by sugars and dicarboxylic acids released by the plant (Figure 19.10*b, c*). The photophysical properties of GFP and other fluorescent proteins isolated from different marine invertebrates (jellyfish, corals, anemones) have since been altered through mutation to yield a broad palette of fluorescent proteins of varying spectral properties (Figure 19.10*a*), offering the experimenter the capability to monitor several species simultaneously.

One drawback to the use of GFP is that to become fluorescent it requires $O_2$, and thus it is not suitable for tracking cells introduced into strictly anoxic habitats. However, flavin-based fluorescent proteins that do not require $O_2$ are available to overcome this limitation. These proteins are derived from bacterial and plant photosensory flavoproteins and are more thermally stable than the GFP, making them useful for tracking mildly thermophilic species.

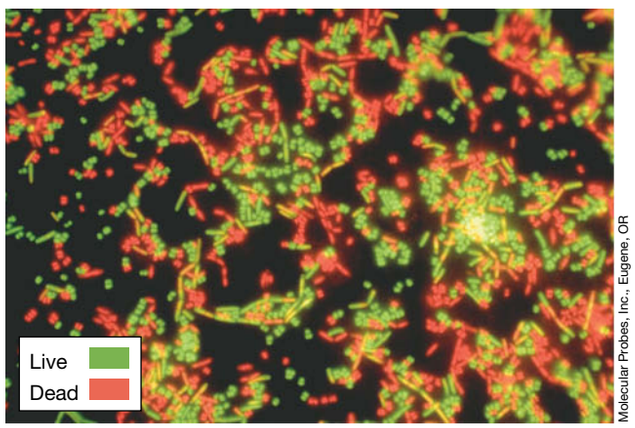



**Figure 19.9 Viability staining.** Live (green) and dead (red) cells of *Micrococcus luteus* (cocci) and *Bacillus cereus* (rods) stained by the LIVE/DEAD BacLight Bacterial Viability Stain.

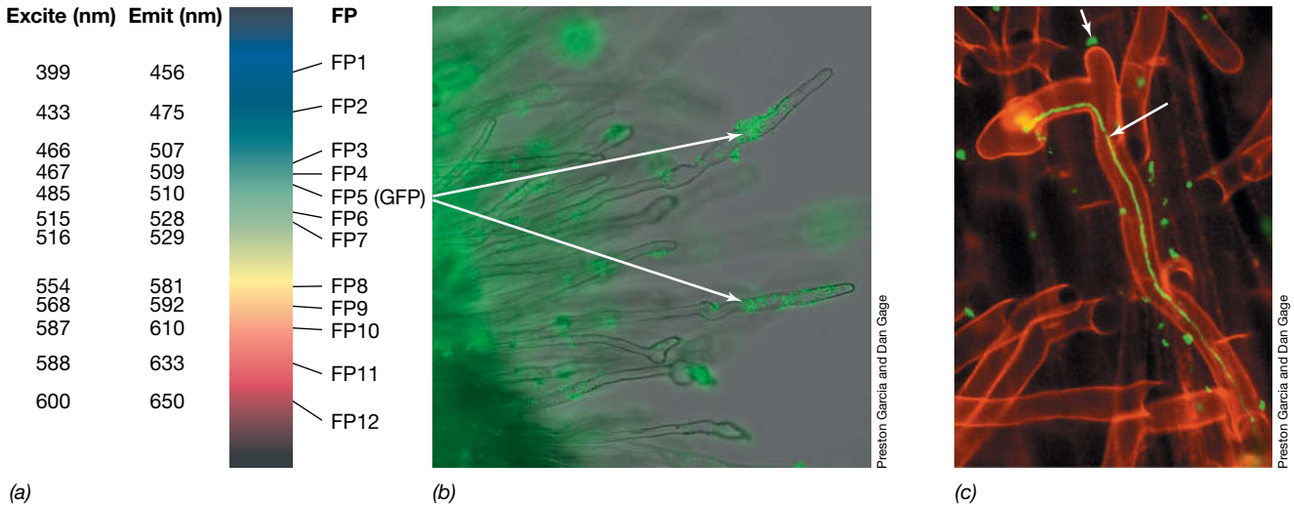



**Figure 19.10 Fluorescent protein reporters.** *(a)* Twelve different fluorescent proteins (FP1–FP12) are known that have distinct excitation (Excite) and emission (Emit) properties. *(b)* Cells of *Sinorhizobium meliloti* (arrows) carrying a plasmid with an α-galactoside-inducible promoter fused to the GFP (FP5); the cells are on clover seedling roots. Green fluorescence indicates that α-galactosides are released and available to support the growth of this bacterium. *(c) S. meliloti* cells (arrows) carrying a plasmid with a succinate-inducible promoter fused to GFP; green fluorescence indicates that succinate or other $C_4$ dicarboxylic acids have been secreted by the plant root hairs.

In addition to these fluorescent tags for tracking microbes, phylogenetic stains (Section 19.5) are widely used for identifying microbes and a wide variety of new fluorescent "super-resolution" microscopy techniques are available for tracking individual molecules within a microbial cell (Section 7.1). Thus, fluorescence technology has come a long way since the days when only DAPI and acridine orange were available for visualizing microbial cells in nature.

## Limitations of Microscopy

The microscope is an essential tool for exploring microbial diversity and for enumerating and identifying microorganisms in natural samples. However, microscopy alone does not suffice for the study of microbial diversity. Prokaryotic cells vary greatly in size (Section 2.2 and Table 2.1). Very small cells can be a major problem and can go totally unnoticed, and some cells are near the limits of resolution of the light microscope. Such cells can easily be overlooked in the examination of natural samples, especially if the sample contains high levels of particulate matter or high numbers of large cells. Also, it is often difficult to differentiate live cells from dead cells or cells in general from certain inert materials in natural samples. However, the biggest limitation with the microscopic methods we have discussed thus far is that none of them reveal the phylogenetic diversity of the microorganisms in the habitat under study.

We will see in the next section and get a preview here (Figure 19.11) of powerful staining methods that can reveal the *phylogeny* of organisms observed in a natural sample. These methods have revolutionized microbial ecology and have helped microbiologists overcome the major limitation of the light microscope in microbial ecology: identifying from a phylogenetic perspective cells observed in a microscopic field. These methods have also taught microbial ecologists an important lesson—when observing unstained or nonspecifically stained natural populations of microorganisms under the microscope, one must remember that the sample almost certainly contains a genetically diverse community, even if many cells "look" the same (Figure 19.11). The simple shapes of bacteria conceal their remarkable diversity.

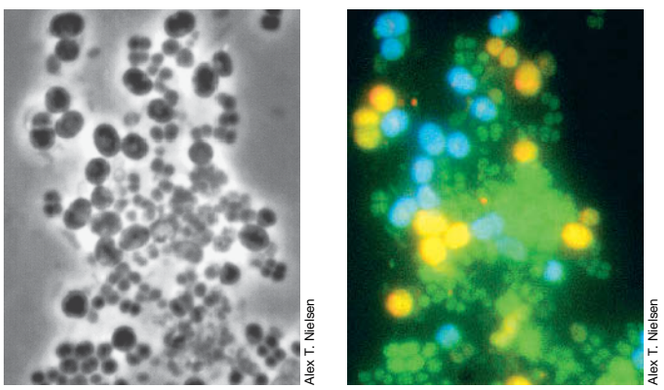



**Figure 19.11 Morphology and genetic diversity.** The photomicrographs shown here, produced by *(a)* phase contrast and *(b)* a technique called phylogenetic FISH (Section 19.5), are of the same field of cells. Although the large oval cells are of a rather unusual size for prokaryotic cells and all look similar by phase-contrast microscopy, the phylogenetic stains reveal that there are two genetically distinct types (one stains yellow and one stains blue). Both cell types are about 2.25 μm in diameter. The smaller, green cells in pairs or clusters are about 1 μm in diameter.

### MINIQUIZ

- How does viability staining differ from stains like DAPI?
- What types of environments limit the application of GFP?
- Why is it incorrect to say that the GFP is a "staining" method?

UNIT 5

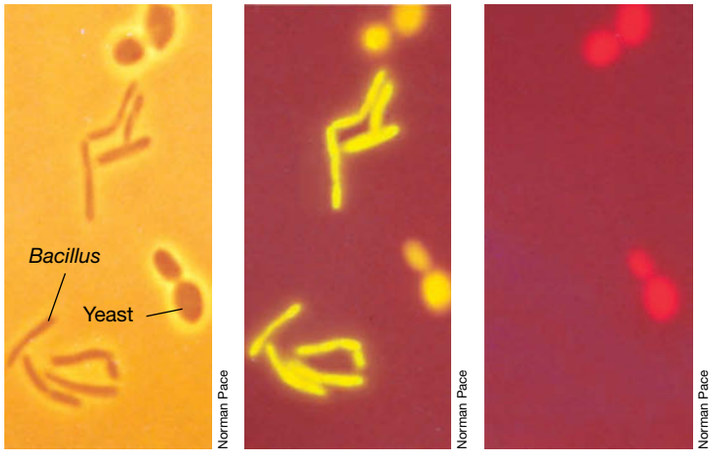



**Figure 19.12 Fluorescently labeled rRNA probes: Phylogenetic stains.** *(a)* Phase-contrast photomicrograph of cells of *Bacillus megaterium* (rod, *Bacteria*) and the yeast *Saccharomyces cerevisiae* (oval cells, *Eukarya*). *(b)* Same field; cells stained with a yellow-green universal rRNA probe (this probe hybridizes with rRNA from organisms of any phylogenetic domain). *(c)* Same field; cells stained with a eukaryal probe (only cells of *S. cerevisiae* react). Cells of *B. megaterium* are about 1.5 μm in diameter and cells of *S. cerevisiae* are about 6 μm in diameter.

## 19.5 Fluorescence In Situ Hybridization (FISH)

Because of their great specificity, nucleic acid probes are powerful tools for identifying and quantifying microorganisms. Recall that a **nucleic acid probe** is a DNA or RNA oligonucleotide complementary to a sequence in a target gene or RNA; when the probe and the target come together they hybridize (Section 12.1). Nucleic acid probes can be made fluorescent by attaching fluorescent dyes to them. The fluorescent probes can often be used to identify organisms that contain a nucleic acid sequence complementary to the probe. This technique is called **fluorescence in situ hybridization (FISH)**, and different applications are described here, including methods that target phylogeny (Figure 19.12) or gene expression (see Figure 19.14).

### Phylogenetic Identification Using FISH

Phylogenetic FISH stains are fluorescing oligonucleotides complementary in base sequence to sequences in ribosomal RNA (16S or 23S rRNA in *Bacteria* and *Archaea* or 18S or 28S rRNA in eukaryotes). Phylogenetic stains penetrate cells without lysing them and hybridize with rRNA directly in the ribosomes. The number of fluorescent probes bound to a cell reflects the number of its ribosomes. As single microbial cells can contain tens of thousands of ribosomes, strong signals can be achieved. Because ribosomes are scattered throughout the cell in most prokaryotic cells, the entire cell becomes fluorescent (Figures 19.11*b* and 19.12).

By targeting sites in the rRNA sequence that are variable between different organisms, phylogenetic stains can be designed to be very specific and react with only one species or a handful of related microbial species. Alternatively, by targeting conserved sequences in the rRNA they can be made more general and react with, for example, all cells of a given phylogenetic domain. Using FISH, an investigator can identify or track an organism of interest or a domain of interest in a natural sample. For example, if one wishes to determine the percentage of a given microbial population that are *Archaea*, an archaea-specific phylogenetic stain may be used in combination with DAPI (Section 19.4) to assess *Archaea* and total numbers, respectively, and a percentage could then be derived by calculation.

FISH technology can also employ multiple phylogenetic probes. With a suite of probes, each designed to react with a particular organism or group and each containing its own fluorescent dye, FISH can image multiple taxa in a habitat in a single experiment (Figure 19.13). If FISH is combined with confocal microscopy (Section 1.7), it is possible to explore microbial populations with depth, as, for example, in a biofilm (Section 20.4). In addition to microbial ecology, FISH is also an important tool in the food industry and in clinical diagnostics for the microscopic detection of specific pathogens in food products or clinical specimens.

### CARD-FISH

Besides characterizing the abundance of different taxa in a habitat, FISH can be used to measure *gene expression* in organisms in a natural sample. Because the target in this case is messenger RNA (mRNA), a form of RNA that is much less abundant in cells than is rRNA, standard FISH techniques cannot be applied. Instead, the signal (fluorescence) must be amplified. A FISH method that enhances the signal is called *catalyzed reporter deposition FISH* (*CARD-FISH*).

In CARD-FISH the specific nucleic acid probe contains a molecule of the enzyme peroxidase conjugated to it instead of a fluorescent dye. After there has been time for hybridization, the preparation is treated with a fluorescently labeled soluble compound called *tyramide*, which is a substrate for peroxidase. Within cells containing the nucleic acid probe, the tyramide is converted by the activity of peroxidase into a very reactive intermediate that

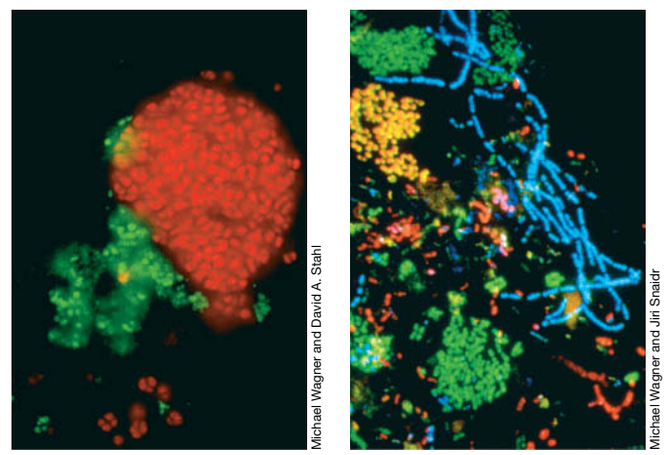



**Figure 19.13 FISH analysis of activated sludge from a wastewater treatment plant.** *(a)* Nitrifying bacteria. Red, ammonia-oxidizing bacteria; green, nitrite-oxidizing bacteria. *(b)* Confocal laser scanning micrograph of a sewage sludge sample treated with three phylogenetic FISH probes, each containing a fluorescent dye (green, red, or blue) that identifies a particular group of *Proteobacteria*. Green-, red-, or blue-stained cells reacted with only a single probe; other cells reacted with two (turquoise, yellow, purple) or three (white) probes.

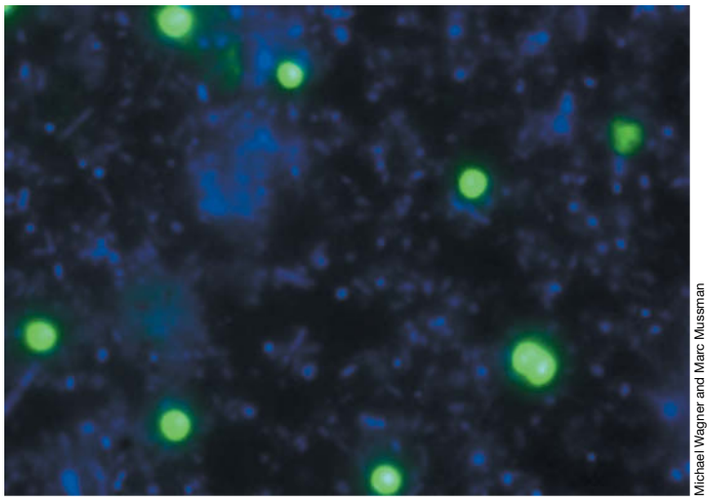



**Figure 19.14 Catalyzed reporter deposition FISH (CARD-FISH) labeling of *Archaea*.** Archaeal cells in this preparation fluoresce intensely (green) relative to DAPI-stained cells (blue).

covalently binds to adjacent proteins; this amplifies the signal sufficiently to be detected by fluorescence microscopy (Figure 19.14). Each molecule of peroxidase activates many molecules of tyramide so that even mRNAs present at very low abundance can be visualized.

Besides detecting mRNA, CARD-FISH is also useful in phylogenetic studies of microbes that may be growing very slowly, such as organisms inhabiting the open oceans where cold temperatures and low nutrient concentrations limit growth rates (Figure 19.14). Because such cells have few ribosomes compared with more actively growing cells, standard FISH often yields only a weak signal.

**MINIQUIZ**

- What structure in the cell is the target for fluorescent probes in phylogenetic FISH?
- FISH and CARD-FISH can be used to reveal different things about cells in nature. Explain.

# III • Culture-Independent Genetic Analyses of Microbial Communities

Microbial biodiversity studies often forgo isolating organisms or even quantifying or identifying them microscopically. Instead, *specific genes* are used as measures of biodiversity and metabolic capacities. Some genes are unique to particular organisms. Detection of such a gene in an environmental sample implies that the organism is present. The major techniques employed in this type of microbial community analysis are the polymerase chain reaction (PCR), DNA fragment analysis by gel electrophoresis (DGGE, T-RFLP, ARISA) or molecular cloning, and DNA sequencing and analysis. In addition, entire genomes extracted from cells present in an environmental sample can be analyzed as a measure of the biodiversity of microbial communities.

## 19.6 PCR Methods of Microbial Community Analysis

We discussed the principle of the polymerase chain reaction (PCR) in Section 12.1. Recall the major steps involved: (1) Two nucleic acid primers are hybridized to a complementary sequence in a target gene; (2) DNA polymerase copies the target gene; and (3) multiple copies of the target gene are made by repeated melting of complementary strands, hybridization of primers, and new synthesis (Figure 12.1). From a single copy of a gene, several million copies can be made for subsequent analyses. PCR finds wide applications in microbial ecology.

### PCR and Microbial Community Analysis

Which genes are suitable as target genes for microbial community analyses? Because genes encoding the small subunit ribosomal (SSU) rRNAs are phylogenetically informative and techniques for their analysis well developed (Section 13.7), they are widely used in community analyses. Moreover, because rRNA genes are universal and contain several regions of high sequence conservation, it is possible to amplify them from all organisms using only a few different PCR primers, even though the organisms may be phylogenetically distantly related. In addition to rRNA genes, genes that encode enzymes for metabolic functions unique to a specific organism or group of related organisms can be the target genes (Table 19.3).

Genes such as those encoding rRNAs that have retained ancestral function while changing in sequence over time as species have diverged are called *orthologs* (Sections 9.5 and 13.7). Organisms that share the same or very closely related orthologous genes are called a **phylotype**. In microbial ecology, the phylotype concept is primarily used to provide a natural (phylogenetic) framework for describing the microbial diversity of a given habitat, regardless of whether the identified phylotypes are cultured organisms or not. Thus, the word *phylotype* is widely used to describe the microbial diversity of a habitat based solely on nucleic acid sequences. It is only when additional physiological and genetic information becomes available, typically after the organism is brought into laboratory culture (Sections 19.2 and 19.3), that proposing a genus and species name for a phylotype becomes possible.

In a typical community analysis experiment, total DNA is isolated from a microbial habitat (Figure 19.15). Commercially available kits that yield high-purity DNA from soil or other complex habitats are available for this purpose. The DNA obtained is a mixture of genomic DNA from all of the microorganisms that were in the sample from the habitat (Figure 19.15). From this mixture, PCR is used to amplify the target gene and make multiple copies of each variant (phylotype) of the target gene. If RNA is isolated instead of DNA (to detect those genes being transcribed), the RNA can be converted into complementary DNA (cDNA) by the enzyme reverse transcriptase (Section 10.11) and the cDNA subjected to PCR as for isolated DNA. However, regardless of whether DNA or RNA is originally isolated, the different phylotypes need to be sorted out following the PCR step before they can be sequenced.

**TABLE 19.3 Genes commonly used for evaluating specific microbial processes in the environment using PCR**

| *Metabolic process*[a] | *Target gene* | *Encoded enzyme* |
|---|---|---|
| Denitrification | *narG* | Nitrate reductase |
| | *nirK, nirS* | Nitrite reductase |
| | *norB* | Nitric oxide reductase |
| | *nosZ* | Nitrous oxide reductase |
| Nitrogen fixation | *nifH* | Nitrogenase |
| Nitrification | *amoA* | Ammonia monooxygenase |
| Methane oxidation | *pmoA* | Methane monooxygenase |
| Sulfate reduction | *apsA* | Adenosine phosphosulfate reductase |
| | *dsrAB* | Sulfite reductase |
| Methane production | *mcrA* | Methyl coenzyme M reductase |
| Degradation of petroleum compounds | *nahA* | Naphthalene dioxygenase |
| | *alkB* | Alkane hydroxylase |
| Anoxygenic photosynthesis | *pufM* | M subunit of photosynthetic reaction center |

[a]All of these metabolic processes are discussed in Chapter 14 and Section 3.12.

Sorting can be accomplished using one of three different methods: (1) physical separation by gel electrophoresis (Section 12.1), (2) clone library construction (Sections 12.2 and 12.9), and (3) next-generation sequencing technology (Section 9.2). We consider these methods now.

## Denaturing Gradient Gel Electrophoresis: Separating Very Similar Genes

One method to resolve phylotypes is **denaturing gradient gel electrophoresis (DGGE)**, which separates genes of the same *size* that differ in their melting (denaturing) profile because of differences in their *base sequence* (Figure 19.16*a, b*). DGGE employs a gradient of a DNA denaturant, typically a mixture of urea and formamide. When a double-stranded DNA fragment moving through the gel reaches a region containing sufficient denaturant, the strands begin to "melt"; at this point, their migration stops (Figures 19.15 and 19.16*b*). Differences in base sequence cause differences in the melting properties of DNA. Thus, the different bands observed in a DGGE gel are phylotypes that can differ in base sequence significantly or by as little as a single base change.

Once DGGE has been performed, the individual bands are excised and sequenced (Figure 19.15). With 16S rRNA as the target gene, for example, the DGGE pattern immediately reveals the number of phylotypes (distinct 16S rRNA genes) present in a habitat (Figure 19.16*c*). The method provides an excellent mechanism to quickly evaluate temporal and spatial shifts in microbial community structure (Figure 19.16*c*). If PCR primers specific for genes other than 16S rRNA are used, such as a metabolic gene (Table 19.3), the variants of this specific gene that exist in the sample can also be assessed. Thus, although the number of bands on a DGGE gel is an overview of the biodiversity in a habitat (Figure 19.16*c*), sequence analysis is still required for identification and to infer phylogenetic relationship.

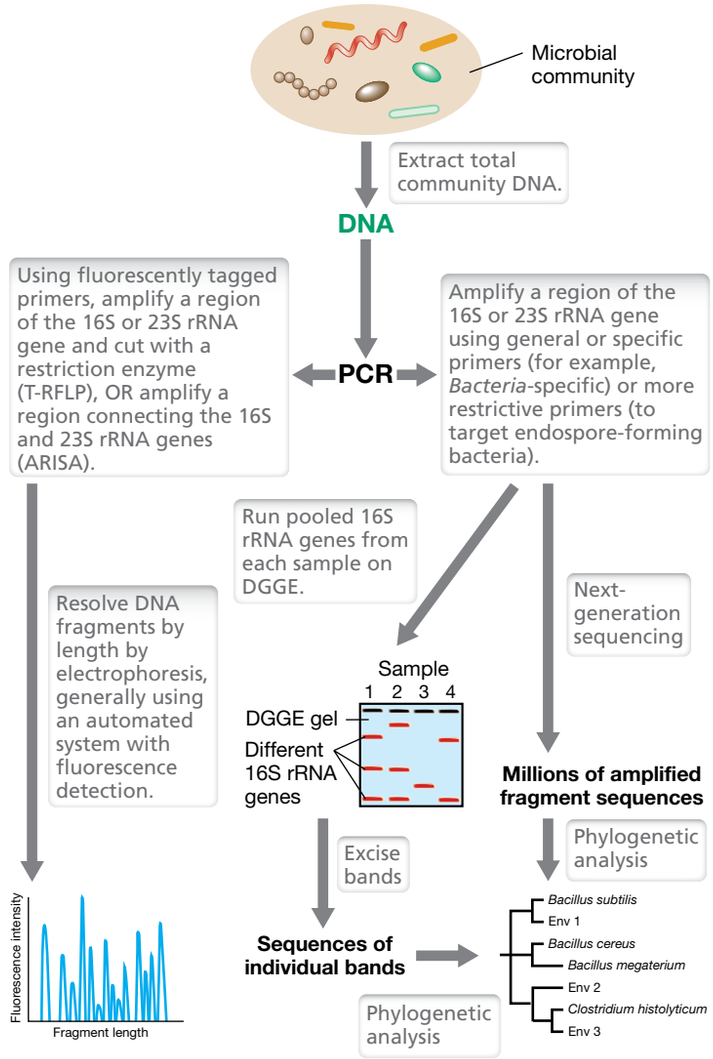



**Figure 19.15 Steps in single-gene biodiversity analysis of a microbial community.** From total community DNA, 16S rRNA genes are amplified using primers that target only *Firmicutes*, a group of gram-positive *Bacteria* that includes the endospore-forming genera *Bacillus* and *Clostridium*. The 16S rRNA gene products obtained from PCR are then either separated by DGGE or sequenced directly by next-generation sequencing; from the sequence data, a phylogenetic tree is generated. "Env" indicates an environmental sequence (phylotype). In T-RFLP analyses, the number of peaks indicates the number of phylotypes.

## T-RFLP and ARISA

A rapid method of microbial community analysis is *terminal restriction fragment length polymorphism* (*T-RFLP*). In this method a target gene (usually an rRNA gene) is amplified by PCR from community DNA using a primer set in which one of the primers is end-labeled with a fluorescent dye. The PCR products are then treated with a restriction enzyme (Section 12.2) that cuts the DNA at specific sequences. This generates a series of DNA fragments of varying length, the number of which depends on how many restriction cut sites are present in the DNA. The fluorescently labeled terminal fragments are then separated by size on an automated DNA sequencer that detects fluorescent fragments (thus, only the terminal dye-labeled fragments are detected). The pattern obtained

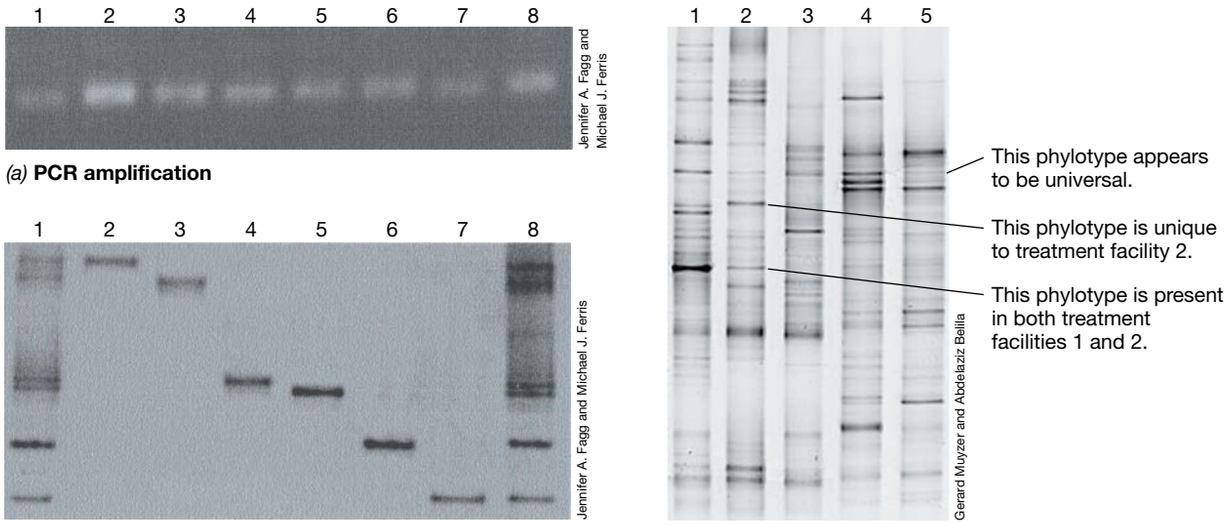



**Figure 19.16 PCR and DGGE gels.** Bulk DNA was isolated from a microbial community and amplified by PCR using primers for 16S rRNA genes of *Bacteria* (*a*, lanes 1 and 8). Six bands later resolved by DGGE (*b*, lanes 2–7) were excised and reamplified and each gave a single band at the same location on the PCR gel (*a,* lanes 2–7). However, by DGGE analysis, each band migrated to a different location on the gel (*b*, lanes 2–7). Note that all bands migrate to the same location in the nondenaturing PCR gel because they are all of the same size, but they migrate to different locations on the DGGE gel because they have different sequences. *(c)* DGGE profiles of microbial communities from different wastewater treatment facilities amplified using primers for the 16S rRNA genes of *Bacteria*.

shows the rRNA sequence variation and general abundance of different sequence types (fragment fluorescence intensity) in the microbial community sampled (Figure 19.15).

DGGE and T-RFLP both measure *single-gene diversity*, but in different ways. The pattern of bands on a DGGE gel reflects the number of same-length sequence variants of a single gene (Figure 19.16), whereas the pattern of bands on a T-RFLP gel reflects variants differing in DNA sequence of a single gene as measured by differences in restriction enzyme cut sites. The information obtained from a T-RFLP analysis, in addition to providing insight into the diversity and population abundances of a microbial community, can also be used to infer phylogeny. Diagnostic information for each fragment includes knowledge of sequences near both ends (primer sequence and restriction enzyme cut site), knowledge that a second restriction site does not exist within the fragment, and fragment length. Using specialized software, this information can be used to search for matching 16S rRNA sequences in public databases. Although this is of some predictive value, closely related sequences are often not differentiated by these criteria. Thus, T-RFLP generally underestimates the diversity within a microbial community.

A technique related to T-RFLP that provides more detailed analysis of microbial communities is *automated ribosomal intergenic spacer analysis* (*ARISA*), which exploits the proximity of the 16S rRNA and 23S rRNA genes in the genomes of *Bacteria* and *Archaea*. The DNA separating these two genes, called the *internal transcribed spacer* (*ITS*) region, differs in length among species and often also differs in length among the multiple rRNA operons of a single species (**Figure 19.17*a***). The PCR primers for ARISA are complementary to conserved sequences in the 16S and 23S rRNA genes that flank the spacer region. Amplification (Figure 19.17*b*) and analysis (Figure 19.17*c*) are conducted as described for T-RFLP, resulting in a complex pattern of bands that can be used for community analysis. However, ARISA differs from T-RFLP in that ARISA does not require a restriction enzyme digestion following PCR amplification. The word "automated" in the ARISA acronym refers to the use of a DNA sequencer that automatically identifies and assigns sizes to each dye-labeled fragment (Figure 19.17*c*), as can also be done in T-RFLP analyses. ARISA has received greatest application in the study of microbial community dynamics by monitoring, for example, changes in the presence and relative abundance of a specific community member through time and space.

## Diversity Studies Using Clone Libraries or Next-Generation Sequencing

Early molecular microbial diversity research relied on the construction of clone libraries to separate individual amplified DNA molecules (*amplicons*); each clone in the library contained a unique sequence that was then used as a template for sequence determination (↪ Sections 9.2 and 12.2). Figure 19.16*a* shows that a 16S rRNA gene amplicon mixture appears as a single band when run on a nondenaturing gel. However, because the amplified target gene came from a *mixture* of different cells, the different phylotypes in the single band need to be sorted out before they are sequenced. Today this is done by next-generation sequencing systems (↪ Section 9.2) rather than by DGGE or by cloning.

Next-generation sequencing does not require a cloning step, as individual DNA fragments are separated and amplified on the sequencing device itself; thus, PCR products can be used directly for sequencing. Since millions of amplification reactions are then conducted simultaneously, the total number of sequencing reads vastly exceeds what is possible by sequencing individual clones obtained in a clone library one at a time (**Figure 19.18**). This tremendous volume of sequence data allows for what has been called

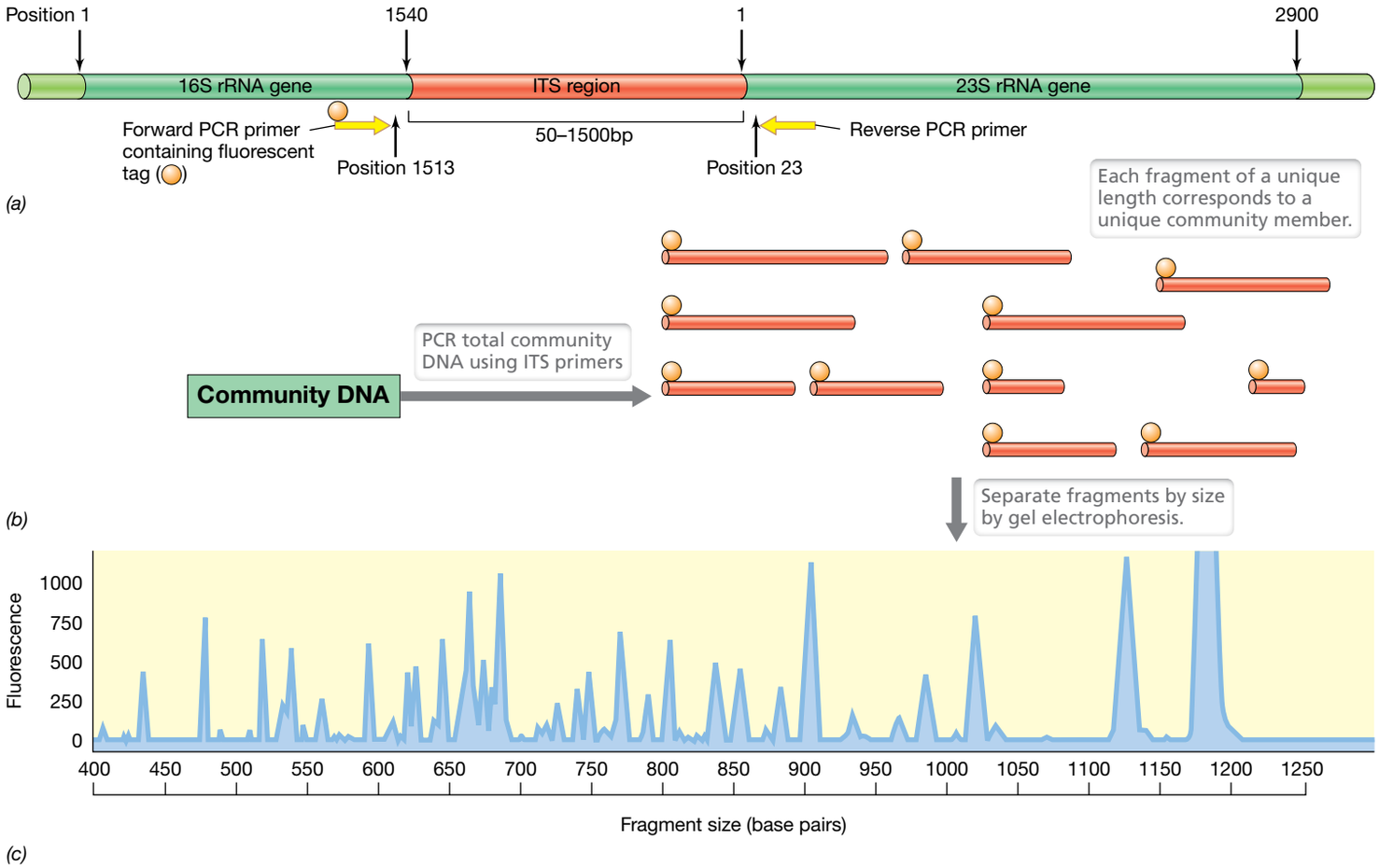



**Figure 19.17 Automated ribosomal intergenic spacer analysis (ARISA).** *(a)* Structure of rRNA operon spanning the 16S rRNA gene (positions 1–1540), an internal transcribed spacer (ITS) region of variable length, and the 23S rRNA gene (positions 1–2900). The PCR primers, one labeled with a fluorescent dye, are complementary to conserved sequences near the ITS region. *(b)* Amplified DNA fragments of different lengths, each corresponding to a community member. *(c)* Fragment analysis determined by an automated DNA sequencer. The peaks, which correspond to different ITS regions, can be identified by cloning and sequencing the amplified products.



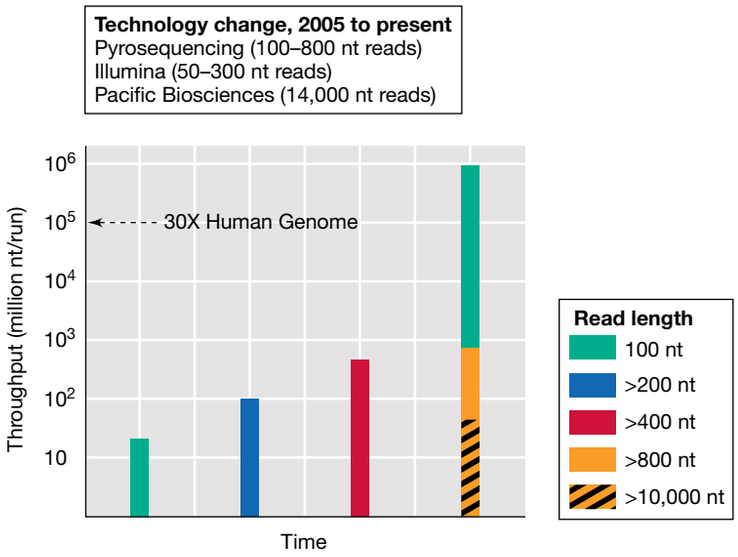
*deep sequence analyses*, meaning that minor phylotypes that were possibly missed by the more limited and expensive clone library method are now revealed (Figure 19.18*b*). For example, if a particular phylotype were present at only 0.01% in a library of cloned sequences, it could take one thousand clones or more to ever detect it. By contrast, next-generation sequencing would detect this low-abundance phylotype along with its more abundant neighbors. This collection of minor phylotypes, which represent a substantial fraction of total diversity but only a minor component of total organism abundance in most environments, has been called the *rare biosphere* (Figure 19.18).

## Results of PCR Phylogenetic Analyses

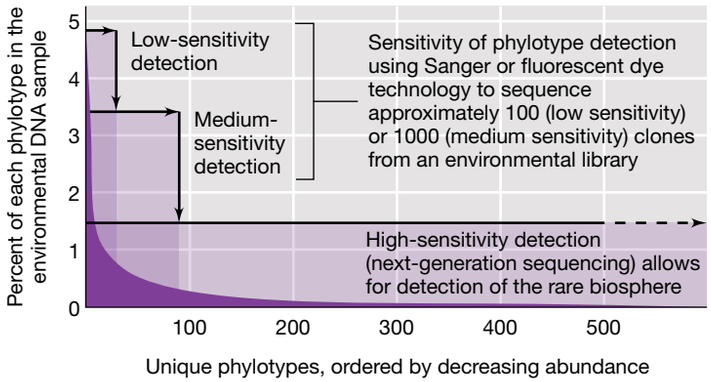
Phylogenetic analyses of microbial communities have yielded surprising results. For example, using the gene encoding 16S rRNA as the target, analyses of natural microbial communities typically show that many phylogenetically distinct *Bacteria* and *Archaea* (phylotypes) are present whose rRNA gene sequences differ from those of all known laboratory cultures (Figure 19.15). Moreover, using quantitative PCR (Section 12.1), a variant of PCR that allows each phylotype to be quantified as well as amplified, it has often been observed that the most abundant phylotypes in a natural microbial community are ones that have thus far defied laboratory culture. These sobering results make it clear that our knowledge of microbial diversity from enrichment cultures is far from complete and that enrichment bias (Section 19.1) is a serious problem in culture-dependent biodiversity studies. Obviously, much work remains to put our abilities to culture microbes on a par with our existing abilities to detect and identify them in nature.

### MINIQUIZ

- What could you conclude from PCR/DGGE analysis of a sample that yielded one band by PCR and one band by DGGE? One band by PCR and four bands by DGGE?
- What surprising finding has come out of many molecular studies of natural habitats using 16S rRNA as the target gene?
- How has next-generation sequencing technology altered our understanding of microbial community diversity?




(a)




(b)

**Figure 19.18 Community diversity analyses using next-generation sequencing technology.** *(a)* Current sequencing platforms (Section 9.2) have the capacity to generate $10^{12}$ nucleotides (nt) of sequence in a single sequencing run (requiring a week or less), with individual read lengths varying from 100 to 800 nucleotides. The three segments in the rightmost bar show that technologies generating longer reads have lower throughput per sequencing run. *(b)* This enormous sequencing capacity revealed many unique phylotypes that were not detected using DGGE or clone library sequencing. Fewer than 100 unique phylotypes would be detected by Sanger (first-generation) sequencing of 1000 clones in a library of 16S rRNA gene PCR amplicons. Jed Fuhrman is acknowledged for input to part *b*.

## 19.7 Microarrays for Analysis of Microbial Phylogenetic and Functional Diversity

We previously considered the use of DNA chips—a type of *microarray*—for assessing overall gene expression in a microbial pure culture (Section 9.9). Specific microarrays can also be constructed for rapid gene-based analyses of biodiversity and the functional potential of natural microbial communities. Microarrays designed for biodiversity studies, called *PhyloChips*, have been developed for screening microbial communities for specific phylogenetic groups. Another type of microarray, the *GeoChip*, has been designed to detect genes encoding metabolic functions of biogeochemical significance, such as genes encoding proteins required for sulfate respiration, ammonia oxidation, denitrification, or nitrogen fixation (Table 19.3).

### PhyloChips and GeoChips

PhyloChips are constructed by affixing rRNA probes or rRNA gene–targeted oligonucleotide probes to the chip surface in a known pattern, and several thousand different probes can be added to a single PhyloChip. As an example, consider a PhyloChip designed to assess the diversity of sulfate-reducing bacteria (Section 15.9) in a sulfidic natural environment, such as marine sediments (**Figure 19.19**). Oligonucleotides complementary to specific sequences in the 16S rRNA genes of all known sulfate-reducing bacteria (over 100 species) would be affixed to the chip. Then, following the isolation of total community DNA from the sample and PCR amplification and fluorescence labeling of the 16S rRNA genes, the environmental DNA would be hybridized to the probes on the PhyloChip. The species of sulfate reducers present in the sample would then be determined by detecting which probes hybridized sample DNA (Figure 19.19). Alternatively, rRNA could be extracted from the microbial community, labeled with a fluorescent dye, and hybridized directly to the PhyloChip without an amplification step.

In contrast to the PhyloChip, the GeoChip (**Figure 19.20**) targets *functional* genes rather than phylogenetic genes. However, because genes encoding enzymes of similar function can vary significantly in primary sequence, these *functional gene microarrays*, as they are called, must contain many thousands of probes in order to achieve reasonable coverage of natural diversity. Even then, such arrays may only sample a fraction of the actual functional diversity in a habitat. The most recent version of the GeoChip functional gene microarray contains over 160,000 probes covering more than 1400 gene families related to carbon, nitrogen, and sulfur cycling. By detecting which of the probes contain hybridized DNA from the natural sample, a rapid

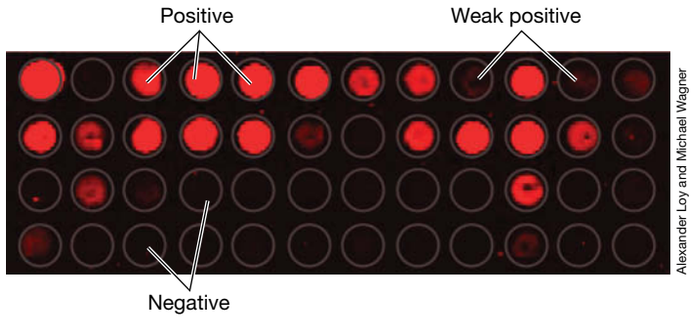
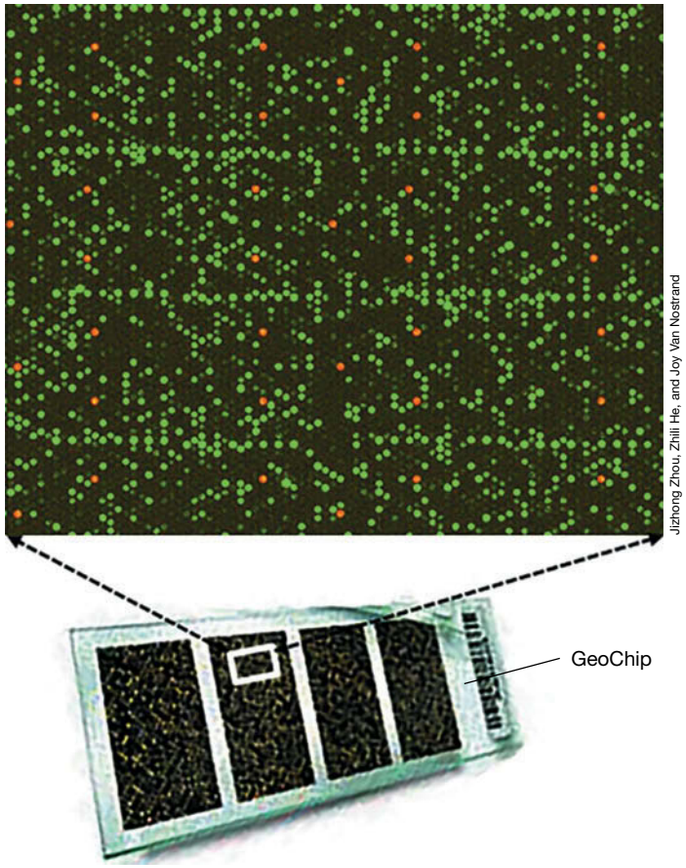



**Figure 19.19 PhyloChip analysis of sulfate-reducing bacteria diversity.** Each spot on the microarray shown has an oligonucleotide complementary to a sequence in the 16S rRNA of a different species of sulfate-reducing bacteria. After the microarray is hybridized with 16S rRNA genes PCR-amplified from a microbial community and then fluorescently labeled, the presence or absence of each species is signaled by fluorescence (positive or weak positive) or nonfluorescence (negative), respectively.




(a)

| Functional category | Genes families | Total probes | Database gene coverage (%) |
|---|---|---|---|
| Carbon cycling | 149 | 26922 | 49 |
| Nitrogen cycling | 32 | 6493 | 52 |
| Sulfur cycling | 27 | 4739 | 64 |
| Phosphorus | 7 | 3260 | 52 |
| Metal homeostasis | 121 | 43432 | 47 |
| Viruses | 115 | 2857 | 55 |
| Other | 81 | 10380 | 42 |
| Organic remediation | 104 | 11591 | 41 |
| Virulence | 639 | 21152 | 45 |
| Secondary metabolism | 68 | 4032 | 56 |
| Electron transfer | 15 | 797 | 65 |
| Stress response | 89 | 26306 | 33 |
| **TOTAL** | **1447** | **161,961** | **44** |

(b)

**Figure 19.20 GeoChip analysis of functional gene diversity.** The current version of the GeoChip contains over 160,000 probes covering more than 365,000 gene sequences in public databases, encompassing most major biogeochemical processes. The image shows green fluorescence of varying intensity (approximating gene abundance) following hybridization of fluorescent dye-labeled environmental DNA to the individual probes in one region of the high-density array of probes. The red spots correspond to repeated applications of a known amount of a reference DNA standard. A red-dye-labeled probe complementary to the reference standard was added to the environmental DNA prior to hybridization. Red fluorescence of equal intensity among the reference standard spots confirms that hybridization was uniform throughout the array.

appraisal of the potential metabolisms operating in a particular habitat can be obtained.

### Advantages and Caveats of Environmental Microarrays

PhyloChips and GeoChips circumvent many of the time-consuming steps of molecular microbial ecology—PCR, DGGE, cloning, and sequencing (Figure 19.15). However, an important caveat to keep in mind is the possibility of *nonspecific hybridization*. That is, gene variants that are closely related in sequence may not be resolved because of overlapping hybridization patterns. Moreover, totally unrelated genes may yield false positive results if they are sufficiently complementary in base sequence to hybridize to the probe. And finally, unlike nucleic acid sequencing, whose costs keep plummeting yearly, designing and manufacturing gene chips are not inexpensive activities. Nevertheless, functional gene arrays offer another important tool for the culture-independent assessment of microbial biodiversity and potential metabolic activities.

**MINIQUIZ**

- What is a PhyloChip and what can it tell you? How does a PhyloChip differ from a GeoChip?
- What are the advantages and disadvantages of microarray technology compared to sequencing PCR products?

## 19.8 Environmental Genomics and Related Methods

A more encompassing approach to the molecular study of microbial communities is **environmental genomics**, also called **metagenomics**. Before the metagenomics era, microbial community analyses typically focused on the diversity of a *single* gene in an environmental sample. By contrast, in environmental genomics, *all* genes in a given microbial community can be sampled, and if the experiment is properly designed, the information obtained can support a much deeper understanding of the structure and function of the community than can single-gene analyses.

### Metagenomics and Reconstructing Environmental Genomes

The goal of a metagenomics study today is to use next-generation DNA sequencing (Section 9.2) to identify as many genes as possible from an environmental DNA sample and determine the phylogeny of the organism(s) to which the genes belong. Although complete and finished genomes are often not the goal of metagenomics, there is increasing interest in assembling individual genomes from large metagenomic datasets to at least the draft stage. Rather than simply generating a list of all genes present in an environment, these nearly complete genomes can better connect functional and phylogenetic aspects of a microbial habitat.

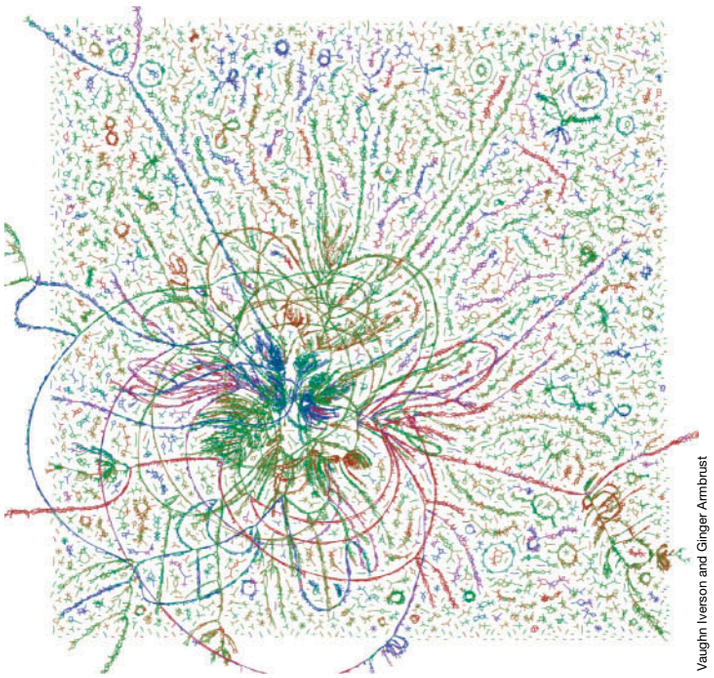



**Figure 19.21 Genome assembly from a coastal marine metagenome consisting of 58.5 *billion* nucleotides of sequence.** This "connection graph" is intended as a visual representation of the complexity and abundance of partial and complete genomes assembled from the water sample. The long strands, colored by differences in the percentage of guanine plus cytosine content, correspond to prokaryotic genomes and the small circular strands are most likely from viruses or plasmids.

An example of this is shown in the "connection graph" of Figure 19.21 that depicts an assembly of genomes from a coastal marine water sample. A total of 58.5 *billion* nucleotides in the metagenome were used to stitch together these complete and near-complete genomes. Such massive metagenomic undertakings often reveal links between physiologies and phylogenies not obtainable in the absence of reconstructed genomes.

A problem with genome assembly from a mixture of environmental DNA sequence reads, however, is that the genomes obtained are unlikely to be either complete or clonal, instead being composed of fragments of DNA from closely related strains of a species (Figure 19.22). This has proven to be a major problem in the assembly of soil microbial genomes from metagenomic data. A single gram of fertile soil contains about $10^{12}$ bacterial and archaeal genes and $10^9$ genomes; complete coverage of these genes is not yet feasible with available technology, even with the sequencing of 300 billion nucleotides in one soil study. Single-cell genomics (Section 9.12) may ultimately overcome this problem (see also Section 19.12), but by definition it only provides information from a single microbial cell.

Of critical importance in evaluating a genome reconstructed from metagenomic DNA is to assess whether it contains all of the genes required by a cell (for example, all necessary tRNA and rRNA genes and genes encoding essential proteins such as DNA and RNA polymerases) and is therefore a legitimate genome candidate. In addition, an assessment of the relative abundance of genes encoding specific functions is equally valuable, since abundance changes suggest interactions among species or a common response to a particular environmental variable. For example, if a high number of genes were recovered in the pathway for nitrogen fixation, this would suggest that the environment sampled was limited in $NH_4^+$, $NO_3^-$, and other forms of fixed nitrogen, thus selecting for nitrogen-fixing bacteria. Figure 19.22 contrasts the environmental genomic approach with single-gene analysis of microbial communities.

Although metagenomics can reveal much about a microbial habitat, there are many things metagenomics cannot tell us about environmental microbial communities. Currently no methods are available for translating metagenomic sequence data into fundamental physiological information about the microbial community, such as the maximum specific growth rate of different species, their saturation constants for nutrients, their optimum, minimum, and maximum pH or temperature for growth, or their speed of recovery from starvation. Moreover, any metabolisms never before encountered are unlikely to be deduced from nucleotide sequence data alone. These realities once again underscore why it is important to culture new microbes from nature; at present, there is simply no substitute for culture-based characterization to define many critical aspects of a microbe's functional biology.

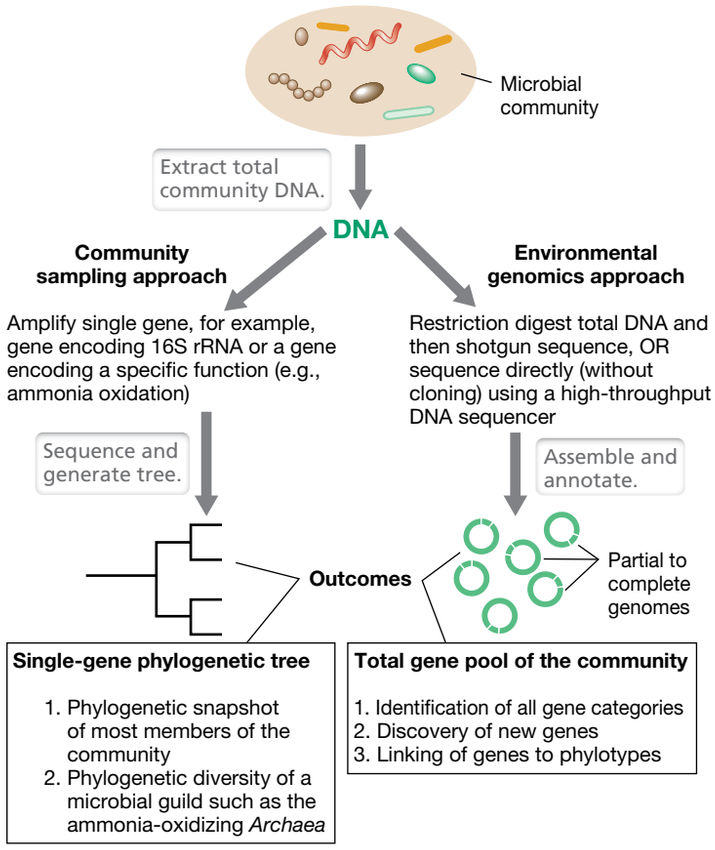



**Figure 19.22 Single-gene versus environmental genomic approaches to microbial community analysis.** In the environmental genomic approach, all community DNA is sequenced, but the assembled genomes may not all be complete. Total gene recovery is variable and depends on several factors including the complexity of the habitat and the amount of sequence determined. Recovery is typically better when diversity is low and sequence redundancy is high.

## Some Examples of Environmental Genomics

Environmental genomics can detect both new genes in known organisms and known genes in new organisms. A large number of interesting microbial communities have been probed using early metagenomic tools. In an early study of bacterial and archaeal diversity in the Sargasso Sea (a low-nutrient region of the Atlantic Ocean near Bermuda), over one billion nucleotides were sequenced and from this over 1800 bacterial and archaeal species were detected, including 148 previously unknown phylotypes and many novel genes. Many of these species had previously been missed using rRNA community analyses that employed PCR and cloning or PCR and DGGE (Section 19.6). Genes that fail to amplify, of course, remain undetected in community analyses, and cloning efficiency is far from 100%. Metagenomics sidesteps these problems by sequencing DNA directly without the need to amplify it or resolve different phylotypes before sequencing.

The Sargasso Sea metagenome study revealed several novel findings such as the presence of ammonia-oxidizing genes in archaeal genomes, a result that led to the discovery of a new group of *Archaea*, the *Thaumarchaeota* (Section 17.5). Moreover, genes encoding *proteorhodopsin*, a light-sensitive proton pump present in certain *Proteobacteria* and related to bacteriorhodopsin of extreme halophiles (Section 17.1), were found in the genomes of several new phylogenetic lineages of *Bacteria*. However, despite this major sequencing undertaking, much was missed. This is because 1 milliliter of seawater contains approximately 5 *trillion* base pairs (bp) of bacterial genomic DNA and would therefore require 5000 times the sequencing effort just to cover each base pair once on average! Hence, even with current technology—which can generate over a trillion bp of sequence in a few days (Figure 19.18)—no one natural environment has yet been sequenced completely.

Genomic/metagenomic approaches have also revealed variations in genes associated with a single phylotype; that is, in strains that contain identical, or nearly identical, rRNA genes. For example, in studies of *Prochlorococcus*, the most abundant cyanobacterium (oxygenic phototroph) in the ocean (Section 15.3), comparison of the genome sequences of cultured strains with *Prochlorococcus* genes obtained from metagenomic analyses of ocean water identified extensive regions shared between the cultured and environmental populations (**Figure 19.23**). This high level of gene conservation confirms that the organisms in culture are typical of environmental populations. In addition, however, these analyses also identified several highly variable regions in which the genomes of cultured strains differed significantly from those of environmental populations. These variable regions were clustered in the genome as *genomic islands* (chromosomal islands, Section 9.7), and likely encode functions that control the growth response of particular *Prochlorococcus* populations to environmental variables such as temperature or light quality and intensity.

## Metatranscriptomics and Metaproteomics

As we discussed in Chapter 9, the genomics era has spawned several additional "omics," in particular, *metatranscriptomics* and *metaproteomics*. **Metatranscriptomics** is analogous to metagenomics but analyzes the sequences of community *RNA* rather than community *DNA*. The isolated RNA is converted into cDNA by reverse transcription (Section 10.11) before sequencing and analysis as for DNA. Whereas metagenomics describes the functional capacities of the community (for example, the relative abundance of specific genes), metatranscriptomics reveals which genes in the community *are actually being expressed* and the relative level of that expression, at a specific time and place. Because the expression of most genes in *Bacteria* and *Archaea* is controlled at the level of transcription (Chapter 6), mRNA abundance can be taken as a census of individual gene expression levels. Thus, gene transcript abundance determined for an entire community can be used to infer the operation of major

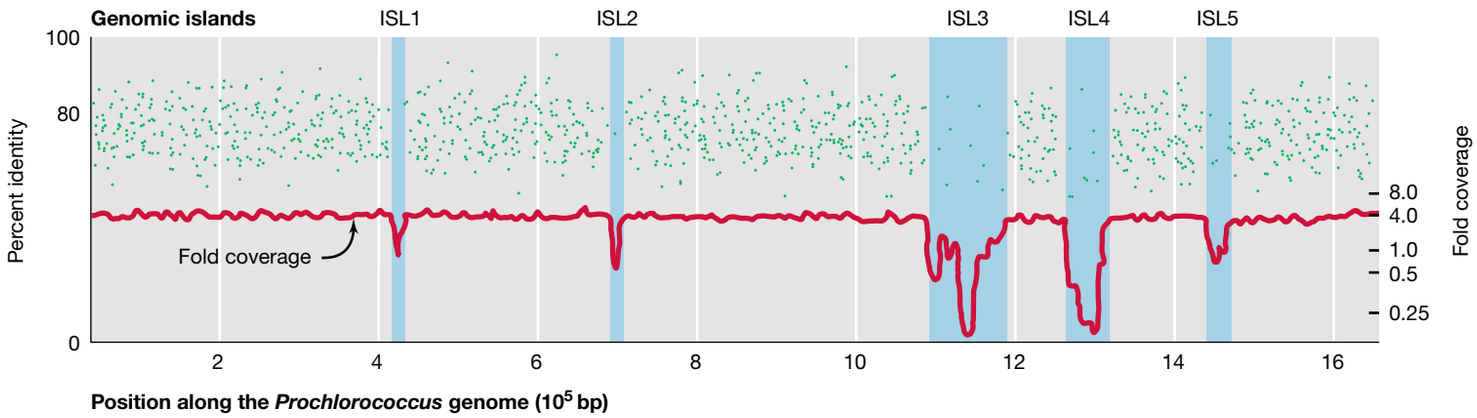



**Figure 19.23 Metagenomic analysis.** Sequences (represented as green dots) from the Sargasso Sea metagenome that align to the genome sequence of a cultured *Prochlorococcus*, showing regions where the cultured strain has genes of high similarity (high % identity) with sequences in the metagenome, and other regions (shaded) where it lacks genes in common (genomic islands, ISL1–ISL5). Since the DNA sequence contained within the genomic islands is thought to encode niche-specific functions, the cultured strain would likely not exhibit the same environmental distribution as strains containing all the island genes. Fold coverage is a measure of how completely the various regions in the *Prochlorococcus* genome are accounted for by similar sequences in the metagenome.

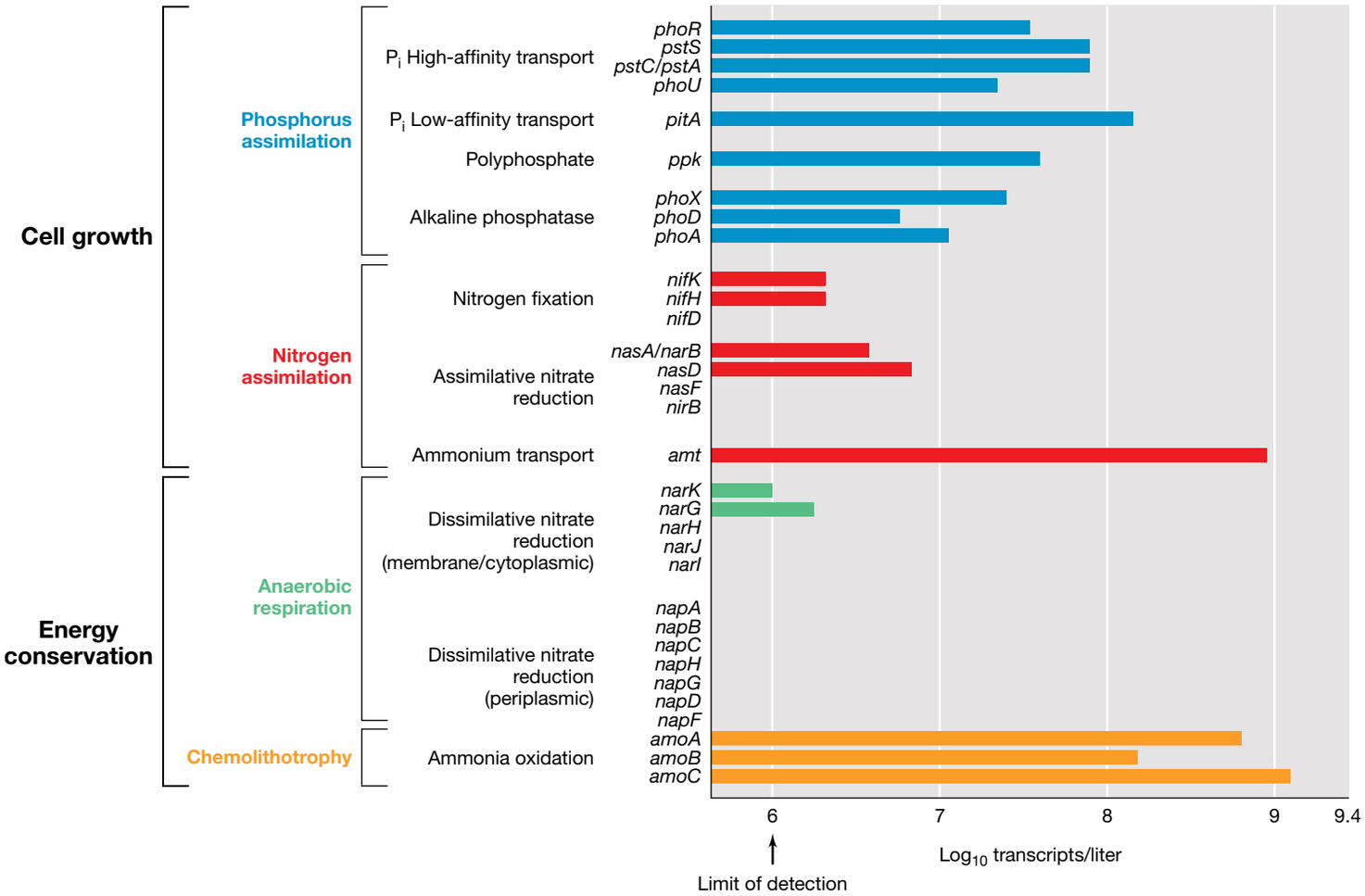



**Figure 19.24 Metatranscriptomic analysis of coastal marine surface waters.** Expression of genes for key steps in the N and P cycle in a seawater sample determined by sequencing environmental mRNA. These data showed that the microbial community was using both inorganic (high expression of P transporters) and organic (alkaline phosphatase) forms of phosphate ($PO_4^{3-}$). Low levels of transcripts for genes required for $NO_3^-$ assimilation contrasted with the high expression of genes for $NH_3$ transport and chemolithotrophic $NH_3$ oxidation. Also, as expected for oxic marine surface waters, there was little expression of genes for $NO_3^-$ respiration. Data courtesy of Mary Ann Moran, University of Georgia Marine Sciences.

metabolic processes catalyzed by that community at the time of sampling (Figure 19.24).

**Metaproteomics**, the measure of the diversity and abundance of different *proteins* in a community, is an even more direct measure of cell function than is metatranscriptomics. This is because different mRNAs have different half-lives and efficiencies of translation, and thus will not all yield the same number of protein copies. However, metaproteomics is more of a technical challenge than is either metagenomics or metatranscriptomics. Protein identification, usually by mass spectrometric characterization of peptides released from enzymatic digestion of the total protein pool using specific proteases (Section 9.10), relies on naturally available material since it is not possible to amplify protein sequences as one does using PCR to amplify nucleic acids for sequencing. Protein identification also requires at least partial separation of the individual peptides in order to reduce complexity of the sample as well as a reference genome or metagenome to identify potential coding sequences. As a consequence, as a tool in microbial ecology, metaproteomics has thus far been mainly restricted to the qualitative characterization of rather simple microbial communities, such as those in some extreme environments, or to the characterization of only very abundant proteins in more complex microbial communities.

## MINIQUIZ

- What is a metaproteome, and how does it differ from a metagenome and from a metatranscriptome?
- How do environmental genomic approaches differ from environmental single-gene analyses, such as that based on 16S rRNA gene analysis for microbial community characterization?
- How can the most metabolically active cell populations in a community be identified using environmental omics methods?

# IV • Measuring Microbial Activities in Nature

Up until now our emphasis has been on measuring microbial phylogenetic and genomic *diversity*. We wrap up this chapter by considering how microbial ecologists measure microbial *activity;* that is, what microorganisms are actually *doing* in their environment. The techniques we consider include the use of radioisotopes, microsensors, stable isotopes, and several genomic methods.

Activity measurements in a natural sample are *collective* estimates of the physiological reactions occurring in the entire microbial community, although several techniques to be discussed later (see Sections 19.10–19.12) allow for a more targeted assessment of physiological activity. Activity measurements reveal both the types and rates of the major metabolic reactions occurring in a habitat, and the various techniques can be used alone or in combination in microbial community analyses. In conjunction with biodiversity estimates and gene expression analyses, these help define the structure and function of the microbial ecosystem, the ultimate goal of microbial ecology. Activity measurements can also provide valuable information for the design of enrichment cultures.

## 19.9 Chemical Assays, Radioisotopic Methods, and Microsensors

In many studies, direct chemical measurements of microbial reactions are sufficient for assessing microbial activity in an environment. For example, the fate of lactate oxidation by sulfate-reducing bacteria in a sediment sample can be tracked easily. If sulfate-reducing bacteria are present and active in a sediment sample, then lactate added to the sediment will be consumed and $SO_4^{2-}$ will be reduced to $H_2S$. Since lactate, $SO_4^{2-}$, and $S^{2-}$ can all be measured with fairly high sensitivity using simple chemical assays, the transformations of these substances relative to one another in a sample can easily be followed (**Figure 19.25*a***).

### Radioisotopes

When very high sensitivity is required, or turnover rates need to be determined, or the fate of portions of a molecule needs to be followed, *radioisotopes* are more useful than strictly chemical assays. For instance, if measuring photoautotrophy is the goal, the light-dependent uptake of radioactive carbon dioxide ($^{14}CO_2$) into microbial cells can be measured (Figure 19.25*b*). If $SO_4^{2-}$ reduction is of interest, the rate of conversion of $^{35}SO_4^{2-}$ to $H_2{}^{35}S$ can be assessed (Figure 19.25*c*). Heterotrophic activities can be measured by tracking the release of $^{14}CO_2$ from $^{14}$C-labeled organic compounds (Figure 19.25*d*), and so on.

Both isotopic and chemical methods are widely used in microbial ecology. To be valid, however, these must employ proper controls because some isotopic transformations might be due to abiotic processes. The *killed cell control* is the key control in such experiments. That is, it is essential to show that the transformation being measured stops when chemical agents or heat treatments that kill microorganisms are applied to the sample. Formalin at a final concentration of 4% is commonly used as a chemical sterilant in microbial ecology studies. This kills all cells, and transformations that are insensitive to the presence of 4% formalin can be ascribed to abiotic processes (Figure 19.25*a*).

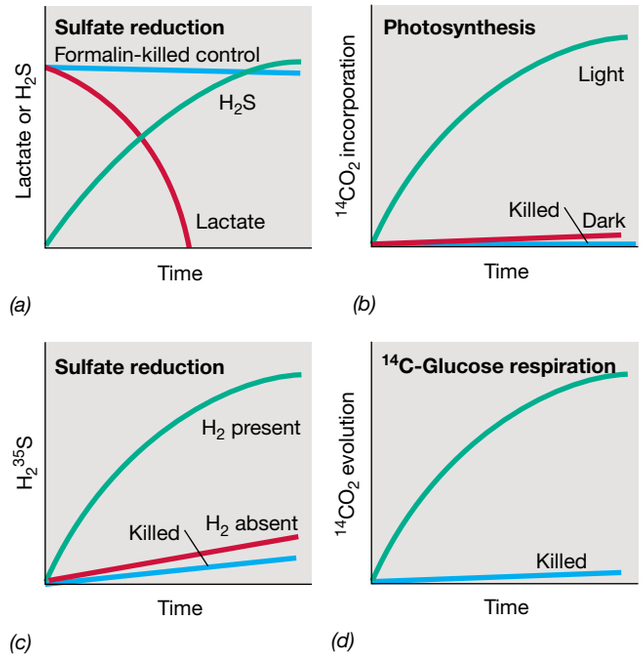



**Figure 19.25 Microbial activity measurements.** *(a)* Chemical measurements of lactate and $H_2S$ transformations during $SO_4^{2-}$ reduction. Radioisotopic measurements: *(b)* photosynthesis measured with $^{14}CO_2$; *(c)* $SO_4^{2-}$ reduction measured with $^{35}SO_4^{2-}$; *(d)* production of $^{14}CO_2$ from $^{14}$C-glucose.

### Microsensors

**Microsensors** in the form of glass needles containing a sensing mechanism at the tip have been used to study the activity of microorganisms in nature. Microsensors have been constructed that measure many chemical species including pH, $O_2$, $NO_2^-$, $NO_3^-$, nitrous oxide ($N_2O$), $CO_2$, $H_2$, and $H_2S$. As the name *micro*-sensor implies, these devices are very small, their tips ranging in diameter from 2 to 100 µm (**Figure 19.26**). The sensors are carefully inserted into the habitat in small increments to follow microbial activities over very short distances.

Microsensors have many applications. For example, $O_2$ concentrations in microbial mats (Figure 20.7*c*), aquatic sediments, or soil particles (Figure 20.3) can be very accurately measured over extremely fine intervals using microsensors. A micromanipulator is used to insert the sensors gradually through the sample such that measurements can be taken every 50–100 µm (**Figure 19.27**). Using a bank of microsensors, each sensitive to a different chemical, simultaneous measurements of several transformations in a habitat can be made.

Microbial processes in the sea are extensively studied because they have a profound impact on nutrient cycles and the overall health of the planet. As it is difficult to reproduce in the laboratory the conditions found at great depths, it is useful to use microsensors on robotic devices to analyze microbial activities on the seafloor. **Figure 19.28** shows deployment of an instrument "lander" equipped with various microsensors so that the distribution of

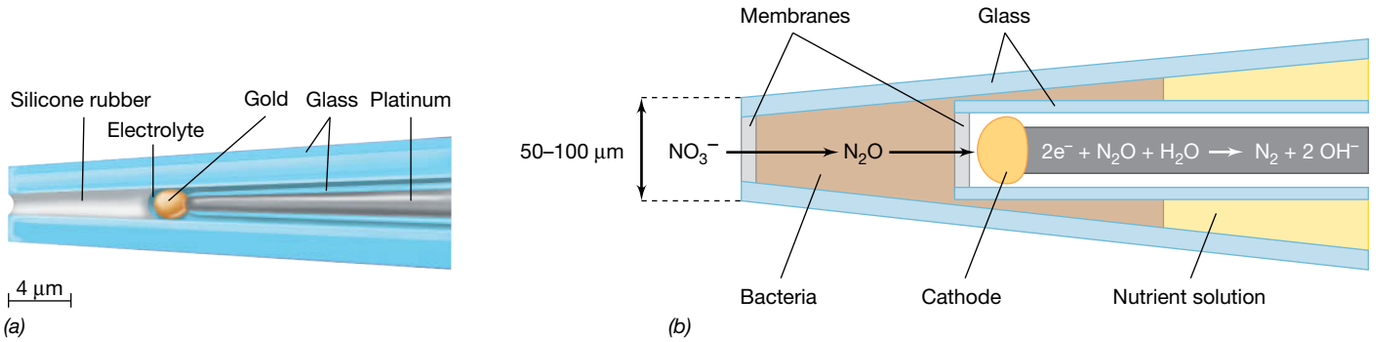



**Figure 19.26 Microsensors.** *(a)* Schematic drawing of an oxygen ($O_2$) microsensor. Oxygen diffuses through the silicone membrane in the microsensor tip and reacts with electrons on the gold surface of the cathode, forming hydroxide ions ($OH^-$); the latter generates a current proportional to the $O_2$ concentration in the sample. Note the scale of the electrode. *(b)* Biological microsensor for the detection of nitrate ($NO_3^-$). Bacteria immobilized at the sensor tip denitrify $NO_3^-$ or $NO_2^-$ to $N_2O$, which is detected by electrochemical reduction to $N_2$ at the cathode. Based on drawings by Niels Peter Revsbech.

chemicals in the sediment can be analyzed and compared with that in overlying ocean water.

One of the biologically most important chemical species in the oceans is $NO_3^-$, but electrochemical sensors cannot measure $NO_3^-$ in seawater, as the high concentrations of salts interfere. To circumvent this problem, a "living" microsensor was designed that contains bacteria within its tip that reduce $NO_3^-$ (or $NO_2^-$) to $N_2O$. The $N_2O$ produced by the bacteria is then detected following its abiotic reduction to $N_2$ at the cathode of the microsensor (Figure 19.26*b*); this provides an electrical impulse signaling the presence of $NO_3^-$. In the oxic layer of marine sediments, $NO_3^-$ is produced from the oxidation of $NH_4^+$ (nitrification, Section 14.11), so there is often a peak of $NO_3^-$ in the sediment

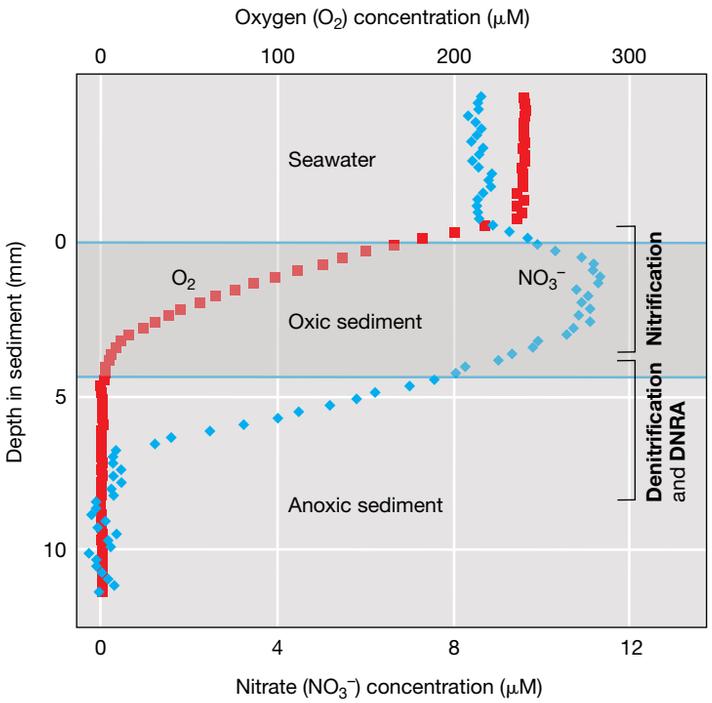



**Figure 19.27 Depth profiles of $O_2$ and $NO_3^-$.** Data obtained using the lander shown in Figure 19.28 equipped with microelectrode sensors for remote chemical characterization of deep-sea sediments. Note the zones of nitrification and denitrification. DRNA, dissimilative reduction of $NO_3^-$ to $NH_4^+$. Based on data and drawings by Niels Peter Revsbech.


**Figure 19.28 Deployment of a deep-sea lander.** The lander is equipped with a bank of microsensors (arrow) to measure distribution of chemicals in marine sediments.

surface layer (Figure 19.27). In the deeper, anoxic layers of the sediment, $NO_3^-$ is consumed by denitrification and dissimilative nitrate reduction to ammonia (DRNA) (Section 14.13), and $NO_3^-$ therefore disappears a few millimeters below the oxic–anoxic interface (Figure 19.27).

**MINIQUIZ**

- How can a microbial ecologist be sure that transformation of a radioisotope is actually caused by microbes?
- If a large pulse of organic matter entered the sediment, how would that change the profiles of $NO_3^-$ and $O_2$ shown in Figure 19.27?

## 19.10 Stable Isotopes and Stable Isotope Probing

Many of the chemical elements have more than one isotope, which differ in their number of neutrons. Certain isotopes are unstable and break down as a result of radioactive decay. Others, called *stable isotopes*, are not radioactive, but are metabolized differently by microorganisms and can be used to study microbial transformations in nature. There are two methods in which stable isotopes can yield information on microbial activities, *isotopic fractionation* and *stable isotope probing*.

### Isotopic Fractionation

The two elements most useful for stable isotope studies in microbial ecology are carbon (C) and sulfur (S), although the heavy isotope of nitrogen, $^{15}N$, is also widely used. Carbon (C) exists in nature primarily as $^{12}C$, but about 5% exists as $^{13}C$. Likewise, S with its four stable isotopes exists primarily as $^{32}S$. Some S is found as $^{34}S$, and very small amounts as $^{33}S$ and $^{36}S$. The relative abundance of these isotopes changes when certain C or S compounds are metabolized by microorganisms because the enzymes that act on these compounds typically favor the *lighter* isotope of C or S. That is, relative to the lighter isotope, the heavier isotope is discriminated against when both are metabolized by an enzyme (Figure 19.29).

For example, when $CO_2$ is fixed into cell material by an autotrophic organism, the cellular C becomes *enriched* in $^{12}C$ and *depleted* in $^{13}C$, relative to an inorganic carbon standard of known isotopic composition. Likewise, the S atom in $H_2S$ produced from the bacterial reduction of $SO_4^{2-}$ is isotopically lighter than $H_2S$ that has formed geochemically. These discriminations are called **isotopic fractionations** (Figure 19.29) and are typically the result of biological activities. Thus this technique can be used as a measure of whether or not a particular transformation has been catalyzed by microorganisms.

The isotopic fractionation of C in a sample is calculated as the extent of $^{13}C$ depletion relative to a standard having an isotopic composition of geological origin. The standard for C isotope analysis is rocks from a Cretaceous (65- to 150-million-year-old) limestone formation (the Pee Dee belemnite). Because the magnitude of fractionation is usually very small, depletion is calculated as "per mil" (‰, or parts per thousand) and reported as the $\delta^{13}C$ (pronounced "delta C 13") of a sample using the following formula:

$$\delta^{13}C = \frac{(^{13}C/^{12}C\,\text{sample}) - (^{13}C/^{12}C\,\text{standard})}{(^{13}C/^{12}C\,\text{standard})} \times 1000‰$$

The same formula is used to calculate the fractionation of S isotopes, in this case using iron sulfide (FeS) mineral from the Canyon Diablo meteorite as the standard:

$$\delta^{34}S = \frac{(^{34}S/^{32}S\,\text{sample}) - (^{34}S/^{32}S\,\text{standard})}{(^{34}S/^{32}S\,\text{standard})} \times 1000‰$$

### Use of Isotopic Fractionation in Microbial Ecology

The isotopic composition of a material can reveal its biological or geological past. For example, plant material and petroleum (which is derived from plant material) have similar isotopic compositions (Figure 19.30). Carbon from both plants and petroleum is isotopically

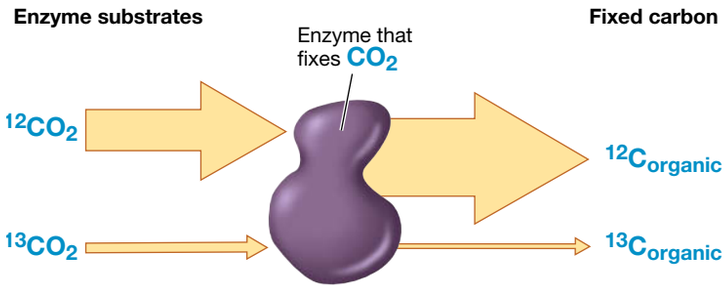



**Figure 19.29 Mechanism of isotopic fractionation with C as an example.** Enzymes that fix $CO_2$ preferentially fix the lighter isotope ($^{12}C$). This results in fixed carbon being enriched in $^{12}C$ and depleted in $^{13}C$ relative to substrate $CO_2$. The size of the arrows indicates the relative abundance of each isotope of carbon.

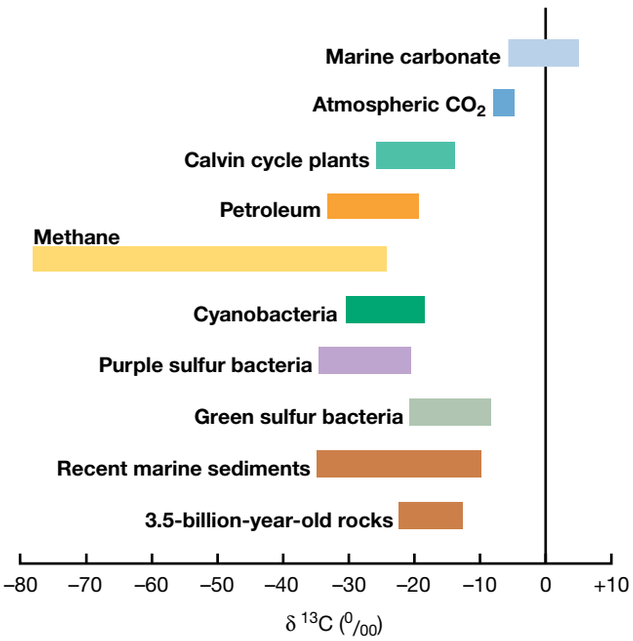



**Figure 19.30 Isotopic geochemistry of $^{13}C$ and $^{12}C$.** Note that C fixed by autotrophic organisms is enriched in $^{12}C$ and depleted in $^{13}C$. Methane formed from the reduction of $CO_2$ with $H_2$ by methanogenic *Archaea* shows extreme isotopic fractionation.

lighter than the $CO_2$ from which it was formed because the biochemical pathway used to fix $CO_2$ discriminated against $^{13}CO_2$ (Figures 19.29 and 19.30). Moreover, methane ($CH_4$) produced by methanogenic *Archaea* (Section 17.2) is isotopically extremely light, indicating that methanogens discriminate strongly against $^{13}CO_2$ when they reduce $CO_2$ to $CH_4$ (Section 14.17). By contrast, carbon in isotopically heavier marine carbonates is clearly of geological origin (Figure 19.30).

Because of the differences in the proportion of $^{12}C$ and $^{13}C$ in carbon of biological versus geological origin, the $^{13}C/^{12}C$ ratio of rocks of different ages has been used as evidence for or against past biological activity in Earth's ancient environments. Organic C in rocks as old as 3.5 billion years shows evidence of isotopic fractionation (Figure 19.30), supporting the idea that autotrophic life existed at this time. Indeed, we now believe that the first life on Earth appeared somewhat before this, about 3.8–3.9 billion years ago (Sections 1.3 and 13.1).

The activity of sulfate-reducing bacteria is easy to recognize from their fractionation of stable S isotopes in sulfides (**Figure 19.31**). As compared with an $H_2S$ standard, sedimentary $H_2S$ is highly enriched in $^{32}S$ (depleted in $^{34}S$, Figure 19.31). Fractionation during sulfate reduction allows one to identify biologically produced S and has been widely used to trace the activities of sulfur-cycling *Bacteria* and *Archaea* through geological time. Sulfur isotopic analyses have also been used as evidence for the lack of life on the Moon. For example, the data in Figure 19.31 show that the isotopic composition of sulfides in lunar rocks closely approximates that of the $H_2S$ standard, which represents primordial Earth, and differs from that of microbially produced $H_2S$.

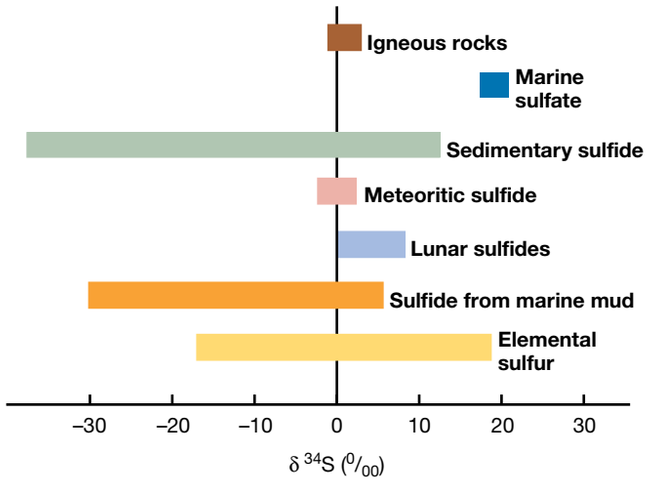



**Figure 19.31 Isotopic geochemistry of $^{34}S$ and $^{32}S$.** Note that $H_2S$ and $S^0$ of biogenic origin are enriched in $^{32}S$ and depleted in $^{34}S$.

## Stable Isotope Probing

Beyond stable isotope fractionation, an alternative stable isotope method called **stable isotope probing (SIP)** can be used to identify an organism or organisms carrying out the transformation of a nutrient labeled with a specific stable heavy isotope, such as $^{13}C$ or $^{15}N$ or even $^{18}O$ (the lighter, more common isotopes of these elements are $^{12}C$, $^{14}N$, and $^{16}O$, respectively). The idea behind SIP is that the label will be selectively incorporated only into the cellular material of organisms actively metabolizing the nutrient. Then, following the isolation and sequencing of isotopically labeled DNA, the organisms carrying out the transformation can be identified.

Stable isotope probing can be used to ask general to more specific questions. For example, if $^{13}C$-labeled benzoate were added to a sediment sample and the sample incubated for an appropriate period, the $^{13}C$ label would end up in the DNA of the organism (or organisms) that metabolized the benzoate (**Figure 19.32**). Thus, although all DNA from the sample would be isolated, the $^{13}C$-DNA is heavier, albeit only slightly heavier, than $^{12}C$-DNA, and this difference is sufficient to separate the DNAs by a special type of centrifugation technique (Figure 19.32). Once the $^{13}C$-DNA is isolated, it can be analyzed for phylogenetic genes or metabolic genes to yield genomic information about the benzoate degrader(s) in the sample. If instead of an organic compound, the experimental study focused on nitrogen fixation (conversion of $N_2$ to cell nitrogen, Section 14.6), $^{15}N_2$ could be supplied to a sample. When nitrogen fixers in the sample incorporate this, they will produce

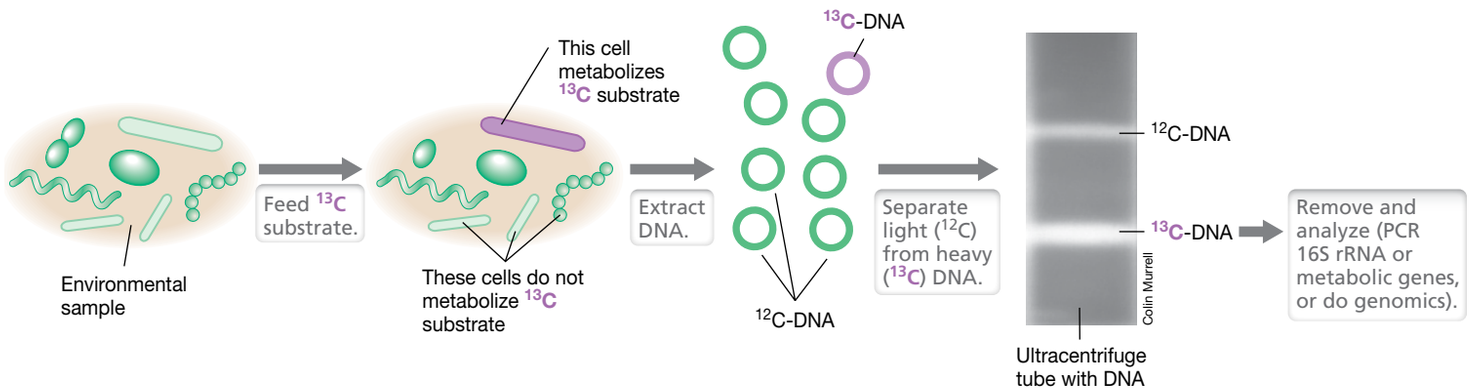



**Figure 19.32 Stable isotope probing.** The microbial community in an environmental sample is fed a $^{13}C$ substrate. Organisms that can metabolize the substrate produce $^{13}C$-DNA as they grow and divide; $^{13}C$-DNA can be separated from lighter $^{12}C$-DNA by density gradient centrifugation (photo). The isolated DNA is then subjected to specific gene analysis or entire genomic analysis.

UNIT 5

slightly heavier DNA than organisms that cannot fix nitrogen, and the heavier DNA can be isolated and analyzed for genes of interest.

SIP can also be used in combination with metagenomics to pinpoint organisms carrying out a specific metabolism (from SIP results) in the context of all the other species and metabolisms present in the sample (as revealed by metagenomic results). Besides a phylogenetic "hit" from the SIP results, additional genomic analyses of the labeled DNA could reveal functional genes required for the specific metabolism. Moreover, the phylogenetic and functional results from the SIP experiment could be further confirmed from the metagenomic profile.

**MINIQUIZ**

- What is the simplest explanation for why lunar sulfides are isotopically similar to those of the primordial Earth?
- What is the expected isotopic composition of carbon in methanotrophs (bacteria that consume $CH_4$)?
- How might exchange of metabolites among members of a microbial community complicate interpretation of an SIP experiment?

## 19.11 Linking Functions to Specific Organisms

The isotopic methods described thus far used samples containing large numbers of cells to infer that specific metabolisms were occurring within a community or in particular species within the community. These methods give an overview of community activities but do not reveal the contribution of individual cells. To do this, new methods have been developed that can measure the activity and the elemental and isotopic composition of single cells. These are powerful methods for connecting cells of a specific microbial population with a specific activity or ecological niche, but in most cases, the phylogeny of the organisms of interest must be known such that the necessary FISH probes (Section 19.5) can be developed.

### Single-Cell Metabolisms Imaged by Secondary Ion Mass Spectrometry (SIMS)

*Secondary ion mass spectrometry* (*SIMS*) is based on the detection of ions released from a sample placed under a focused high-energy primary ion beam, for example, of cesium ($Cs^+$); from the data generated, the elemental and isotopic composition of released materials can be obtained. When the primary ion beam impacts the sample, most chemical bonds are broken and atoms or polyatomic fragments are ejected from a very thin layer (1–2 nm) of the surface as either neutral or charged particles (secondary ions), a process called *sputtering*. These secondary ions are directed to a mass spectrometer, an instrument that can determine their mass-to-charge ratio.

NanoSIMS instruments are SIMS devices designed to yield information on single cells. The instrument is equipped with $Cs^+$ and $O_2$ primary beam sources with a resolution of 50 nm for the $Cs^+$ ion beam and 200 nm for the $O_2$ beam. The $O_2$ beam generates positive secondary ions and is used to analyze metals (e.g., Fe, Na, Mg) while the $Cs^+$ beam generates negative secondary ions for the analysis of major cellular elements (C, N, P, S, O, H) and halogens. The NanoSIMS instrument also records where on the specimen the ion beam is directed such that a two-dimensional image of the distribution of specific ions on the sample surface is obtained. In addition, by focusing the ion beam on the same spot during repeated cycles of sputtering, material can be slowly burned away to expose deeper regions of the sample. This high-resolution SIMS analysis is where the term *NanoSIMS* got its name. NanoSIMS instruments have multiple detectors that provide for the simultaneous analysis of ions of different mass-to-charge ratios originating from the same sample location (**Figure 19.33*a***).

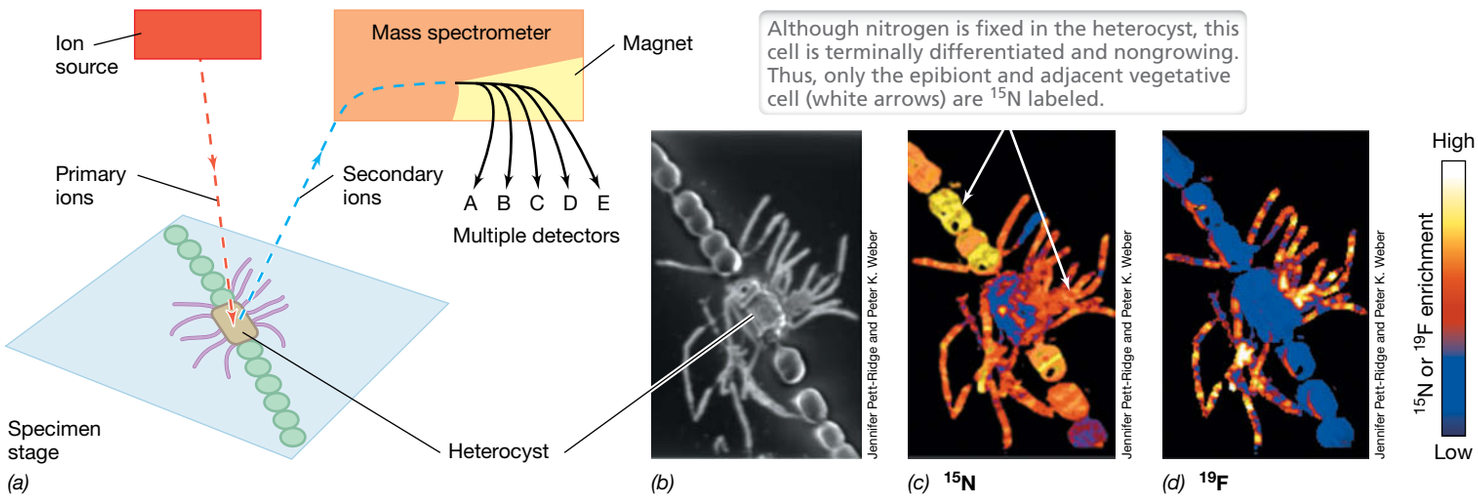



**Figure 19.33 NanoSIMS technology.** *(a)* Schematic of NanoSIMS operation showing the beams of primary (red) and secondary (blue) ions and five different detectors, each of which identifies ions of a different mass-to-charge ratio. *(b–d)* Demonstration of interspecies nutrient transfer from a filamentous cyanobacterium (*Anabaena*) to a *Rhizobium* species attached to the cyanobacterial heterocyst. The coculture was incubated with $^{15}N_2$, and the transfer of $^{15}N$-labeled compounds from *Anabaena* to *Rhizobium* was imaged using a combination of EL-FISH and NanoSIMS. *(b)* Total $^{12}C$ abundance shown by gray tones. *(c)* $^{15}N$ enrichment. *(d)* $^{19}F$ abundance conferred by a probe that hybridizes only to the attached rhizobial cells (EL-FISH).

When NanoSIMS is combined with FISH (Section 19.5) in a technology called *FISH-SIMS*, the incorporation of different elements, natural isotopes, or isotope-labeled substrates can be tracked into individual cells of specific cell populations. A variation on the FISH-SIMS method that simplifies the identification of cells scanned by NanoSIMS uses probe-conferred deposition of a halide (Br, Fl, I), either through direct incorporation of the halide into an oligonucleotide probe or by using a halide-containing tyramide substrate (see CARD-FISH, Section 19.5). Halogens possess a high ionization yield compared with other elements and are thus easy to detect and are typically of low natural abundance. Using this technology, one of the NanoSIMS detectors is dedicated to identifying cells to which the probe has hybridized (Figure 19.33*d*) by halogen ionization while the remaining detectors are used for assessing elemental composition (Figure 19.33*c*).

Because of its excellent spatial resolution, NanoSIMS technology is being increasingly used to examine metabolite transfer among single cells of interacting microorganisms. For example, labeling with $^{15}N_2$ followed by NanoSIMS was used to demonstrate the transfer of $N_2$ fixed by filamentous cyanobacteria to attached heterotrophic bacteria (Figure 19.33*c*). Labeling with $^{15}NH_4$ and $^{13}C$-labeled $CO_2$ or organic substrates is also being used to explore the assimilation of key nutrients and the transfer of metabolites among microbial species in both aquatic and soil microbial communities.

## Raman Microspectroscopy

*Raman microspectroscopy* can be used to characterize the molecular and isotopic composition of single cells by nondestructive illumination with monochromatic light generated by a laser. Raman is a form of spectroscopy that measures light scattering and can yield both qualitative and quantitative results. Compositional analysis is based on photon scattering after interaction with different cellular components. Although most of the scattered photons have the same energy as the incident photons, a small fraction of scattered photons are shifted in wavelength (relative to the incident wavelength) to either a longer wavelength (a phenomenon known as *Stokes Raman scattering*) or shorter wavelength (*anti-Stokes scattering*).

Raman spectrometers separate the more abundant Stokes scattered photons for analysis and, when combined with confocal microscopy (Section 1.7), can generate a compositional spectrum of a single microbial cell (**Figure 19.34**). Although the spectrum is complex, several compounds and molecules have characteristic peaks that can identify specific cell types, physiological states, or metabolic activities following incorporation of compounds labeled with stable isotopes.

Major advantages of Raman microspectroscopy include the following: It is nondestructive and can be used on living cells; water does not cause interference; it can be combined with FISH; and cells of interest can be further manipulated, for example, through capture by laser tweezers (Section 19.3). In the example of Raman shown in Figure 19.34, the incorporation of phenylalanine (an amino acid) by an obligate chlamydial symbiont of an amoeba has been tracked following addition of phenylalanine labeled with $^{15}N$ and $^{13}C$ to the amoeba growth medium. A less specific but still useful application of Raman is based on the incorporation of heavy protons from deuterated water ($^2H_2O$). Since water is a reactant in many biochemical reactions, metabolically active microorganisms that incorporate the heavy hydrogen atom can be identified by Raman microspectroscopy for further processing, including characterization by single-cell genomics (Section 19.12).

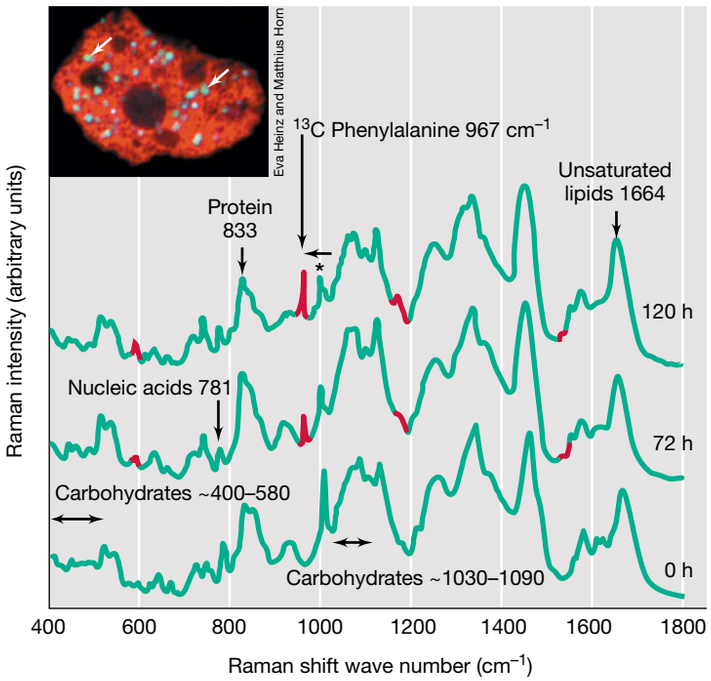
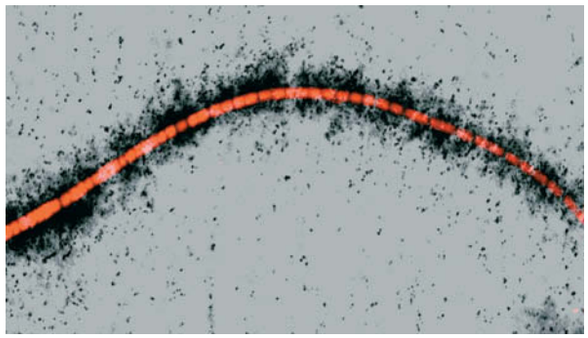


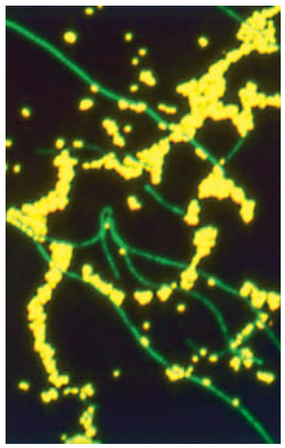

**Figure 19.34 Raman microspectroscopic analysis of single cells.** A cell of *Acanthamoeba* containing FISH-stained chlamydial symbionts (inset photo, arrows point to blue chlamydial cells) and Raman spectroscopy of isolated chlamydial cells. After incubation for 72 and 120 h in medium containing $^{13}C$ phenylalanine, symbionts were released by lysis of the amoeba and their Raman spectra recorded. Peaks diagnostic for labeled phenylalanine are shown in red. Since the Raman wave number of $^{13}C$ phenylalanine at 967 cm$^{-1}$ is well resolved from other spectral features, the ratio of 1003 cm$^{-1}$ (unlabeled phenylalanine, at *) to 967 cm$^{-1}$ (labeled phenylalanine) peak areas corresponds to the relative amount of labeled phenylalanine incorporated by single cells (note how this increases with time). Other red peaks correspond to spectral peaks specific for different chemical bonds in $^{13}C$ phenylalanine. Also shown are selected peaks and their wave numbers for other cellular components of the symbiont.

## Radioisotopes in Combination with FISH: Microautoradiography-FISH

Radioisotopes are used as measures of microbial activity in a microscopic technique called **microautoradiography (MAR)**. In this method, cells from a microbial community are exposed to a substrate containing a radioisotope, such as an organic compound or $CO_2$. Heterotrophs take up the radioactive organic compounds and autotrophs take up the radioactive $CO_2$. Following incubation in the substrate, cells are affixed to a slide and the slide is dipped in photographic emulsion. While the slide is left in darkness for a period, radioactive decay from the incorporated substrate induces formation of silver grains in the emulsion; these appear as black




(a)

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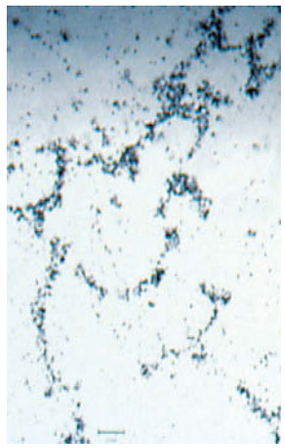



(c)

**Figure 19.35 MAR-FISH.** Fluorescence in situ hybridization (FISH) combined with microautoradiography (MAR). *(a)* An uncultured filamentous cell belonging to the *Gammaproteobacteria* (as revealed by FISH) is shown to be an autotroph (as revealed by MAR-measured uptake of $^{14}CO_2$). *(b)* Uptake of $^{14}$C-glucose by a mixed culture of *Escherichia coli* (yellow cells) and *Herpetosiphon aurantiacus* (filamentous green cells). *(c)* MAR of the same field of cells shown in part *b*. The radioactivity of incorporated glucose exposes the film and shows that glucose was assimilated mainly by cells of *E. coli*.

dots above and around the cells. Figure 19.35*a* shows a MAR experiment in which an autotrophic cell has taken up $^{14}CO_2$.

Microautoradiography can be done simultaneously with FISH (Section 19.5) in **MAR-FISH**, a powerful technique that combines identification with activity measurements. MAR-FISH allows a microbial ecologist to determine (by MAR) which organisms in a natural sample are metabolizing a particular radiolabeled substance while at the same time identifying these organisms (by FISH) (Figure 19.35). MAR-FISH thus goes a step beyond phylogenetic identification by revealing physiological information about the organisms, as is also true of NanoSIMS. Such data are useful not only for understanding the activity of the microbial ecosystem but also for guiding enrichment cultures. For example, knowledge of the phylogeny and morphology of an organism metabolizing a particular substrate in a natural sample can be used to design an enrichment protocol to isolate the organism. In addition, MAR-FISH results can be quantified by counting the silver grains as a measure of the amount of substrate consumed by single cells, allowing the activity distribution in a community to be described. The technique is limited only by the availability of suitable radioactive isotopes. For example, although C-labeled substrates work well, it is not feasible to track N incorporation using MAR-FISH because the radioactive isotope $^{13}$N has a very short half-life. However, it is feasible to track N incorporation using the nonradioactive stable isotope $^{15}$N with NanoSIMS, as we saw earlier (Figure 19.33).

**MINIQUIZ**

- How could NanoSIMS be used to identify a nitrogen-fixing bacterium?
- Why is Raman microspectroscopy suited for the selective isolation of microorganisms and NanoSIMS is not?
- How does MAR-FISH link microbial diversity and activity?

## 19.12 Linking Genes and Cellular Properties to Individual Cells

We have seen in the previous section how the combination of FISH with MAR or FISH with NanoSIMS allows for analyses of both microbial diversity and activity. Coupled with advanced DNA sequencing methods that can determine a genome sequence from the DNA contained in a single cell (Section 9.12), these techniques are at the cutting edge of microbial ecology today. Improvements in single-cell DNA sequencing technology, combined with high-throughput analysis and isolation of single cells by flow cytometry, now allow for gene identification and selected physiological analyses (e.g., size and intrinsic fluorescence) to be performed on selected populations and single cells in the environment.

### Flow Cytometry and Multiparametric Analyses

Because of the large population sizes of natural microbial communities, methods that rely on microscopy can examine only a very small part of a whole community. It is difficult to assess cell numbers by counting cells microscopically, and this problem is compounded if populations are present in low numbers. However, flow cytometry (Section 19.3) offers an alternative to more labor-intensive microscopic methods.

Flow cytometers can examine specific cell parameters such as size, shape, or fluorescent properties as the cells pass through a detector at rates of many thousands of cells per second (Figure 19.36). Fluorescence may be intrinsic (for example, chlorophyll fluorescence of phototrophic microorganisms); or it may be conferred by DNA staining, or by differential staining of live versus dead cells (vital stains), or by fluorescent DNA probes (FISH), all methods discussed in this chapter.

A major advantage of flow cytometry is the ability to carry out *multiparametric analyses*, that is, the capacity to combine multiple parameters in the analysis of a microbiological sample or to sort cells in order to find a specific population. A good example of this was the discovery in the late 1980s of a novel and abundant community of marine cyanobacteria, all species of the genus *Prochlorococcus*. *Prochlorococcus* cells are smaller and have different fluorescent properties than another common marine cyanobacterium, *Synechococcus*. Based on differences in size and fluorescence, flow cytometry resolved these two populations and *Prochlorococcus* was subsequently shown to be the predominant

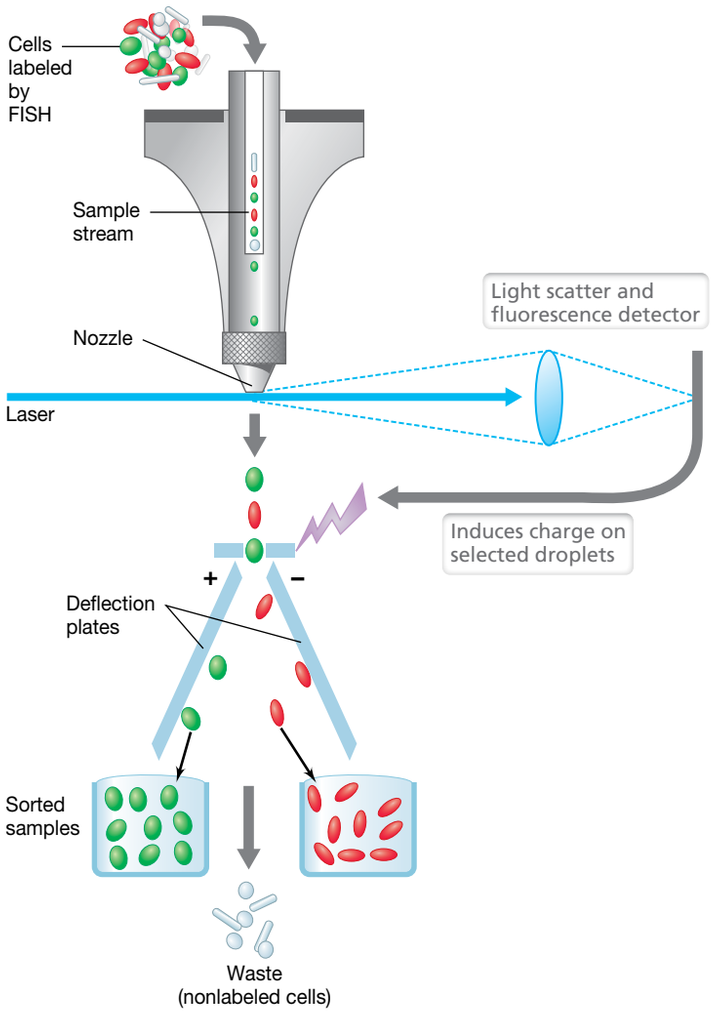



**Figure 19.36 Flow cytometric cell sorting.** As the fluid stream exits the nozzle, it is broken into droplets containing no more than a single cell. Droplets containing desired cell types (detected by fluorescence or light scatter) are charged and collected by redirection into collection tubes or microtiter plates by positively or negatively charged deflection plates.

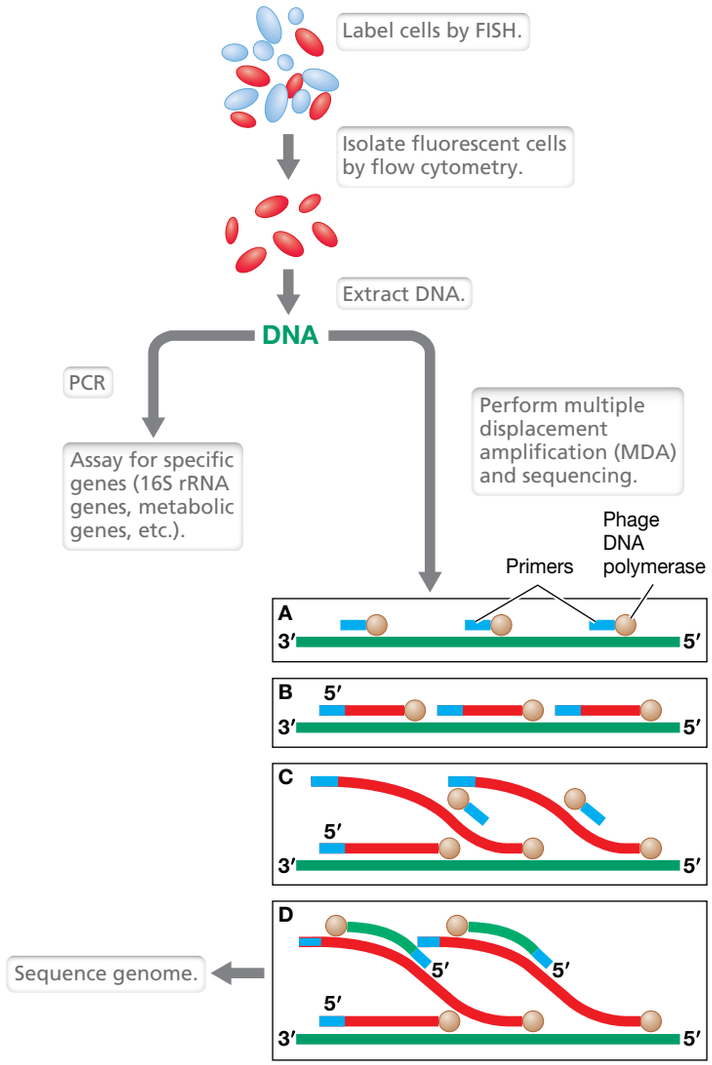



**Figure 19.37 Genetic analysis of sorted cells.** DNA is recovered from a specific population of cells following FISH labeling and flow cytometric sorting (Figure 19.36). DNA is characterized by PCR amplification and sequencing of specific genes, or by amplification of the entire genome by multiple displacement amplification (MDA) followed by sequencing. For MDA, an amount of DNA sufficient for full genome sequence determination is produced using short DNAs of random sequence as primers (A) to initiate genome replication by a bacteriophage DNA polymerase. The bacteriophage polymerase copies DNA from multiple points in the genome and also displaces newly synthesized DNA (B, C), thereby freeing additional DNA for primer annealing and (D) initiation of polymerization.

oxygenic phototroph in ocean waters between 40°S and 40°N latitude, reaching concentrations greater than $10^5$ cells/ml. Metagenomics has also been used to identify the unique genomic features of different natural populations of *Prochlorococcus* (Figure 19.23). These findings have led to the conclusion that *Prochlorococcus* is the most abundant phototrophic organism on Earth. We discuss the biology of *Prochlorococcus* in more detail in Section 20.10.

## Single-Cell Genomics

A major stumbling block in a PCR-based gene recovery method is the requirement that a specific gene that will react with the primers used in the amplification be identified prior to analysis. Newer methods of DNA amplification now provide an alternative method for associating specific genes with a specific organism without the problems and biases associated with PCR. These methods employ *single-cell genomics* (↬ Section 9.12), one of the most recent tools to enter the microbial ecologist's toolbox.

**Multiple displacement amplification (MDA)** (Figure 19.37) is key to single-cell genomics because it can amplify chromosomal DNA from a single cell isolated from a natural environment using a cell sorting technique, such as flow cytometry (Figure 19.36). MDA uses a specific bacteriophage DNA polymerase to initiate replication of cell DNA at random points in the chromosome, displacing the complementary strand as each polymerase molecule synthesizes new DNA. The phage polymerase has strong strand displacement activity, resulting in the synthesis of numerous high-molecular-weight DNA products. The number of genome copies produced by amplification is sufficient to determine the complete, or nearly complete, genome sequence using

next-generation sequencing systems. In this way, both phylogenetic and metabolic functions can be inferred from the genome sequence and PCR is not required.

As could be predicted, MDA requires stringent control over purity to eliminate contaminating DNA, but when combined with high-throughput DNA sequencing methods, MDA provides a powerful tool for linking specific metabolic functions to individual cells that have never been grown in laboratory culture. Information about the metabolic capacities of these uncultured organisms can then be used to develop strategies to recover them by either classical enrichment culture and isolation methods (Sections 19.1 and 19.2) or by any of the several single-cell isolation culturing techniques now available to tease out individual cells and get them growing in the laboratory (Section 19.3).

**MINIQUIZ**

- How can stable isotope probing reveal the identity of an organism that carries out a particular process?
- What key method is required to do genomics on a single cell?
- Compared with microscopy, what are the advantages and disadvantages of flow cytometry for characterizing a microbial community?



# Chapter Review

## I • Culture-Dependent Analyses of Microbial Communities

**19.1** The enrichment culture technique is a means of obtaining microorganisms from natural samples. Successful enrichment and isolation prove that an organism of a specific metabolic type was present in the sample, but do not indicate its ecological importance or abundance. Enrichments following dilution of the sample often yield different organisms than enrichments with undiluted samples.

**Q What exactly constitutes an enrichment culture? What should an effective enrichment culture duplicate?**

**19.2** Once a successful enrichment culture has been established, pure cultures can often be obtained by conventional microbiological procedures, including streak plates, agar dilution, and liquid dilution methods.

**Q Describe how an appropriate inoculum can be used to successfully start an enrichment culture.**

**19.3** Several methods are available to isolate and culture single cells. Laser tweezers allow one to isolate a cell from a microscope field and move it away from contaminants. Flow cytometric sorting combined with high-throughput culturing technology allow for isolated cells to be cultured in a large variety of culture media simultaneously to identify the resources and conditions best suited to the growth of the isolated cell.

**Q It is thought that every microbe has a fundamental niche and a realized niche. How does a fundamental niche differ from a realized niche?**

## II • Culture-Independent Microscopic Analyses of Microbial Communities

**19.4** DAPI, acridine orange, and SYBR Green are general stains for quantifying microorganisms in natural samples. Some stains can differentiate live versus dead cells. The GFP makes cells autofluorescent and is a means for tracking cells introduced into the environment and reporting gene expression. In natural samples, morphologically identical cells may actually be genetically distinct.

**Q What are dyes that stain DNA used for? Is background staining a problem when using such dyes?**

**19.5** FISH methods have combined the power of nucleic acid probes with fluorescent dyes and are thus highly specific in their staining properties. FISH methods include phylogenetic stains and CARD-FISH.

**Q Why is CARD-FISH more suitable than FISH for characterizing very slowly growing microorganisms in the environment?**

## III • Culture-Independent Genetic Analyses of Microbial Communities

**19.6** PCR can be used to amplify specific target genes such as rRNA genes or key metabolic genes for subsequent analysis of community structure and potential functions. DGGE and T-RFLP can identify the different variants of these genes among the species in a community. Application of ARISA is limited to amplification of the internal transcribed spacer region separating the 16S and 23S rRNA genes.

**Q Which method, ARISA or T-RFLP, would provide more detail about microbial community complexity? Why?**

**19.7** Microarrays comprised of thousands of DNA probes are used to screen communities for specific phylogenetic groups as well as genes encoding key biochemical processes.

**Q Why might a microarray be superior to using high-throughput sequencing to identify a rare population member in a complex microbial community? What are the advantages and limitations of FISH and PhyloChips for analysis of microbial communities?**

**19.8** Environmental genomics (metagenomics) is based on cloning (except in direct sequencing), sequencing, and analysis of the collective genomes of the organisms present in a microbial community. Metatranscriptomics and metaproteomics are offshoots of metagenomics whose focus is mRNA and proteins, respectively.

**Q Give an example of how environmental genomics has discovered a known metabolism in a new organism. Why is it difficult to use environmental genomics to discover new biochemical properties?**

## IV • Measuring Microbial Activities in Nature

**19.9** The activity of microorganisms in natural samples can be assessed very sensitively using radioisotopes or microsensors, or both. The measurements obtained give the net activity of the microbial community.

**Q What are the major advantages of radioisotopic methods in the study of microbial ecology? What type of controls (discuss at least two) would you include in a radioisotopic experiment to show $^{14}CO_2$ incorporation by phototrophic bacteria or to show $^{35}SO_4^{2-}$ reduction by sulfate-reducing bacteria?**

**19.10** Natural isotopic composition, the result of isotopic fractionation by enzymes that discriminate against the heavier form of an element, can reveal the biological origin and/or biochemical mechanisms involved in the formation of various substances. Stable isotope probing (SIP) uses compounds labeled with isotopes not naturally abundant to identify microorganisms metabolizing and assimilating the compound added to a community.

**Q Will autotrophic organisms contain more or less $^{12}C$ in their organic compounds than was present in the $CO_2$ that fed them? Why would SIP using $^{15}NO_3^-$ not be useful for identifying bacteria carrying out nitrate respiration?**

**19.11** A variety of advanced technologies such as NanoSIMS, MAR-FISH, and Raman microspectroscopy make it possible to examine metabolic activity, gene content, and gene expression of single cells in natural microbial communities. NanoSIMS employs secondary ion mass spectrometry technology while MAR-FISH combines the uptake of radiolabeled substrates (MAR) along with phylogenetic identification (FISH). Raman microspectroscopy is a nondestructive method (retaining cell viability) for identifying metabolically active microorganisms in the environment.

**Q What can MAR-FISH tell you that FISH alone cannot? How might you combine SIP and NanoSIMS to identify novel methane-consuming cells in a natural community?**

**19.12** Flow cytometry combined with cell sorting can rapidly evaluate many thousands of single cells in a natural environment for basic cellular properties (size, shape) or gene content (using specific fluorescent probes). Single-cell genomics incorporates methods for analyzing the genome of individual cells isolated from a natural microbial community, for example by flow cytometry.

**Q How would you use cytometric cell sorting to evaluate genome sequence variation among a minor population of marine bacteria?**



# Application Questions

1. Design an experiment for measuring the activity of sulfur-oxidizing bacteria in soil. If only certain species of the sulfur oxidizers present were metabolically active, how could you tell this? How would you prove that your activity measurement was due to biological activity?
2. You wish to know whether *Archaea* exist in a lake water sample but are unsuccessful in culturing any. Using techniques described in this chapter, how could you determine whether *Archaea* exist in the sample, and if they do, what proportion of the cells in the lake sample are *Archaea*?
3. Design an experiment to solve the following problem: Determine the rate of methanogenesis ($CO_2 + 4\,H_2 \rightarrow CH_4 + 2\,H_2O$) in anoxic lake sediments and whether or not it is $H_2$-limited. Also, determine the morphology of the dominant methanogen (recall that these are *Archaea*, Section 17.2). Finally, calculate what percentage the dominant methanogen is of the total archaeal and total prokaryotic populations in the sediments. Remember to specify necessary controls.
4. Design a SIP experiment that would allow you to determine which organisms in a lake water sample were capable of oxidizing the hydrocarbon hexane ($C_6H_{14}$). Assume that four different species could do this. How would you combine SIP with other molecular analyses to identify these four species?

# Chapter Glossary

**Acridine orange** a nonspecific fluorescent dye used to stain DNA in microbial cells in a natural sample

**DAPI** a nonspecific fluorescent dye that stains DNA in microbial cells; used to obtain total cell numbers in natural samples

**Denaturing gradient gel electrophoresis (DGGE)** an electrophoretic technique capable of separating nucleic acid fragments of the same size that differ in base sequence

**Enrichment bias** a problem with enrichment cultures in which "weed" species tend to dominate in the enrichment, often to the exclusion of the most abundant or ecologically significant organisms in the inoculum

**Enrichment culture** a culture that employs highly selective laboratory methods for obtaining microorganisms from natural samples

**Environmental genomics (metagenomics)** the use of genomic methods (sequencing and analyzing genomes) to characterize natural microbial communities

**Flow cytometry** a technique for counting and examining microscopic particles by suspending them in a stream of fluid and passing them by an electronic detection device

**Fluorescence in situ hybridization (FISH)** a method employing a fluorescent dye covalently bonded to a specific nucleic acid probe for identifying or tracking organisms in the environment

**Fluorescent protein** any of a large group of proteins that fluoresce different colors, including the green fluorescent protein, for tracking genetically modified organisms and determining conditions that induce the expression of specific genes

**Fundamental niche** the range of environments in which a species will be sustained when it is not resource-limited, such as may result from competition with other species

**Green fluorescent protein (GFP)** a protein that glows green and is widely used in genetic analysis

**High-throughput culturing methods** the use of microtiter plates whose wells contain various culture media that can be inoculated with single cells whose growth or target gene content is measured robotically

**Isotopic fractionation** the discrimination by enzymes against the heavier isotope of the various isotopes of C or S, leading to enrichment of the lighter isotopes

**Laser tweezers** a device for obtaining pure cultures by optically trapping a single cell with a laser beam and moving it away from surrounding cells into sterile growth medium

**MAR-FISH** a technique that combines identification of microorganisms with measurement of metabolic activities

**Metaproteomics** the measurement of whole-community protein expression using mass spectrometry to assign peptides to the amino acid sequences encoded by unique genes

**Metatranscriptomics** the measurement of whole-community gene expression using RNA sequencing

**Microautoradiography (MAR)** the measurement of the uptake of radioactive substrates by visually observing the cells in an exposed photographic emulsion

**Microbial ecology** the study of the interaction of microorganisms with each other and their environment

**Microfluidic devices** miniaturized systems for fluid handling that are increasingly used for high-throughput culturing of microorganisms

**Microsensor** a small glass sensor or electrode for measuring pH or specific compounds such as $O_2$, $H_2S$, or $NO_3^-$ that can be immersed into a microbial habitat at microscale intervals

**Most-probable-number (MPN) technique** the serial dilution of a natural sample to determine the highest dilution yielding growth

**Multiple displacement amplification (MDA)** a method to generate multiple copies of chromosomal DNA from a single organism

**Nucleic acid probe** an oligonucleotide, usually 10–20 bases in length, complementary in base sequence to a nucleic acid sequence in a target gene or RNA

**Phylotype** one or more organisms with the same or related sequences of a phylogenetic marker gene

**Realized niche** the range of natural environments supporting a species when that organism is confronted with factors such as resource limitation, predation, and competition from other species

**Stable isotope probing (SIP)** a method for characterizing an organism that incorporates a particular substrate by supplying the substrate in $^{13}C$ or $^{15}N$ form and then isolating heavy isotope–enriched DNA and analyzing the genes

**Winogradsky column** a glass column packed with mud and overlaid with water to mimic an aquatic environment, in which various bacteria develop over a period of months

# Microbial Ecosystems

20




## microbiologynow

### Microbes of the Abyss

The biology of deep ocean regions, the abyssal zone (below 4000 m) and hadal zone (below 6000 m), has stirred the imagination for centuries. These regions remain among the least explored of Earth's biosphere because their study requires very specialized equipment. Only a few remotely operated vehicles (ROVs), autonomous underwater vehicles, and human-occupied submersibles can dive to the deepest marine waters, which are at depths over 10,000 m; pressures at such depths are around 16,000 pounds per square inch (110,000 kilopascals). For example, the Japanese ROV Kaiko (40% scale model in photo) retrieved sediment samples from the Mariana Trench (Pacific Ocean, depth of 10,900 m) (see Kaiko in action in Figure 20.32), but unfortunately was lost in a later dive when its tethering cable snapped.

Animals and microbes that inhabit the deep sea experience extreme pressure, low levels of nutrients, and near-freezing temperatures. The microbiology of these regions was first explored decades ago using high-pressure cultivation methods to isolate the first pressure-loving bacteria (piezophiles). Cultivation was complemented by limited culture-independent molecular surveys of diversity based on 16S rRNA gene sequencing. However, recent advances in metagenomics and high-throughput sequencing have revolutionized studies of deep-sea microbes and their adaptive strategies, such as alterations in membrane and protein structure that allow growth under such extreme conditions.

Diversity studies have shown that both free-living and particle-attached archaeal and bacterial piezophiles are abundant in the abyssal and hadal zones and that most have not yet been cultured. For example, *Bacteria* related to the phyla *Marinimicrobia* and *Gemmatimonadetes* are enriched at these great depths. Since no member of the *Marinimicrobia* has been isolated and the few *Gemmatimonadetes* available are from soils or wastewater, a more complete understanding of the unique properties of these hadal *Bacteria* awaits their future cultivation. Ammonia-oxidizing *Archaea* related to *Nitrosopumilus*, an organism common in upper marine waters, are the most abundant free-living *Archaea* in hadal waters, highlighting their global dominance in both marine and terrestrial environments.

The presence of these novel *Archaea* and *Bacteria* at great depths points to a unique hadal microbiology that we are only now beginning to understand. Future ROV descents such as those pioneered by Kaiko should bring deep-sea microbiology into better focus.

**Source:** Tarn, J., et al. 2016. Identification of free-living and particle-associated microbial communities present in hadal regions of the Mariana Trench. *Front. Microbiol. 7:* Article 665.

Microorganisms do not live alone in nature but instead interact with other organisms and with their environment. In so doing, microorganisms carry out many essential activities that support all life on Earth. In this chapter we explore some of the major habitats of microorganisms; these include soil, freshwater, and the oceans. In addition to these, microbes have also established more specific, and often very intimate, associations with plants and animals. We examine a few examples of such microbial partnerships and symbioses in Chapter 23.

# I • Microbial Ecology

We begin with a broad overview of the science of microbial ecology, including ways that organisms interact with each other and their environments and the difference between species *diversity* and species *abundance*. These basic ecological concepts pervade this and the next three chapters.

## 20.1 General Ecological Concepts

The distribution of microorganisms in nature resembles that of macroorganisms in the sense that a given species resides in certain places but not others; that is, everything is not everywhere. Also, environments differ in their abilities to support diverse microbial populations, from the highly diverse microbial world of undisturbed fertile soil to the rather restricted world of some highly extreme environments.

### Ecosystems and Habitats

An **ecosystem** is a dynamic complex of plant, animal, and microbial communities and their abiotic surroundings, all of which interact as a functional unit. An ecosystem contains many different **habitats**, parts of the ecosystem best suited to one or a few populations. Although microorganisms are present in any habitat containing plants and animals, many microbial habitats are unsuitable for plants and animals. For example, microorganisms are ubiquitous on Earth's surface and even deep within it; they inhabit boiling hot springs and solid ice, acidic environments near pH 0, saturated brines, environments contaminated with radionuclides and heavy metals, and the interior of porous rocks that contain only traces of water. Therefore, some ecosystems are mostly or even exclusively microbial.

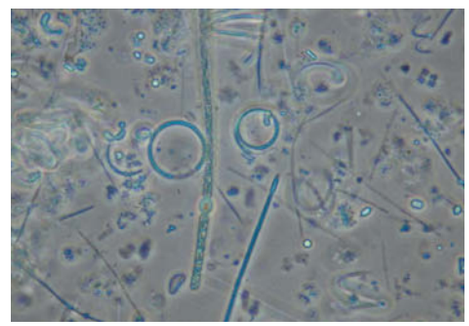
Collectively, microorganisms show great metabolic diversity and are the primary catalysts of nutrient cycles in nature (Chapter 21). The *types* of microbial activities possible in an ecosystem are a function of the species present, their population sizes, and the physiological state of the microorganisms in each habitat. By contrast, the *rates* of microbial activities in an ecosystem are controlled by the nutrients and growth conditions that prevail. Depending on several factors, microbial activities in an ecosystem can have minimal or profound impacts and can diminish or enhance the activities of both the microorganisms themselves and the macroorganisms that may coexist with them.

### Species Diversity in Microbial Habitats

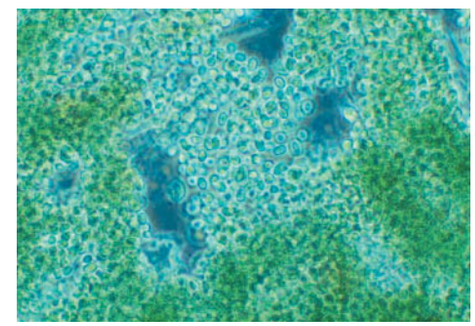
A group of microorganisms of the same species that reside in the same place at the same time constitutes a microbial **population** and may be descendants of a single cell. A microbial population differs from a microbial **community**. A community consists of populations of one species living in association with populations of one or more other species. The microbial species that reside in a certain habitat are those best able to grow with the nutrients and conditions that prevail there.

The diversity of microbial species in a community can be expressed in two ways. One is **species richness**, the total *number* of different species present. Identifying cells is, of course, basic to determining microbial species richness, but this need not require their isolation and culture. Species richness may also be expressed in molecular terms by the diversity of phylotypes (for example ribosomal RNA genes, Section 19.6) observed in a given community. **Species abundance**, by contrast, is the *proportion* of each species in the community (compare Figure 20.1*b* and *c*). Species richness and abundance can change quickly over a short time as shown by the change in abundance of cyanobacteria in a lake receiving nutrient-rich agricultural runoff (**Figure 20.1*a***). One goal of microbial ecology is to understand species richness and abundance in microbial communities along with the community's

(a)



(b)



(c)

Hans Paerl, University of North Carolina at Chapel Hill

**Figure 20.1 Microbial species diversity: Richness versus abundance.** *(a)* Collecting samples from Lake Taihu, China, following a bloom of the cyanobacterium *Microcystis*. *(b)* High species richness in St. John's River, Florida, shown by microscopy of planktonic microorganisms including cyanobacteria, diatoms, green algae, flagellates, and bacteria. *(c)* Shift of St. John's River community to low richness but high abundance following a bloom of the cyanobacterium *Microcystis*.

**TABLE 20.1 Resources and conditions that govern microbial growth in nature**

| *Resources* |
|---|
| Carbon (organic, $CO_2$) |
| Nitrogen (organic, inorganic) |
| Other macronutrients (S, P, K, Mg) |
| Micronutrients (Fe, Mn, Co, Cu, Zn, Mn, Ni) |
| $O_2$ and other electron acceptors ($NO_3^-$, $SO_4^{2-}$, $Fe^{3+}$) |
| Inorganic electron donors ($H_2$, $H_2S$, $Fe^{2+}$, $NH_4^+$, $NO_2^-$) |
| *Conditions* |
| Temperature: cold ⟶ warm ⟶ hot |
| Water potential: dry ⟶ moist ⟶ wet |
| pH: 0 ⟶ 7 ⟶ 14 |
| $O_2$: oxic ⟶ microoxic ⟶ anoxic |
| Light: bright light ⟶ dim light ⟶ dark |
| Osmotic conditions: freshwater ⟶ marine ⟶ hypersaline |

associated activities and the abiotic environment. Once all of these factors are known, microbial ecologists can model the ecosystem by perturbing it in some way and observing whether predicted changes match experimental results.

The microbial species richness and abundance of a community are functions of the conditions that prevail and the kinds and amounts of nutrients available in the habitat. Table 20.1 lists common nutrients and conditions relevant to microbial growth. In some microbial habitats, such as undisturbed organic-rich soils, high species richness is common (see Figure 20.12), with most species present at only moderate abundance. Nutrients in such a habitat are of many different types, and this helps select for high species richness. In other habitats, such as some extreme environments, species richness is often very low and abundance of one or a few species very high. This is because the physical and chemical (physicochemical) in the environment exclude all but a handful of species, and key nutrients are present at such high levels that the highly adapted species can grow to high cell densities. Bacteria that catalyze acid mine runoff from the oxidation of iron are a good example. These organisms thrive in highly acidic, iron-rich but organic-poor waters, where the acidic conditions and the dearth of organic carbon limit species richness. However, the elevated levels of ferrous iron ($Fe^{2+}$) present, which is oxidized to $Fe^{3+}$ in energy-yielding reactions (↔ Section 14.10), fuel high species abundance. We examine the activities of acidophilic iron-oxidizing microorganisms in Sections 21.5 and 22.1.

**MINIQUIZ**

- What is the difference between species richness and species abundance?
- How does an ecosystem differ from a habitat?
- What are the characteristics of a microbial population?
- How does a microbial population differ from a microbial community?

## 20.2 Ecosystem Service: Biogeochemistry and Nutrient Cycles

In any ecosystem whose resources and growth conditions are suitable, microorganisms will grow to form populations. Metabolically similar microbial populations that exploit the same resources in a similar way are called **guilds**. A habitat that is shared by a guild and supplies the resources and conditions the cells require for growth is called a **niche**. Sets of guilds form microbial communities (Figure 20.2). Microbial communities interact with macroorganisms and abiotic factors in the ecosystem in a way that defines the workings of that ecosystem.

### Energy Inputs to the Ecosystem

Energy enters ecosystems as sunlight, organic carbon, and reduced inorganic substances. Light is used by phototrophs to make ATP and synthesize new organic matter (Figure 20.2). In addition to carbon (C), new organic matter contains nitrogen (N), sulfur (S), phosphorus (P), iron (Fe), and the other elements of life (↔ Section 3.1). This newly synthesized organic material along with organic matter that enters the ecosystem from the outside (called *allochthonous* organic matter) fuels the catabolic activities of chemoorganotrophic organisms. These activities oxidize the organic matter to $CO_2$ by respiration or ferment it to various reduced substances. If chemolithotrophs are present and metabolically active in the ecosystem, they can conserve energy from the oxidation of inorganic electron donors, such as $H_2$, $Fe^{2+}$, $S^0$, or $NH_3$ (Chapters 14 and 15),

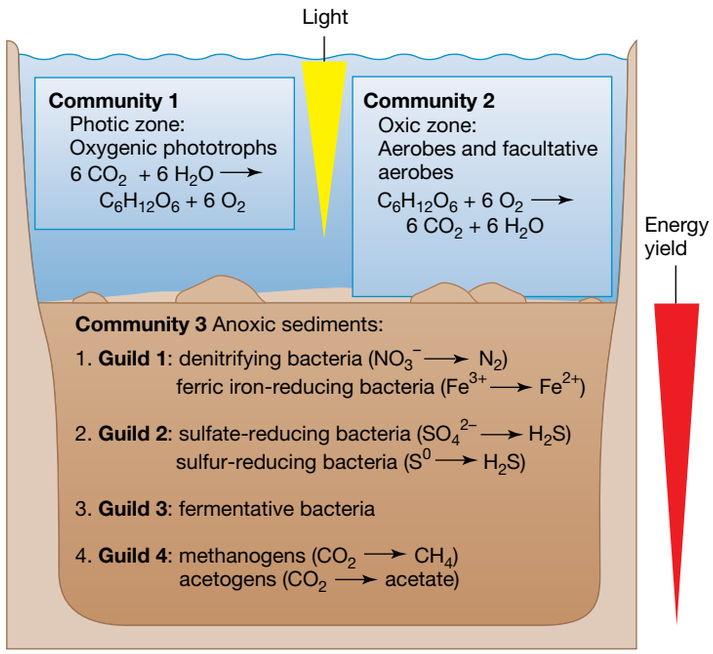



**Figure 20.2 Populations, guilds, and communities.** Microbial communities consist of populations of cells of different species. A freshwater lake ecosystem would likely have the communities shown here. The reduction of $NO_3^-$, $Fe^{3+}$, $SO_4^{2-}$, $S^0$, and $CO_2$ are examples of anaerobic respirations. The region of greatest activity for each of the different respiratory processes would differ with depth in the sediment. As more energetically favorable electron acceptors are depleted by microbial activity near the surface, less favorable reactions occur deeper in the sediment.

and contribute new organic matter through their autotrophic activities (Figure 20.2).

### Biogeochemical Cycling

Microorganisms play an essential role in cycling elements, in particular C, N, S, and Fe, between their different chemical forms. The study of these transformations is part of **biogeochemistry**, an interdisciplinary science that includes biology, geology, and chemistry. Figure 20.2 shows how the activities of different guilds of microorganisms influence the chemistry of one environment, a lake ecosystem. The sequence of changing chemistry with increasing depth in the sediments corresponds to the layers of different microbial guilds. The location of each guild in the ecosystem is primarily determined by the availability of electron donors and acceptors, both of which tend to decrease with increasing depth in the sediments.

A *biogeochemical cycle* defines the transformations of an element that are catalyzed by either biological or chemical means (or both). Many different microorganisms participate in biogeochemical cycling reactions, and in many cases, microorganisms are the *only* biological agents capable of regenerating forms of the elements needed by other organisms, particularly plants. Thus, biogeochemical cycles are often also *nutrient cycles*, reactions that generate important nutrients for other organisms.

Most biogeochemical cycles proceed by oxidation–reduction reactions as the element moves through the ecosystem and are often tightly *coupled*, with transformations in one cycle affecting one or more other cycles. For example, hydrogen sulfide ($H_2S$) is oxidized by phototrophic and chemolithotrophic microbes to sulfur ($S^0$) and sulfate ($SO_4^{2-}$), the latter being a key nutrient for plants. Phototrophs and chemolithotrophs are also autotrophs, and thus affect the carbon cycle by producing new organic carbon from $CO_2$. However, $SO_4^{2-}$ can be reduced to $H_2S$ by the sulfate-reducing bacteria, organisms that consume organic carbon, and this reduction closes the biogeochemical sulfur cycle while regenerating $CO_2$. The cycling of nitrogen is also a microbial process and is key to the regeneration of forms of nitrogen usable by plants and other organisms. The nitrogen cycle is driven by both chemolithotrophic and chemoorganotrophic bacteria, organisms that produce and consume organic carbon, respectively. We considered the microbiology of biogeochemical cycles and their coupled nature in Chapters 14 and 15 and will revisit this theme in more detail in Chapter 21.

**MINIQUIZ**

- How does a microbial guild differ from a microbial community?
- What is a biogeochemical cycle? Give an example based on sulfur. Why are biogeochemical cycles also called nutrient cycles?

# II • The Microbial Environment

Microorganisms define the limits of life throughout aquatic and terrestrial environments on our planet. Specific conditions required by a particular organism or group of organisms may be subject to rapid change as a result of inputs to and outputs from their habitat or as a result of microbial activities or physical disturbances. Thus, within one environment there can be multiple habitats, some relatively stable and others changing rapidly over time and space.

## 20.3 Environments and Microenvironments

Besides living in the common habitats of soil and water, microorganisms thrive in extreme environments and also reside on and within the cells of other organisms. The intimate associations developed between microorganisms and other organisms will be presented in Chapters 23 and 24. Here we focus on terrestrial and aquatic microbial habitats.

### The Microorganism, Niches, and the Microenvironment

The habitat in which a microbial community resides is governed by physicochemical conditions that are determined in part by the metabolic activities of the community. For example, the organic material used by one species may have been a metabolic by-product of a second species. Another example is oxygen ($O_2$), which can become limiting if biological consumption exceeds the rate at which it is supplied.

Because microbes are very small, they directly experience only a tiny local environment; this small space is called their **microenvironment**. For example, for a typical 3-µm rod-shaped bacterium, a distance of 3 mm is equivalent to that which a human would experience over a distance of 2 km! As a consequence of the smallness of microorganisms, the variable metabolic activities of nearby microbes, and the changes in physicochemical conditions over short intervals of time and distance, numerous microenvironments can exist within a given habitat. The conditions supporting growth within a microenvironment correspond to the general requirements for growth we considered in Chapter 5.

Ecological theory states that for every organism there exists at least one niche, the *realized niche* (also called the *prime niche*), where it will be most successful. The organism dominates the realized niche but may also inhabit other niches; in other niches it is less ecologically successful than in its realized niche but it may still be able to compete. The full range of environmental conditions under which an organism can exist is called its *fundamental niche* (we considered the realized and fundamental niche in the context of enrichment culture and isolation in Sections 19.1–19.3). The word "niche" should not be confused with the word "microenvironment" because the microenvironment describes conditions at a specific location and can change rapidly. In other words, the general conditions that describe a specific niche may be transient at many places in a microenvironment.

Another important consequence of microbes being so small is that diffusion often determines the availability of resources. Consider, for example, the distribution of an important microbial nutrient such as $O_2$ in a soil particle. Microsensors (Section 19.9) can be used to measure oxygen concentrations throughout small soil particles. As shown in the data from an actual microsensor experiment (**Figure 20.3**), soil particles are not homogeneous in terms of their $O_2$ content but instead contain many adjacent microenvironments. The outer layer of the soil particle may be fully oxic (21% $O_2$) while the center, only a very short distance away (in human terms, but of course a great distance from a microbial standpoint), may be anoxic ($O_2$-free). The microorganisms near the outer edges

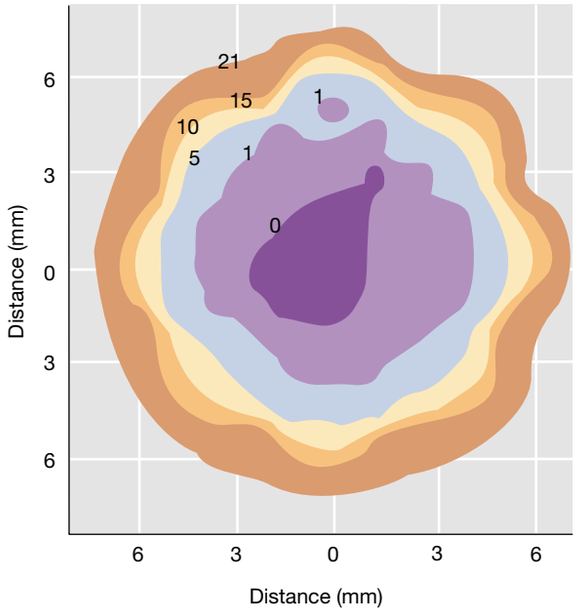



**Figure 20.3 Oxygen microenvironments.** Contour map of $O_2$ concentrations in a small soil particle as determined by a microsensor (Section 19.9). The axes show the dimensions of the particle. The numbers on the contours are percentages of $O_2$ concentration (air is 21% $O_2$). Each zone can be considered a different microenvironment.

consume all of the $O_2$ before it can diffuse to the center of the particle. Thus, anaerobic organisms could thrive near the center of the particle, microaerophiles (aerobes that require very low oxygen levels) farther out, and obligately aerobic organisms in the outermost region of the particle. Facultatively aerobic bacteria (organisms that can grow either aerobically or anaerobically) could be distributed throughout the particle (Section 5.14). Nutrient transfer is particularly important in thick assemblages of cells, such as biofilms and microbial mats, and we explore this in Section 20.4.

Physicochemical conditions in a microenvironment are subject to rapid change in both time and space. For example, the $O_2$ concentrations shown in the soil particle in Figure 20.3 represent "instantaneous" values. Measurements taken in the same particle following a period of intense microbial respiration or disturbance due to wind, rain, or disruption by soil animals could differ dramatically from those shown. During such events certain populations may temporarily dominate the activities in the soil particle and grow to high numbers, while others remain dormant or nearly so. However, if the microenvironments shown in Figure 20.3 are eventually reestablished, the various microbial activities characteristic of different regions of the soil particle will eventually return as well.

## Nutrient Levels and Growth Rates

Resources (Table 20.1) typically enter an ecosystem intermittently. A large pulse of nutrients—for example, an input of leaf litter or the carcass of a dead animal—may be followed by a period of nutrient deprivation. Because of this, microorganisms in nature often face a "feast-or-famine" existence. It is thus common for them to produce storage polymers as reserve materials when resources are abundant and draw upon these reserves in periods of starvation. Examples of storage materials are poly-β-hydroxyalkanoates, polysaccharides, and polyphosphate (Section 2.8).

Extended periods of exponential microbial growth in nature are probably rare. Microorganisms typically grow in spurts, linked closely to the availability and types of resources. Because all relevant physicochemical conditions in nature are rarely optimal for microbial growth at the same time, growth rates of microorganisms in nature are usually well below the maximum growth rates recorded in the laboratory. For instance, the generation time of *Escherichia coli* in the intestinal tract of a healthy adult eating at regular intervals is about 12 h (two doublings per day), whereas in pure culture it can grow much faster, with a minimum generation time of about 20 min under optimal conditions. In addition, research-based estimates indicate that most cultured soil bacteria typically grow in nature at less than 1% of the maximal growth rate measured in the laboratory.

These slower growth rates in nature than in laboratory culture reflect the facts that (1) resources and growth conditions (Table 20.1) are frequently suboptimal; (2) the distribution of nutrients throughout the microbial habitat is not uniform; and (3) except in rare instances, microorganisms in nature grow in mixed populations rather than pure culture. An organism that grows rapidly in pure culture may grow much slower in a natural environment where it must compete with other organisms that may be better suited to the resources and growth conditions available.

## Microbial Competition and Cooperation

Competition among microorganisms for resources in a habitat may be intense, with the outcome dependent on several factors, including rates of nutrient uptake, inherent metabolic rates, and ultimately, growth rates. A typical habitat contains a mixture of different species (Figures 20.1 and 20.2), with the density of each population dependent on how closely its niche resembles its realized niche.

Some microbes work together to carry out transformations that neither can accomplish alone—a process called *syntrophy*—and these microbial partnerships are particularly important for anoxic carbon cycling (Sections 14.23 and 21.2). Metabolic cooperation can also be seen in the activities of organisms that carry out *complementary* metabolisms. For example, we have previously considered metabolic transformations that are carried out by two distinct groups of organisms, such as those of the nitrifying *Bacteria* and *Archaea* (Sections 14.11, 15.13, and 17.5). Together, these nitrifiers oxidize ammonia ($NH_3$) to nitrate ($NO_3^-$). Because nitrite ($NO_2^-$), the product of ammonia-oxidizing nitrifiers, is the substrate for the nitrite-oxidizing bacteria, the two groups of organisms often live in nature in tight association within their habitats (Figure 19.13).

Winogradsky discovered nitrification in 1890 (Section 1.11), but until lately no single organism capable of complete ammonia oxidation was known. However, the recent discovery of *Nitrospira* species (*Bacteria*) whose genomes encode enzyme systems for both ammonia and nitrite oxidation shows that a single species can catalyze both oxidations, a process called *comammox*. With the use of molecular tools (Chapter 19) to survey various habitats for comammox bacteria, related organisms have been identified in wetlands, riverbeds, aquifers and lake sediments, and wastewater treatment systems. However, no marine comammox species have been identified, and thus the overall significance of comammox organisms in the global nitrogen cycle is unclear at this point.

**MINIQUIZ**

- What characteristics define the realized niche of a particular microorganism?
- Why can many different physiological groups of organisms live in a single habitat?

## 20.4 Surfaces and Biofilms

Surfaces are important microbial habitats, typically offering greater access to nutrients, protection from predation and physicochemical disturbances, and a means for cells to remain in a favorable habitat, modify the habitat from their own activities, and not be washed away. Moreover, flow across a colonized surface increases transport of nutrients to the surface, providing more resources than are available to planktonic cells (cells that live a floating existence) in the same environment. A surface may also be provided by another organism or by a nutrient such as a particle of organic matter. For example, plant roots become heavily colonized by soil bacteria living on organic exudates from the plant, as revealed when fluorescent stains are used (Figure 20.4*a*).

Virtually any natural or artificial surface exposed to microorganisms will be colonized. For example, microscope slides have been used as experimental surfaces to which organisms can attach and grow. A slide can be immersed in a microbial habitat, left for a period of time, and then retrieved and examined microscopically (Figure 20.4*b*). Clusters of a few cells that develop from a single colonizing cell—called *microcolonies*—form readily on such surfaces, much as they do on natural surfaces in nature. Periodic microscopic examination of immersed slides has been used to measure growth rates of attached organisms in nature.

Surface colonization may be sparse, consisting only of microcolonies not visible to the naked eye, or may consist of so many cells that microbial accumulation becomes visible as, for example, in a stagnant toilet bowl. Surface growth can be particularly problematic in a hospital setting where microbial colonization of indwelling devices such as catheters and intravenous lines can cause serious infection. In a few extreme environments that lack small animal grazers (for example, hot springs), microbial accumulation on a surface can be many centimeters in thickness. Called **microbial mats** (Section 20.5), such accumulations often contain highly complex yet very stable assemblages of phototrophic, chemolithotrophic, autotrophic, and heterotrophic microbes.

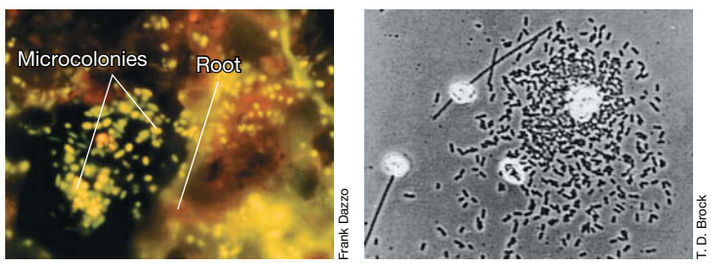



**Figure 20.4 Microorganisms on surfaces.** *(a)* Fluorescence photomicrograph of a natural microbial community living on plant roots in soil and stained with acridine orange. Note microcolony development. *(b)* Bacterial microcolonies developing on a microscope slide that was immersed in a river. The bright particles are mineral matter. The short, rod-shaped cells are about 3 μm long.

### Biofilms

As bacterial cells grow on surfaces they commonly form **biofilms**—assemblages of bacterial cells attached to a surface and enclosed in an adhesive matrix that is the product of excretion by cells and cell death (Figure 20.5). The matrix is typically a mixture of polysaccharides, proteins, and nucleic acids that bind the cells together. Biofilms trap nutrients for microbial growth and help prevent the detachment of cells on dynamic surfaces, such as in flowing systems (Figure 20.5*c*). We examined some of the genetic regulatory features of biofilm formation in Section 7.9 and so here primarily consider their ecological and medical consequences.

Biofilms typically contain multiple layers of cells embedded in the porous matrix material, and the cells in each layer can be examined by confocal scanning laser microscopy (Section 1.7; Figure 20.5*b*). Biofilms may contain one or two species or, more commonly, many species of bacteria. The biofilms that form on tooth and soft surfaces of the mouth, for example, contain between 100 and 200 different phylotypes (Section 19.6), including species of both *Bacteria* and *Archaea*; in total, the human mouth is a habitat for approximately 700 phylotypes (Sections 24.3 and 25.2). Biofilms are thus functional and growing microbial communities and not just cells trapped in a sticky matrix.

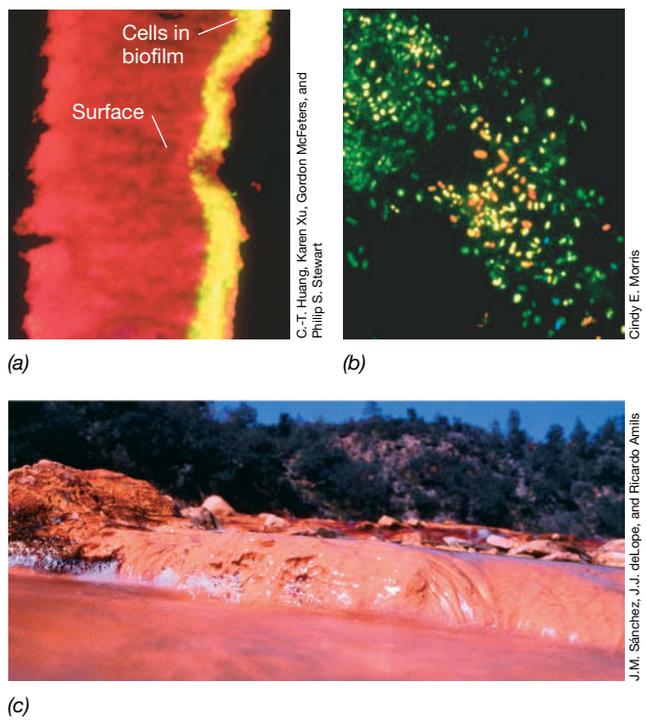



**Figure 20.5 Examples of microbial biofilms.** *(a)* A cross-sectional view of an experimental biofilm made up of cells of *Pseudomonas aeruginosa*. The yellow layer (about 15 μm in depth) contains cells and is stained by a reaction showing activity of the enzyme alkaline phosphatase. *(b)* Confocal scanning laser microscopy of a natural biofilm (top view) on a leaf surface. The color of the cells indicates their depth in the biofilm: red, surface; green, 9-μm depth; blue, 18-μm depth. *(c)* A biofilm of iron-oxidizing bacteria attached to rocks in the Rio Tinto, Spain. As $Fe^{2+}$-rich water passes over and through the biofilm, the organisms oxidize $Fe^{2+}$ to $Fe^{3+}$.

Wherever submerged surfaces are present in natural environments, biofilm growth is almost always more extensive and diverse than the planktonic growth in the liquid that surrounds the surface. Biofilms differ from planktonic communities in supporting critical transport and transfer processes, which generally control growth in biofilm environments. For example, if consumption of $O_2$ by populations near the surface exceeds diffusion of $O_2$ into deeper regions of the biofilm, the deeper regions will become anoxic, opening up new niches for colonization by obligate anaerobes or facultative aerobes. This is similar to the depletion of $O_2$ in the interior of a soil particle that was depicted in Figure 20.3.

One of the most clinically and industrially relevant properties of biofilm microbial communities is their inherent tolerance to antibiotics and other antimicrobial chemicals. A given species growing in a biofilm can be up to 1000 times more tolerant of an antimicrobial substance than planktonic cells of the same species. Reasons for this greater tolerance include slower growth rates in biofilms, reduced penetration of antimicrobial substances through the extracellular matrix, and the expression of genes that increase tolerance to stress. This tolerance of antimicrobial substances may explain why biofilms are responsible for many untreatable or difficult-to-treat chronic infections and are also hard to eradicate in industrial systems, such as wastewater plants, where surface growth (fouling) by microbes may impair important processes.

## *Pseudomonas aeruginosa* and Cystic Fibrosis

*Pseudomonas aeruginosa* is a notorious biofilm former (**Figure 20.6**). In this bacterium (and many other *Bacteria*), elevated levels of the small molecule cyclic di-guanosine monophosphate (c-di-GMP, Figure 7.19) initiate the production of extracellular polysaccharide, decrease flagellar function, and prepare cells for cell–cell and cell–surface interactions. Over time, in nutrient-rich conditions, *P. aeruginosa* cells can develop mushroom-shaped microcolonies that can be over 0.1 mm high and contain millions of cells enmeshed in a sticky polysaccharide matrix (Figure 20.6). The final architecture of the biofilm is determined by multiple factors in addition to signaling molecules, including nutritional factors and local flow environment (Section 7.9).

*P. aeruginosa* biofilms also form in human lungs in those with the genetic disease *cystic fibrosis*. Once in the biofilm state, *P. aeruginosa* is difficult to treat with antibiotics and the biofilm helps the bacteria persist in individuals with this disease. Like most biofilms, that which develops in the lungs of cystic fibrosis patients is composed of more than one bacterial species. So, in addition to *intra*species signaling, *inter*species signaling likely contributes to both initiating and maintaining the cystic fibrosis biofilm as well as biofilms of other types.

## Why Bacteria Form Biofilms

At least three reasons have been proposed for the formation of biofilms. First, biofilms are a means of microbial self-defense that increase survival. Biofilms resist physical forces that could otherwise remove cells only weakly attached to a surface. Biofilms also resist phagocytosis by protozoa and cells of the immune system, and retard the penetration of toxic molecules such as antibiotics. These advantages improve the chances for survival of cells in the biofilm. Second, biofilm formation allows cells to remain in a favorable niche. Biofilms attached to nutrient-rich surfaces, such as animal tissues, or to surfaces in flowing systems (Figure 20.5*c*) fix bacterial cells in locations where nutrients may be more abundant or are constantly being replenished. Third, biofilms form because they allow bacterial cells to live in close association with each other. This facilitates cell-to-cell communication, offers more opportunities for nutrient and genetic exchange, and in general increases chances for survival.

Biofilms form on virtually any surface capable of supporting bacterial growth, and this suggests that biofilms are the "default" growth mode for bacteria in natural habitats, environments that typically differ dramatically in nutrient levels from the rich liquid culture media used in the laboratory. If this is true, planktonic growth may be the atypical growth mode and the norm only for those bacteria adapted to life at extremely low nutrient concentrations (discussed in Sections 20.7, 20.9, and 20.11).

## Common Biofilms and Their Control

Biofilms have significant implications in human medicine and commerce. In the body, bacterial cells within a biofilm are protected from attack by the immune system, and antibiotics and other antimicrobial agents sometimes fail to penetrate the biofilm. Besides cystic fibrosis, biofilms have been implicated in several medical and dental conditions, including periodontal disease, chronic wounds, kidney stones, tuberculosis, Legionnaires' disease, and *Staphylococcus* infections (Figure 5.4*a*). Medical implants are ideal surfaces for biofilm development. These include both short-term devices, such as urinary catheters, as well as long-term implants, such as artificial joints. It is estimated that 10 million people a year in the United States experience biofilm infections from implants or intrusive medical procedures.



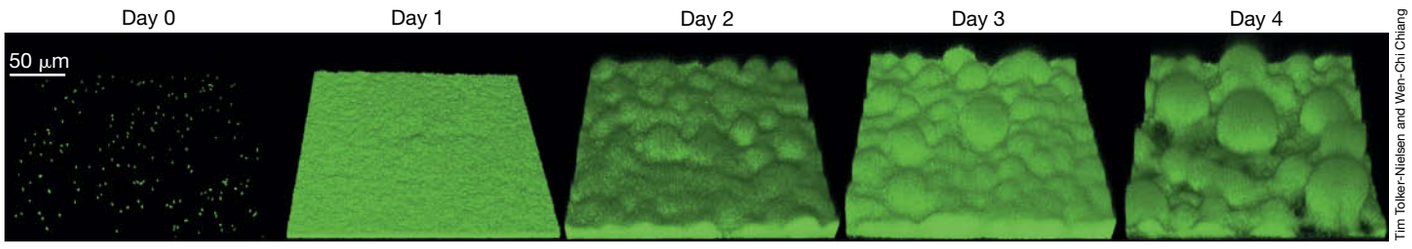



**Figure 20.6** ***Pseudomonas aeruginosa*** **biofilm development.** Confocal scanning laser micrographs of a developing *Pseudomonas aeruginosa* biofilm in a flow cell continuously irrigated with nutrient-rich medium. *P. aeruginosa* cells first attach to the glass surface (day 0), then rapidly grow and move on the surface to cover the entire surface (day 1); by day 4 mushroom-shaped microcolonies over 0.1 mm high have developed.

Biofilms explain why routine oral hygiene is so important for maintaining dental health. Dental plaque is a typical biofilm and contains acid-producing bacteria responsible for dental caries (Sections 24.9 and 25.2 and Figures 25.7 and 25.8).

Biofilms can slow the flow of water, oil, or other liquids through pipelines and can accelerate corrosion of the pipes themselves. Biofilms also initiate the degradation of submerged objects, such as structural components of offshore oil platforms, boats, and shoreline installations. The safety of drinking water may be compromised by biofilms that develop in water distribution pipes, many of which in the United States are nearly 100 years old (Section 22.9). Water-pipe biofilms mostly contain harmless microbes, but if pathogens successfully colonize a biofilm, standard chlorination practices may fail to kill them. Periodic releases of pathogenic cells can then lead to outbreaks of disease. For example, it is thought that *Vibrio cholerae*, the causative agent of cholera (Section 32.3), may be propagated in this manner.

Biofilm control is big business, and thus far, only a limited number of tools exist to fight biofilms. Collectively, industries commit huge financial resources to treating pipes and other surfaces to keep them free of biofilms. New antimicrobial agents that can penetrate biofilms, as well as drugs that eliminate biofilm formation by interfering with intercellular communication, are being developed. A class of chemicals called *furanones*, for example, has shown promise as biofilm preventives on abiotic surfaces.

**MINIQUIZ**

- Why might a biofilm be a good habitat for bacterial cells living in a flowing system?
- Give an example of a medically relevant biofilm that forms in virtually all healthy humans.
- How is it possible for both aerobes and obligate anaerobes to coexist in the same biofilm?

## 20.5 Microbial Mats

Microbial mats are among the most visibly conspicuous of microbial communities and can be thought of as extremely thick biofilms. Supported by phototrophic or chemolithotrophic bacteria, these layered microbial communities can be several centimeters thick (**Figure 20.7*a*, *b***). The layers are composed of species of different microbial guilds whose activities are governed by light availability and other resources (Table 20.1). The combination of microbial metabolism and nutrient transport controlled by diffusion results in steep concentration gradients of different microbial nutrients and metabolites, creating unique niches at different depth intervals in the mats. The most abundant and versatile phototrophic mat builders are filamentous cyanobacteria, which are oxygenic phototrophs and many of which tolerate extreme environmental conditions. For example, some species of cyanobacteria grow in waters as hot as 73°C or as cold as 0°C, and others tolerate salinities in excess of 12% and pH values as high as 10.

### Cyanobacterial Mats

Cyanobacterial mats (Figure 20.7*a*, *b*) are complete microbial ecosystems, containing large numbers of **primary producers** (cyanobacteria and other phototrophic bacteria) that use light energy to synthesize new organic material from $CO_2$. These along with populations of consumers in the mat community mediate all key nutrient cycles.

Microbial mats have existed for over 3.5 billion years (Section 13.1) but are found today only in aquatic environments where environmental stresses such as high temperatures or high salt concentrations restrict grazing by small animals and insects. Well-studied microbial mats are found in hypersaline solar evaporation basins; such basins have either formed naturally, such as Solar Lake (Sinai, Egypt), or have been constructed for the recovery of sea salt (Figure 20.7*a*). Because microbial mats are restricted to extreme environments, most are found in remote

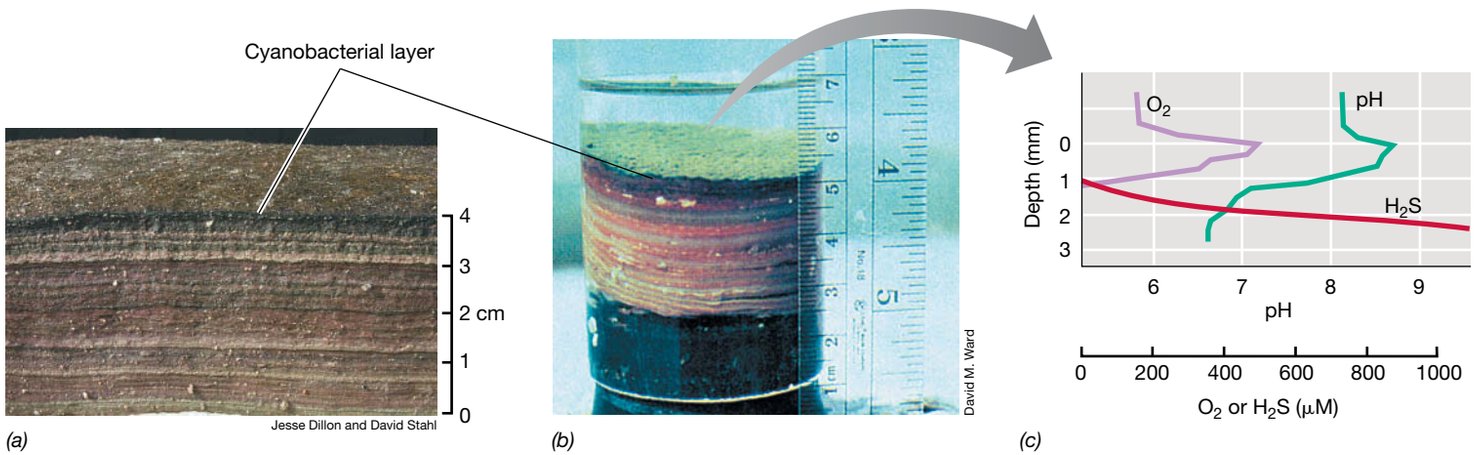



**Figure 20.7 Microbial mats.** *(a)* Mat specimen collected from the bottom of a hypersaline pond at Guerrero Negro, Baja California (Mexico). Most of the bottom of this shallow pond is covered with mats built by the major primary producer, the filamentous cyanobacterium *Microcoleus chthonoplastes*. *(b)* Microbial mat core from an alkaline Yellowstone National Park (USA) hot spring. The upper (green) layer contains mainly cyanobacteria, while the reddish layers contain anoxygenic phototrophic bacteria. *(c)* Daylight oxygen ($O_2$), $H_2S$, and pH profiles through a hot spring mat core such as that shown in part *b*.

locations and many are not readily accessible to study. In contrast, however, the cyanobacterial mats that colonize the outflow channels of hot springs in Yellowstone National Park (USA), Iceland, and many other thermal regions in the world are easily accessible and have been widely studied (Figure 20.7*b, c*).

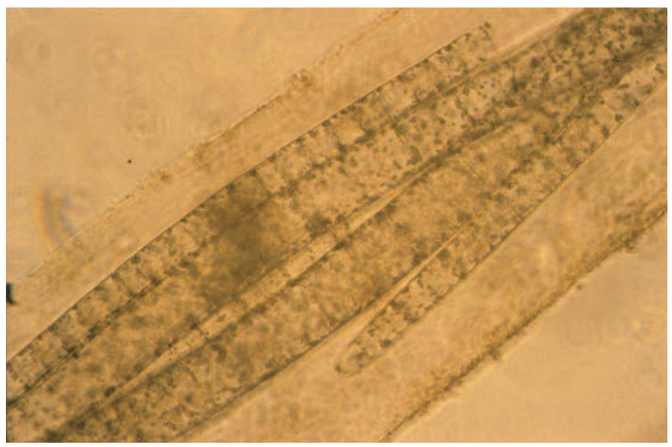
The chemical and biological structure of a microbial mat can change dramatically during a 24-h period (called a *diel cycle*) as a consequence of changing light intensity. Using microsensors (Section 19.9) it is possible to measure pH, $H_2S$, and $O_2$ repeatedly over a diel cycle in zones in the mat separated vertically by only a few micrometers. During the day, there is intense oxygen production in the cyanobacterial surface layer of microbial mats and active sulfate reduction throughout the lower regions. Near the zone where $O_2$ and $H_2S$ begin to mix, intense metabolic activity by phototrophic and chemolithotrophic sulfur bacteria may consume these substrates rapidly over very short vertical distances. Detecting the rate of these changes reveals the zones of greatest microbial activity (Figure 20.7*c*). These gradients disappear at night when photosynthesis stops and the entire mat turns anoxic and $H_2S$ accumulates. Some mat organisms rely on motility to follow the shifting chemical gradients. For example, sulfur-oxidizing filamentous phototrophic bacteria such as *Chloroflexus* and *Roseiflexus* (Section 15.7) follow the up-and-down movement of the $O_2$–$H_2S$ interface on a diel basis.

## Chemolithotrophic Mats

The most common types of chemolithotrophic mats are composed of filamentous sulfur-oxidizing bacteria, such as *Beggiatoa* and *Thioploca* species, which grow on marine sediment surfaces at the interface between $O_2$ supplied from the overlying water and $H_2S$ produced by sulfate-reducing bacteria living in the sediment. In these dark habitats, photosynthesis cannot occur and so the bacteria oxidize $H_2S$ to support energy conservation and autotrophic reactions (Sections 14.9 and 15.11).

Chemolithotrophic mats composed of sulfur-oxidizing *Thioploca* species on sediments of the Chilean and Peruvian continental shelf are thought to be the most extensive microbial mats of any type on Earth (**Figure 20.8**). *Thioploca* has developed a

(a)



(b)

(c)

Andreas Teske and Markus Huettel

**Figure 20.8 *Thioploca* mats.** *(a, c)* Filaments of the large sulfur-oxidizing chemolithotroph *Thioploca* extend into the water above the sediment (87 m depth) in the Bay of Concepción off the Chilean coast. *(b) Thioploca* form bundles of 10 to 20 filaments (trichomes) held together by a gelatinous sheath, each bundle approximately 1.5 mm in diameter and 10–15 cm in length. Two species of *Thioploca* commonly inhabit the same bundle: *T. chileae*, about 20 μm in diameter, and *T. araucae*, about 40 μm in diameter. Individual trichomes glide independently within the sheaths and can extend up to 3 cm into the water.

remarkable strategy to bridge spatially separated resources. These mat bacteria contain large internal vacuoles that store high concentrations of nitrate ($NO_3^-$) as an electron acceptor to support the anaerobic respiration of $H_2S$. Much like a scuba diver filling tanks with oxygen to dive into the water, cells of *Thioploca* migrate up to the sediment surface (Figure 20.8*a*, *b*) to charge internal vacuoles with $NO_3^-$ from the water column and then return ("dive") into the anoxic sediment (gliding at speeds of 3–5 mm per hour) to use their stored $NO_3^-$ as an electron acceptor for $H_2S$ oxidation.

The physical and biological structures of both biofilms and microbial mats are determined by metabolic interactions among the microbes within them and the diffusion of nutrients. Thus, as biofilms form on a surface they become increasingly more complex, and in so doing generate new niches for organisms of differing physiologies. This diversity reaches a maximum in mature microbial mats (Figure 20.7*a*, *b*), as molecular community sampling (Section 19.6) has shown these structures to be among the most complex microbial communities yet discovered.

**MINIQUIZ**

- What is a microbial mat and what major nutrient changes occur in mats during a diel cycle?
- How would motile aerobic bacteria in a microbial mat respond to changing $O_2$ concentrations over a diel cycle?

# III • Terrestrial Environments

Extensive microbial habitats exist in two terrestrial environments on Earth that are similar in lacking sunlight, being periodically or permanently anoxic, and sharing several other physicochemical conditions in common. These two habitats are soils and water enclosed in soils and bedrock. We cover these microbial habitats in the next two sections, and in each case we begin with the abiotic part of the environment and conclude with a discussion of the microbial communities that live there.

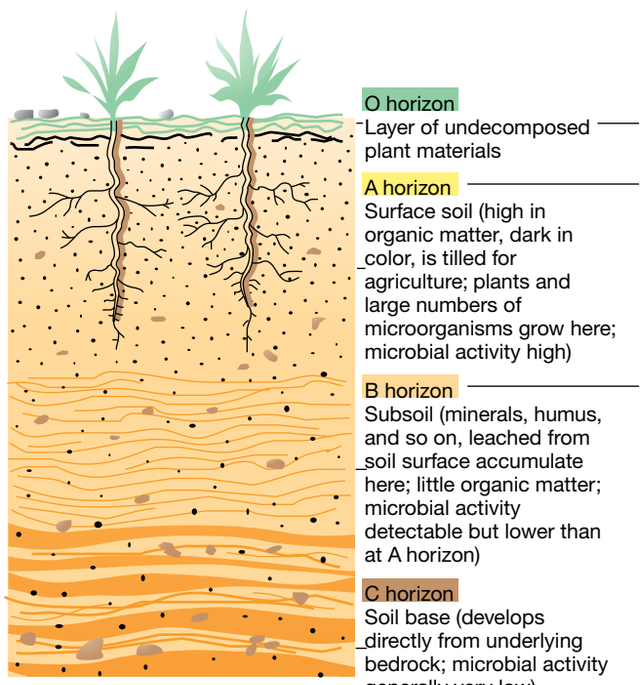
## 20.6 Soils

The word *soil* refers to the loose outer material of Earth's surface, a layer distinct from the bedrock that lies underneath (**Figure 20.9**). Soil develops over long periods through complex interactions among the parent geological materials (rock, sand, glacial drift materials, and so on), the topography, climate, and the presence and activities of living organisms.

Soils can be divided into two broad groups: *Mineral soils* are derived from the weathering of rock and other inorganic materials, and *organic soils* are derived from sedimentation in bogs and marshes. Most soils are a mixture of these two basic types. Although mineral soils, which are the primary focus of this section, predominate in most terrestrial environments, there is increasing interest in the role that organic soils play in carbon storage. A detailed understanding of carbon storage (sinks) and sources (such as release of $CO_2$) is of great relevance to the science of climate change.The carbon cycle is a major focus of Chapter 21.

### Soil Composition and Formation

Vegetated soils have at least four components. These include (1) inorganic mineral matter, typically 40% or so of the soil volume; (2) organic matter, usually about 5%; (3) air and water, roughly 50%; and (4) microorganisms and macroorganisms, about 5%. Particles of various sizes are present in soil. Soil scientists classify soil particles on the basis of size: Those in the




(a)

Michael T. Madigan

(b)

**Figure 20.9 Soil.** *(a)* Profile of a mature soil. The soil horizons are zones defined by soil scientists. *(b)* Photo of a soil profile, showing O, A, and B horizons. This soil from Carbondale, Illinois (USA), is rich in clay and very compact. Such soils are not as well drained as those rich in sand. Note the clear color delineation between the organic-rich A horizon and the less-organic-rich B horizon.

range of 0.1–2 mm in diameter are called *sand*, those between 0.002 and 0.1 mm *silt*, and those less than 0.002 mm *clay*. Different textural classes of soil are then given names such as "sandy clay" or "silty clay" based on the percentages of sand, silt, and clay they contain. A soil in which no one particle size dominates is called a *loam*.

Physical, chemical, and biological processes all contribute to the formation of soil. An examination of almost any exposed rock reveals the presence of algae, lichens, or mosses. These organisms are phototrophic and produce organic matter, which supports the growth of chemoorganotrophic bacteria and fungi. More complex chemoorganotrophic communities composed of *Bacteria*, *Archaea*, and eukaryotes then develop as the extent of the earlier colonizing organisms increases. Carbon dioxide produced during respiration becomes dissolved in water to form carbonic acid ($H_2CO_3$), which slowly dissolves the rock, especially rocks containing limestone ($CaCO_3$). In addition, many chemoorganotrophs excrete organic acids, which also promote the dissolution of rock into smaller particles.

Freezing, thawing, and other physical processes assist in soil formation by forming cracks in the rocks. As the particles generated combine with organic matter, a crude soil forms in these crevices, providing sites needed for pioneering plants to become established. The plant roots penetrate farther into the crevices, further fragmenting the rock; the excretions of the roots promote development in the **rhizosphere** (the soil that surrounds plant roots and receives plant secretions) of high microbial cell abundance (Figure 20.4*a*). When the plants die, their remains are added to the soil and become nutrients for more extensive microbial development. Minerals are rendered soluble, and as water percolates, it carries some of these substances deeper into the soil.

As weathering proceeds, the soil increases in depth and becomes able to support the development of larger plants and small trees. Soil animals such as earthworms colonize the soil and play an important role in keeping the upper layers of the soil mixed and aerated. Eventually, the movement of materials downward results in the formation of soil layers, called a *soil profile* (Figure 20.9). The rate of development of a typical soil profile depends on climatic and other factors, but it can take hundreds to thousands of years.

## Water Availability: Vegetated and Dryland Soils as Microbial Habitats

The limiting nutrients in soils are often inorganic nutrients such as phosphorus and nitrogen, key components of several classes of macromolecules. Another major factor affecting microbial activity in soil is the availability of water, and we have previously emphasized the importance of water for microbial growth (Section 5.13).

Water is a highly variable component of soil, and a soil's water content depends on soil composition, rainfall, drainage, and plant cover. Water is held in the soil in two ways—by adsorption onto surfaces or as free water in thin sheets or films between soil particles (**Figure 20.10**). The water present in soils has materials

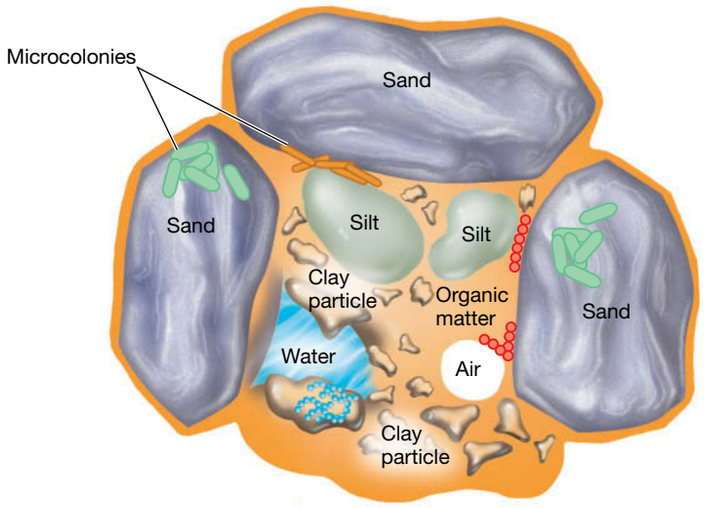



**Figure 20.10 A soil microbial habitat.** Very few microorganisms are free in the soil solution; most of them reside in microcolonies attached to the soil particles. Note the relative size differences among sand, clay, and silt particles.

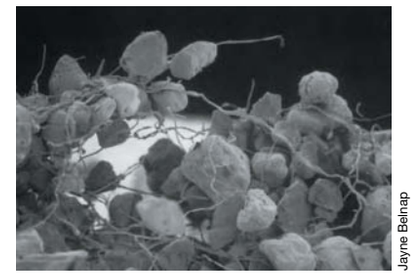
dissolved in it, and the mixture is called the *soil solution*. In well-drained soils, air penetrates readily, and the oxygen concentration of the soil solution can be high, similar to that of the soil surface. In waterlogged soils, however, the only oxygen present is that dissolved in water, and this can be rapidly consumed by the resident microbiota. Such soils then become anoxic, and, as described for freshwater environments (Section 20.8), show profound changes in their biological activities. There is also water in the larger channels in soil, where bulk flow is important for rapid transport of microorganisms and their substrates and products.

## Arid Soils

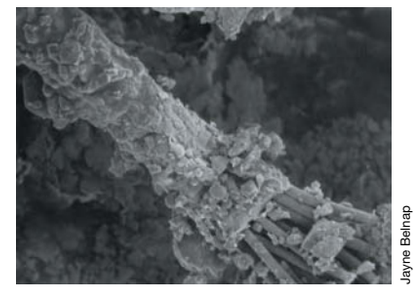
The greatest microbial activity in soils is in the organic-rich surface layers in and around the rhizosphere (Figure 20.4*a*). However, some soils are so dry that plant coverage is greatly limited and only special microbial communities can thrive. These are *arid soils*, and approximately 35% of Earth's landmass is permanently or seasonally arid. Aridity can be defined by the *aridity index*, expressed as the ratio of precipitation to potential evapotranspiration (P/PET). Evapotranspiration is the sum of water loss through evaporation and plant transpiration. A region is deemed arid if there is a P/PET of less than 1; that is, water entering through precipitation (and fog and dew) is less than that lost through evapotranspiration.

Arid soils are among the most extreme environments on Earth, with temperature highs in excess of 60°C and lows of −24°C, high insolation (exposure to solar rays), and low water activity. Although arid regions are typically nearly devoid of leafy plants, they sustain important microbial communities that assemble in and stabilize soil near the surface and reside within and on the surfaces of rocks. The dominant microorganisms present in these carbon-limited environments are cyanobacteria, with lesser numbers of green algae, fungi, heterotrophic bacteria, lichens, and mosses.


(a)




(b)




(c)

**Figure 20.11 Biological soil crust (BSC).** *(a)* BSC on the Colorado Plateau shown adjacent to lighter disturbed soils. *(b, c)* Scanning electron micrographs of filamentous cyanobacteria (*Microcoleus* species) that bind sand grains together with their sheath material. The sand grains in part *b* are about 100 μm in diameter and the filaments in part *c* about 5 μm in diameter.

Dryland microbial habitats include *biological soil crusts* (BSCs) (**Figure 20.11**), ventral surfaces of translucent stones (*hypolithic* colonists), exposed rock surfaces (*epilithic* colonists), and the interior pore spaces, cracks, and fissures of rocks (*endolithic* colonists). The soil crusts are dominated by cyanobacterial *Microcoleus* species (Figure 20.11*b*, *c*), whereas coccoid *Chroococcidiopsis* species are the predominant endolithic population. The rock colonists play an important role in weathering and soil formation as described above; here we primarily consider the BSC communities.

The BSC serves a critical function in soil stabilization of desert ecosystems. Stabilization is critical because of the very slow rate of desert soil formation (<1 cm per 1000 years). Here, the filamentous cyanobacteria (*Microcoleus*) and fungi provide soil cohesion, which is further stabilized aboveground by lichens and mosses when present. Importantly, this microbial network functions to eliminate soil erosion from wind and water. The BSCs are major determinants of water infiltration and influence local hydrological cycles and water availability to vegetation. Remarkably, when moisture and temperature conditions are optimal, the photosynthetic rates of BSC are comparable to those of vascular plant leaves. Cyanobacteria and other nitrogen-fixing bacteria (Sections 7.8, 14.6, and 15.3) provide nitrogen, and much of the fixed nitrogen is released immediately and made available to other soil biota.

The disruption of BSCs is a major contributor to *desertification*, a process exacerbated by climate change and human activities. Dust storms resulting from BSC destruction reduce soil fertility, and when heavy dust is deposited on nearby snowfields it accelerates melt and evapotranspiration rates, thereby reducing freshwater inputs to rivers. Once compromised, soil crusts have recovery times varying from 15 to 50 years. Given the expansive terrestrial presence of BSCs, their importance to human and ecosystem function, and the projected increase in aridity associated with climate change, a better understanding of BSC formation and the rehabilitation of compromised BSCs is important for a healthy planet Earth.

## A Phylogenetic Snapshot of Soil Bacterial and Archaeal Diversity

As we saw in Figure 20.3, even a single soil particle can contain many different microenvironments and can thus support the growth of several physiological types of microorganisms. To examine soil particles directly for microbes, fluorescence microscopes are often used, the organisms in the soil having been previously stained with a fluorescent dye. To visualize a specific microorganism in a soil particle, fluorescent staining, such as with fluorescent gene probes (Sections 19.4, 19.5), can also be used. Microorganisms can also be observed on soil surfaces directly by scanning electron microscopy (Figure 20.11*b*, *c*).

We learned in Chapter 19 that sequence analyses of 16S ribosomal RNA (rRNA) genes obtained from the environment are commonly used as a measure of bacterial and archaeal diversity (Section 19.6). As yet, no natural communities have been so thoroughly characterized by these techniques that *all* resident species have been identified. However, within limits, the method is widely considered to be a valid measure of microbial diversity and avoids the more serious problems of enrichment bias that plague culture-dependent diversity studies (Section 19.1). Here and in later sections of this chapter we present a "phylogenetic snapshot" of major microbial habitats, with the goal of emphasizing trends and patterns rather than absolute details.

Molecular community sampling of a typical vegetated surface has shown typically *thousands* of different species of *Bacteria* and *Archaea* in a single gram of soil, likely reflecting the numerous

microenvironments present there. A "species" is operationally defined here as a 16S rRNA gene sequence obtained from a microbial community that differs from all other sequences by more than 3% (↪ Section 13.8). Such an environmental sequence is called a *phylotype*. Besides very large species numbers, soil microbial diversity studies have also showed that diversity varies with soil type and geographical location. For example, analysis of an Alaska forest soil, an Oklahoma prairie soil, and a Minnesota farm soil (all sites in the USA) revealed approximately 5000, 3700, and 2000 different phylotypes, respectively. The Alaska and Minnesota soils showed similar distributions at the phylum level of taxonomy (for example, *Proteobacteria, Acidobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia*, and *Planctomycetes*) but shared only about 20% of their species in common. This indicates that although the *proportions* of the dominant phyla in different soils are relatively constant, the *actual species present* within a phylum may vary considerably between different soils. In addition, lower bacterial diversity was observed in the farm soil than the Alaska soil, an indication that modern intensive agricultural practices that rely heavily on fertilization, low plant diversity, and the chemical suppression of unwanted plants and animals negatively affect bacterial diversity.

**Figure 20.12** shows the general composition of prokaryotic soil communities based on pooled 16S rRNA sequence data taken

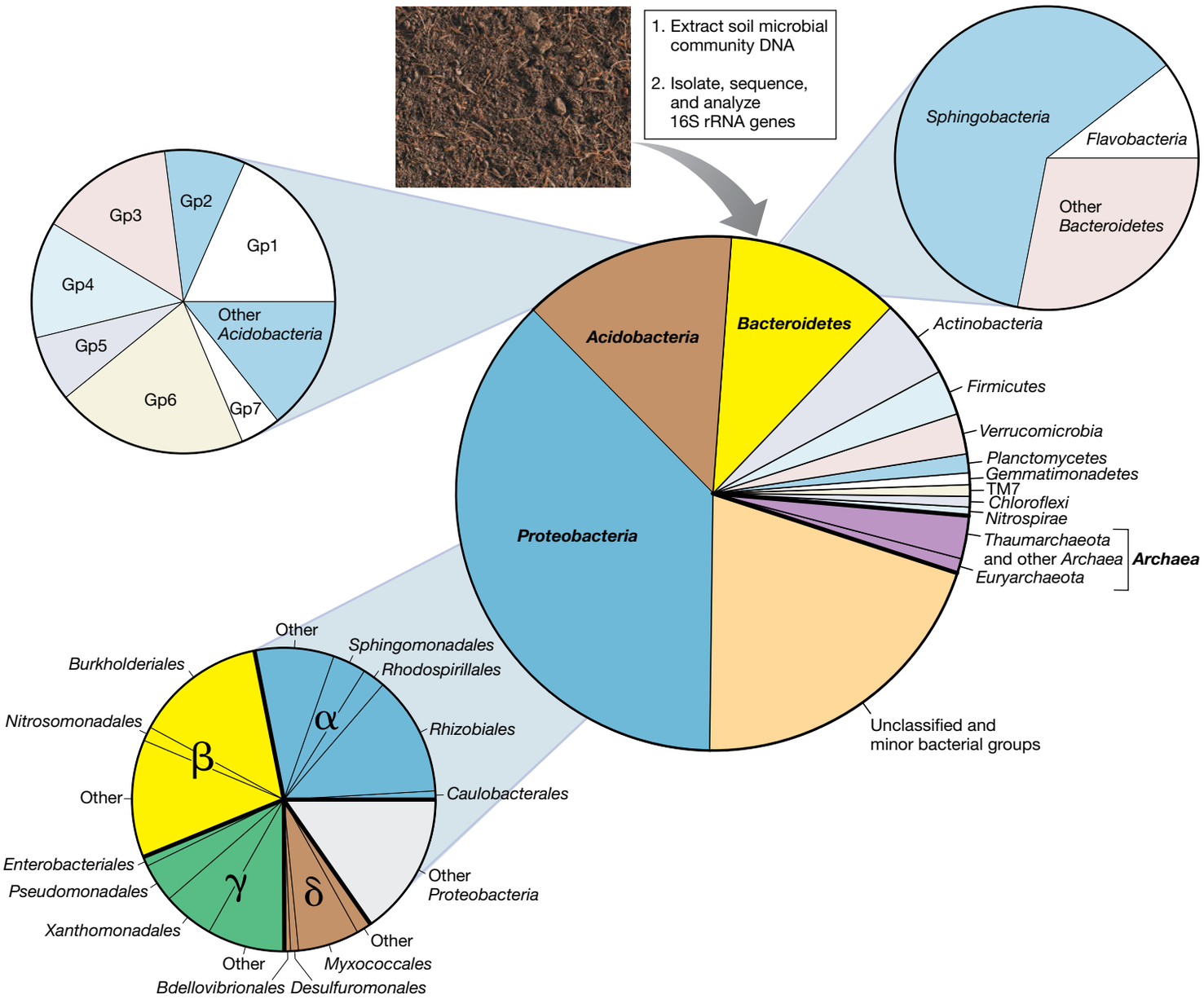



**Figure 20.12 Soil bacterial and archaeal diversity.** The results are pooled analyses from several studies of the 16S rRNA gene content of soil environments. Many of these groups are covered in Chapters 15 and 16 (*Bacteria*) or 17 (*Archaea*). For *Proteobacteria*, *Acidobacteria*, and *Bacteroidetes*, major subgroups are indicated (Gp, group). Note high species richness as indicated by the large proportion of the total community composed of unclassified and minor bacterial groups. Also note the relatively low proportion of the total prokaryotic soil diversity represented by *Archaea* and that many soil *Archaea* are not close relatives of known *Euryarchaeota* or *Crenarchaeota*. Data assembled and analyzed by Nicolas Pinel.

from several soils. As can be seen, *Proteobacteria* (Chapters 15 and 16) make up nearly half of the total phylotypes recovered, with all major subgroups except for *Epsilonproteobacteria* well represented. *Acidobacteria* and *Bacteroidetes* are also abundant groups; *Actinobacteria* and *Firmicutes* are less so. In addition to these, a major proportion of soil phylotypes are unclassified species or members of minor bacterial groups. This underscores the high bacterial diversity typical of soil ecosystems. In contrast to *Bacteria*, the diversity of *Archaea* in soil is minimal, with relatively few sequences within each major phylum of *Archaea* (*Euryarchaeota, Thaumarchaeota,* and *Crenarchaeota*) represented. However, since there have been fewer selective surveys of archaeal diversity in soils, their diversity may be greater than now recognized.

A similar study to that shown in Figure 20.12 but performed on hydrocarbon-polluted soil showed that the general taxonomic makeup of polluted and unpolluted soils is similar: *Proteobacteria* comprise the largest fraction in both soil types, followed by significant representation of *Acidobacteria, Bacteroidetes, Actinobacteria*, and *Firmicutes*. However, there was a significant shift in fractional representation of these taxa in the two soils. Polluted soils are enriched in *Actinobacteria, Gammaproteobacteria,* and *Euryarchaeota* but diminished in *Bacteroidetes, Acidobacteria*, and unclassified *Bacteria* relative to unpolluted soils (Figure 20.12). Hydrocarbon-polluted soils contained a single dominant *Bacteroidetes* phylotype, whereas unpolluted soils contained several phylotypes of *Bacteroidetes* (Figure 20.12). Notably, *Thaumarchaeota* are absent from all surveys of hydrocarbon-polluted soils, suggesting that hydrocarbon pollutants suppress ammonia-oxidizing *Thaumarchaeota* (*Archaea*, Section 17.5).

Although the *functional* significance of the observed diversity of microbial communities in polluted versus unpolluted soils is unknown, the shifts observed signal that the two soils will likely differ in their capacity to process carbon and nitrogen and to carry out other important nutrient cycling events. However, despite this lack of a functional connection, different 16S rRNA gene surveys of soils agree on two things: (1) undisturbed, unpolluted soils support very high prokaryotic diversity, and (2) soil perturbations trigger measurable shifts in community composition toward species that are more competitive in the disturbed soil and are accompanied by an overall reduction in diversity.

**MINIQUIZ**

- Which phylum of *Bacteria* dominates bacterial diversity in vegetated soil?
- What factors govern the extent and type of microbial activity in soils?
- Which region of soil is the most microbially active?

Esta van Heerden

(a)

Terry Hazen

(b)

**Figure 20.13 Sampling the deep subsurface.** *(a)* Sampling hot (55°C) fissure water from a depth of 3000 m in the Tau Tona South African gold mine. *(b)* Drilling to 600 m in Allendale, South Carolina (USA), for the U.S. Department of Energy (DOE) Deep Subsurface Microbiology Program. Subsurface microbiology is both expensive and challenging from the standpoint of obtaining uncontaminated samples from deep underground. However, it is clear that some very interesting *Bacteria* and *Archaea* inhabit earth's deep subsurface (see Figures 20.14 and 20.15).

## 20.7 The Terrestrial Subsurface

In the soils and rocks of Earth's subsurface, there is water. This underground water, called *groundwater*, is a vast but little-explored microbial habitat. As recently as three decades ago most microbiologists were of the opinion that significant microbial numbers were limited to the top 100 m or so of Earth's crust. However, from research made possible by the development of improved drilling and aseptic sampling technology, it is now known that microbial life extends down at least 3 *kilometers* into the Earth in regions containing trapped water. The microbiology of relatively shallow groundwater is quite similar to the microbiology of soils. However, microorganisms in deep subsurface waters exist at temperatures that can exceed 50°C and in anoxic and nutrient-depleted surroundings; thus, their microbial diversity is distinct from that of soil.

### *Bacteria* in the Deep Subsurface

Subsurface microbiology initially focused on relatively shallow and easily accessible aquifer systems, revealing diverse populations of *Archaea* and *Bacteria* and a limited presence of protozoa and fungi. An *aquifer* is an underground layer of water-bearing permeable material, such as fractured rock or gravel. Microorganisms in aquifers are metabolically active and greatly influence the chemistry of groundwater. For example, the presence of ferrous iron ($Fe^{2+}$) in groundwater is largely attributable to the activity of microorganisms such as *Geobacter* that reduce ferric iron ($Fe^{3+}$) as an electron acceptor (Section 14.15).

Research on the deep microbial biosphere has been facilitated by mining and drilling operations that expose water in fractured rock at great depths. For example, samples collected from a nearly 3-km-deep gold-mining operation in South Africa (**Figure 20.13a**) revealed chemolithotrophic and autotrophic *Bacteria* and *Archaea*. DNA extracted from fissure water deep in this mine showed that a $H_2$-oxidizing, sulfate-reducing bacterium was virtually the only bacterium present. Genome analysis of the organism, as yet uncultured but given the provisional name *Desulforudis audaxviator*, indicated that it should be thermophilic and should be capable of autotrophic growth using $H_2$ as the electron donor for anaerobic respiration and $CO_2$ fixation. In addition, the organism contained genes encoding a nitrogen fixation system (Section 14.6), meaning that it should be able to live in an anoxic environment on a diet of a few minerals, $CO_2$, $SO_4^{2-}$, $N_2$, and $H_2$.

*D. audaxviator* would be well suited to long-term isolation in the deep subsurface, as would other autotrophic and nitrogen-fixing bacteria that could use $H_2$ as electron donor. Possible subsurface sources of $H_2$ for this include the radiolysis of water by uranium, thorium, and other radioactive elements, and geochemical processes such as the release of $H_2$ from the oxidation of iron silicate minerals in aquifers. $H_2$ can satisfy the needs of bacteria that carry out many different types of bacterial anaerobic respirations, including sulfate reduction, acetogenesis, and ferric iron reduction (Chapter 14), and examples of all these physiologies have been identified from genomic analyses of subsurface materials. Hence, *Bacteria* capable of these physiologies undoubtedly inhabit the subsurface microbial ecosystem along with *D. audaxviator*.

### *Archaea* in the Deep Subsurface

Many novel lineages of *Archaea* appear to be adapted to the extremely nutrient-limited environments of the terrestrial subsurface and deep marine sediments (Section 20.13). In addition to archaeal species that affiliate with phyla having cultivated representatives (*Euryarchaeota, Crenarchaeota, Thaumarchaeota*, Chapter 17), novel phyla so far identified only through PCR-based and metagenomic surveys (Sections 19.6 and 19.8) include the *Aigarchaeota* and *Bathyarchaeota* (**Figure 20.14**). These surveys have also revealed a remarkable diversity of extremely small *Archaea* having cells 0.15–1.2 µm in diameter, containing small genomes (~500–1000 genes), and living in the subsurface and also in a variety of other nutrient-depleted environments

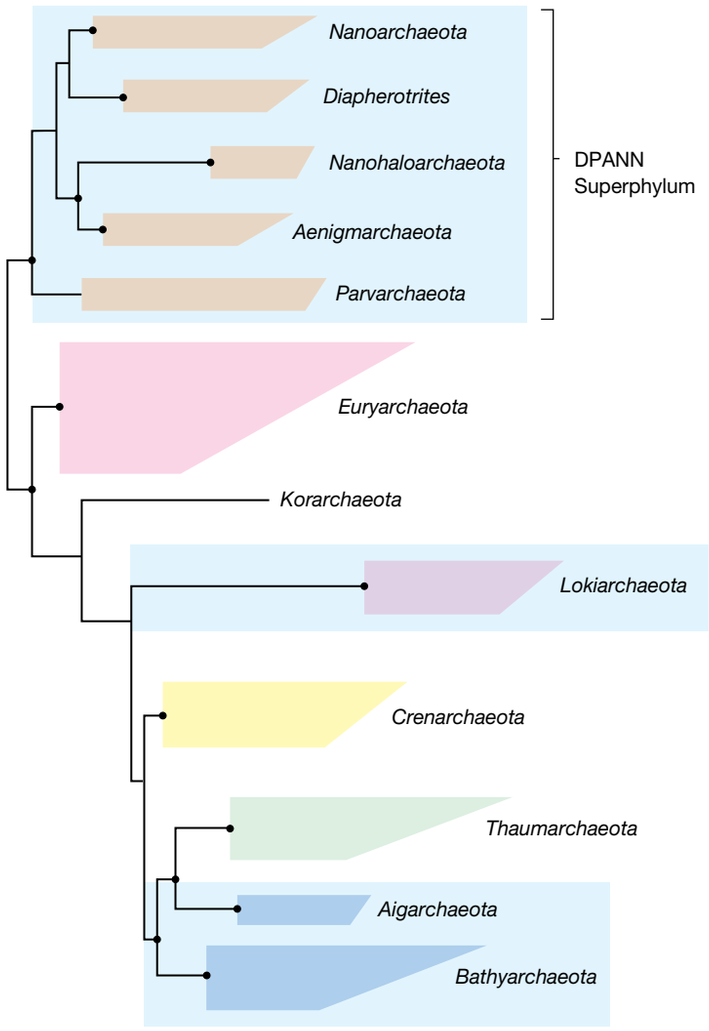



**Figure 20.14 Diversity and abundance of subsurface *Archaea*.** Phylogeny of archaeal clades found predominantly in the terrestrial and marine deep subsurfaces (this is a consensus tree based on 16S rRNA gene sequences and other conserved marker genes). The novel phyla previously unknown until studies of the deep subsurface are highlighted in light blue. The *Lokiarchaeota* may be the closest known relatives of eukaryotic cells (see page 399 for more on the *Lokiarchaeota*–eukaryote connection).

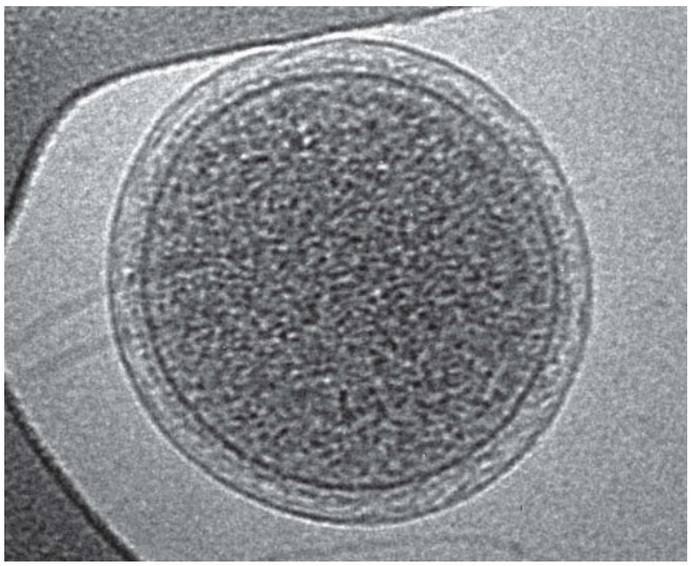



**Figure 20.15 Small *Archaea*.** Electron micrographic section of a cell of a small species of *Archaea* inhabiting acid mine drainage (Section 22.2). The small *Archaea* found in this acidic environment affiliate with the *Diapherotrites* and *Parvarchaeota* phyla (Figure 20.14). This cell is approximately 0.4 μm in diameter.

(**Figure 20.15**); small cells of *Bacteria* also inhabit these environments (see Explore the Microbial World, "Tiny Cells," in Chapter 2).

The tiny *Archaea* form a single deep evolutionary divergence within the *Archaea* that encompass multiple phyla (a "superphylum," Figure 20.14). The first described member of this superphylum (called DPANN as an acronym from the beginning initials of the five phyla within this lineage) was the thermophile *Nanoarchaeum equitans*, a small parasitic species of limited metabolic capacity that grows in obligate physical association with a host archaeon, *Ignicoccus* (Section 17.6). Whether other tiny *Archaea* will show a similar lifestyle is unknown, but metagenomic sequences suggest that some of the subsurface species may be autotrophs and thus more metabolically capable than *Nanoarchaeum*. Nevertheless, any physiology inferred solely from genome sequences is only a hypothesis that must be confirmed with studies of cultured species.

At least two major metagenomic surprises emerged from these deep subsurface studies. First was the discovery of genomes from subsurface *Bathyarchaeota* (Figure 20.14) that contained genes encoding the enzymes that catalyze reactions of methanogenesis and the acetyl-CoA pathway. These metabolisms are the mechanisms of energy conservation and autotrophy, respectively, in methane-producing (methanogenic) *Archaea*. If future laboratory cultures of *Bathyarchaeota* confirm these results they would show that methanogenesis, a major metabolic process thought for some time now to be restricted to species in a single phylum of *Archaea* (*Euryarchaeota*, Figure 20.14 and Chapter 17), is actually more broadly distributed within this domain.

Another remarkable finding from the deep biosphere was the discovery of an archaeal clade given the name *Lokiarchaeota* (Figure 20.14). It has long been known that *Archaea* and *Eukarya* diverged at some point in the distant past (Section 13.3 and Figure 13.9), but the closest archaeal ancestors to the *Eukarya* had thus far remained unclear. However, based on metagenomic analyses, the *Lokiarchaeota* are hypothesized to be the lineage from which the eukaryotic domain emerged. This phylogenetic connection was supported by criteria other than just ribosomal RNA gene sequences, including the fact that *Lokiarchaeota* genomes encode a eukaryotic-type cytoskeleton that include proteins such as actin (Section 2.16) as well as several eukaryotic-like proteins with specific membrane functions and the potential for taking up substances by engulfment or membrane invagination. Thus, analysis of the deep subsurface biosphere has not only greatly expanded the known phylogenetic diversity of *Archaea*, but has possibly revealed new information about the origin of eukaryotes.

### Growth Rates and the Future of Subsurface Microbiology

Bacterial numbers in uncontaminated groundwater vary by several orders of magnitude ($10^2$–$10^8$ per ml), reflecting limited nutrient availability, mostly in the form of dissolved organic carbon. Measured and estimated generation times for deep subsurface bacteria vary by many orders of magnitude, from days to centuries, as determined by the physicochemical environment, the physiology of the resident populations, and nutrient availability. For example, microorganisms appear to be attached to surfaces or within biofilms in the nutrient-depleted subsurface, but it is unknown whether these are genetically and physiologically distinct from microbes in planktonic populations and the extent to which they exploit different metabolic strategies.

The many unanswered questions in subsurface microbiology have encouraged the establishment of permanent science laboratories at great depths in the Earth. For example, the Sanford Underground Research Facility in Lead, South Dakota (USA) (2400 m deep), is supported by government and private agencies for research in physics, geology, and microbiology. The Integrated Ocean Drilling Program, an international effort, has probed for microbial populations at great depths below the seafloor. Results thus far have shown *Archaea* and *Bacteria* as far down as 2000 m below the seafloor (Section 20.13) and in rocks more than 100 *million* years old. Although this may sound ancient, such ages are actually relatively young compared with viable bacteria that have been recovered from salt crystals nearly a half *billion* years old. Obviously, bacterial cells can remain viable for enormously long periods of time.

**MINIQUIZ**

- Why are possible sources of biologically available energy in the terrestrial subsurface?
- What environmental factors determine the abundance and type of cells in the deep subsurface?
- What information obtained from the *Lokiarchaeota* metagenome associate it with the origin of eukaryotes?

# IV • Aquatic Environments

Freshwater and marine environments differ in many ways including salinity, average temperature, depth, and nutrient content, but both provide many excellent habitats for microorganisms.

In this part of the chapter we focus first on freshwater microbial habitats. We then consider two marine environments: (1) coastal and ocean waters, and (2) the deep sea. Much new information is emerging about marine microorganisms from studies using the molecular tools of microbial ecology, especially genetic stains, microbial community sampling, and metagenomics (Chapter 19).

## 20.8 Freshwaters

Freshwater environments vary significantly in the resources and conditions (Table 20.1) available for microbial growth because some lakes and streams are isolated and nearly pristine while others are highly polluted from agricultural, industrial, or residential runoff. Both oxygen-producing and oxygen-consuming organisms are present in aquatic environments, and the balance between photosynthesis and respiration (Figure 20.2) controls the natural cycles of oxygen, carbon, and other nutrients (nitrogen, phosphorus, metals).

Among microorganisms, oxygenic phototrophs include the algae and cyanobacteria. These can either be *planktonic* (floating) and distributed throughout the water columns of lakes, sometimes accumulating in large numbers at a particular depth, or *benthic*, meaning they are attached to the bottom or sides of a lake or stream. Oxygenic phototrophs, which obtain their energy from light and use water as an electron donor to reduce $CO_2$ to organic matter (Chapter 14), are the main primary producers in freshwater aquatic ecosystems.

The activity and diversity of chemoorganotrophic aquatic microbial communities depend to a major extent on primary production, in particular its rates and temporal and spatial distributions. Oxygenic phototrophs produce new organic material as well as $O_2$. If primary production rates are very high, the resultant excessive organic matter production can lead to bottom-water $O_2$ depletion from respiration and the development of anoxic conditions. This in turn stimulates anaerobic metabolisms such as anaerobic respirations and fermentations (Chapter 14). Like oxygenic phototrophs, anoxygenic phototrophs can also fix $CO_2$ into organic material. But these organisms use reduced substances other than water, such as $H_2S$ or $H_2$, as electron donors in photosynthesis (Section 14.3). Organic matter produced by anoxygenic phototrophs can also support and enhance respiration, accelerating the spread of anoxia.

### Oxygen Relationships in Freshwater Environments

The biological and nutrient structure of lakes is greatly influenced by seasonal changes in physical gradients of temperature and salinity. In many lakes in temperate climates the water column becomes stratified, separated into layers of differing physical and chemical characteristics that constitute a **stratified water column**. During the summer, warmer and less dense surface layers, called the **epilimnion**, are separated from the colder and denser bottom layers (the **hypolimnion**). The *thermocline* is the transition zone from epilimnion to hypolimnion (Figure 20.16).

In the late fall and early winter, lake surface waters become colder and thus more dense than the bottom layers. This, combined with wind-driven mixing, causes the cooled surface water to sink and the lake to "turn over," mixing surface and bottom waters and their nutrients. The separation of a relatively well-mixed surface layer from a relatively static bottom layer limits the transfer of nutrients between layers until fall turnover once again mixes the water column.

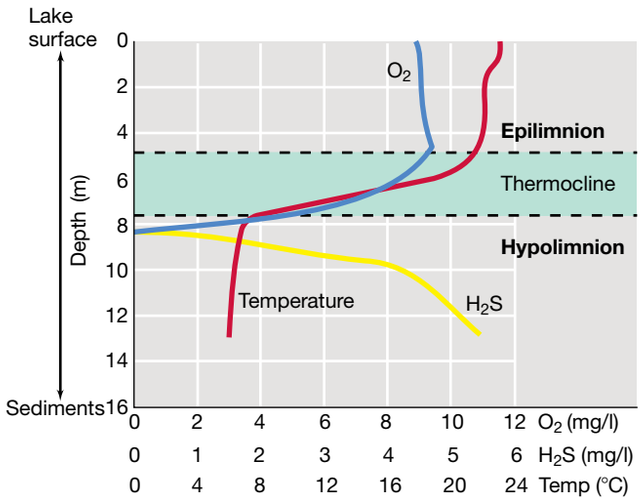



**Figure 20.16 Development of anoxic conditions in a temperate lake due to summer stratification.** The colder bottom waters are more dense and contain $H_2S$ from bacterial sulfate reduction. The thermocline is the zone of rapid temperature change. As surface waters cool in the fall and early winter, they reach the temperature and density of hypolimnetic waters and sink, displacing bottom waters and effecting lake turnover. Data are from a small freshwater lake in northern Wisconsin (USA).

During periods of stratification, transfer between surface and bottom waters is controlled not by mixing but by the much slower process of diffusion. As a result, bottom waters can experience seasonal periods of either low or no dissolved $O_2$. Although $O_2$ is one of the most plentiful gases in the atmosphere (21% of air), it has relatively limited solubility in water, and in a large body of water its exchange with the atmosphere is slow. Whether a body of water actually becomes $O_2$-depleted depends on several factors, including the amount of organic matter present and the degree of mixing of the water column. Organic matter that is not consumed in surface layers sinks to the depths and is decomposed by anaerobes (Figure 20.2). Lakes may contain high levels of dissolved organic matter because inorganic nutrients that run off the surrounding land can trigger algal and cyanobacterial blooms; these organisms typically excrete various organic compounds and also release complex organic compounds when they die and decay. The combination of water body stratification during early summer, high organic loading, and limited $O_2$ transfer results in $O_2$ depletion of the bottom waters (Figure 20.16), making them unsuitable for aerobic organisms such as plants and animals.

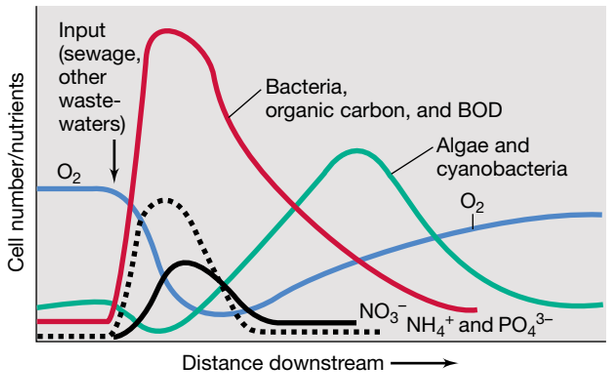
The annual turnover cycle allows the bottom waters of a lake to pass from oxic to anoxic and back to oxic. Microbial activity and community composition is altered with these changes in oxygen content, but other factors that accompany fall turnover of the water column, especially changes in temperature and nutrient levels, govern microbial diversity and activity as well. If organic matter is sparse, as it is in pristine lakes or in the open ocean, there may be insufficient substrate available for chemoorganotrophs to consume all the oxygen. The microorganisms that dominate such environments are typically **oligotrophs**, organisms adapted to growth under very dilute conditions (Section 20.11). Alternatively, where currents are strong or there is turbulence because of wind mixing, the water column may be well mixed, and consequently oxygen may be transferred to the deeper layers.

Oxygen levels in rivers and streams are also of interest, especially those that receive inputs of organic matter from urban, agricultural, or industrial pollution. Even in a river well mixed by rapid water flow and turbulence, large organic inputs can lead to a marked oxygen deficit from bacterial respiration (**Figure 20.17*a***). As the water moves away from a point source input, for example, from an input of sewage, organic matter is gradually consumed, and the oxygen content returns to previous levels. As in lakes, nutrient inputs to rivers and streams from sewage or other pollutants can trigger massive blooms of cyanobacteria and algae (Figure 20.1) and aquatic plants (Figure 20.17*b*), thereby diminishing overall water quality and growth conditions for aquatic animals.

## Biochemical Oxygen Demand

The microbial oxygen-consuming capacity of a body of water is called its **biochemical oxygen demand (BOD)**. The BOD of water is determined by taking a sample, aerating it well to saturate the water with dissolved $O_2$, placing it in a sealed bottle, incubating it in the dark (usually for 5 days at 20°C), and determining the residual oxygen in the water at the end of incubation. A BOD determination gives a measure of the amount of organic material in the water that can be oxidized by the microbes present in the water. As a lake or river recovers from an input of organic matter or from excessive primary production, the initially high BOD becomes lower and is accompanied by a corresponding increase in dissolved oxygen in the ecosystem (Figure 20.17*a*). Another related measure of the organic material in a body of water is the *chemical oxygen demand* (*COD*). This determination uses a strong oxidizing agent, such as acidic potassium dichromate, to oxidize the organic matter to $CO_2$; the amount of organic matter present is proportional to the amount of dichromate consumed. COD is often used as a rapid measure of water quality and of its potential BOD.

We thus see that in freshwaters the oxygen and carbon cycles are linked, with the levels of organic carbon and oxygen being inversely related. Although oxygenic photosynthesis produces $O_2$, the corresponding production of organic matter leads to $O_2$ deficiencies. Anoxic aquatic environments, which are typically rich in organic material, are the end result of respiratory processes that remove dissolved oxygen from the ecosystem, leaving the remaining organic material to be mineralized by organisms employing the anaerobic energy metabolisms we discussed in Chapter 14. It is also important to recognize the importance of storms, floods, and droughts in determining delivery, transport, and cycling of organic matter and inorganic nutrients in freshwater systems, including streams, rivers, lakes, and reservoirs. These less predictable changes also affect microbial productivity, diversity, distribution, and interactions in freshwater systems.




(a)


(b)

**Figure 20.17 Effect of the input of organic-rich wastewaters into aquatic systems.** *(a)* In a river, bacterial numbers increase and $O_2$ levels decrease with a spike of organic matter. The rise in algae and cyanobacteria is a response to inorganic nutrients, especially $PO_4^{3-}$. *(b)* Photo of a eutrophic (nutrient-rich) lake, Lake Mendota, Madison, Wisconsin (USA), showing algae, cyanobacteria, and aquatic plants that bloom in response to nutrients from agricultural runoff. (See also Figure 20.1.)

## A Phylogenetic Snapshot of Freshwater Prokaryotic Diversity

The importance of *Bacteria* and *Archaea* in lakes, streams, and rivers to the production, regeneration, and mobilization of nutrients is well recognized. However, only more recently have molecular methods been used to identify the participating microbial populations, their interactions, and seasonal patterns. As we saw for studies of soil diversity (Section 20.6), 16S ribosomal RNA gene sequencing is used as a culture-independent method to identify and quantify microbial phylotypes (Section 19.6). Since most molecular studies of freshwater systems have focused on lakes, the emerging picture of lake community structure is examined here.

**Figure 20.18** shows the major prokaryotic groups that inhabit lake surface samples (the epilimnion). Five major bacterial phyla are routinely observed: *Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria*, and *Verrucomicrobia*. *Archaea* affiliated with *Euryarchaeota, Crenarchaeota*, and *Thaumarchaeota* are also present. This phylum-level composition shares features in common with the ocean, where *Proteobacteria* and *Bacteroidetes* also comprise the greater part of diversity (see Figure 20.29). However, compared with lakes, in the oceans the diversity of *Betaproteobacteria* is lower and *Gammaproteobacteria* and *Alphaproteobacteria* are the more diverse subgroups of the *Proteobacteria* (see Figure 20.29).

A functional interpretation of lake prokaryotic community structure is constrained by the limited availability of cultured representatives. Freshwater *Thaumarchaeota* affiliate with known ammonia-oxidizing species and can be inferred to oxidize ammonia, but the metabolic features of freshwater *Euryarchaeota* are not yet known. *Actinobacteria* are chemoorganotrophic bacteria that in lakes may be responsible for the breakdown of nucleic acid and proteins. In addition, metagenomic analyses (Section 19.8) have shown that at least some *Actinobacteria* contain genes related to those that encode bacteriorhodopsin, a membrane-integrated protein that converts light energy into ATP (Section 20.11 and

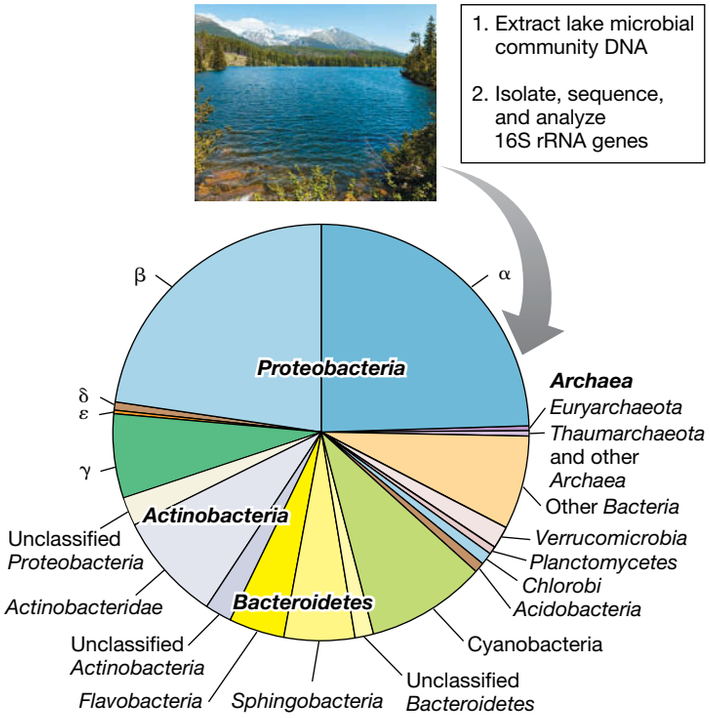



**Figure 20.18 Freshwater lake bacterial and archaeal diversity.** Distribution of 16S ribosomal RNA gene sequences by phylum determined from analysis of a collective dataset of 16S genes detected in the epilimnion of several freshwater lakes. Data assembled and analyzed by Nicolas Pinel.

Section 17.1) in extremely halophilic *Archaea*. The *Actinobacteria* analog is called *actinorhodopsin*. Hence some *Actinobacteria* may be able to harvest light as an energy source.

*Bacteroidetes* are well represented in lake ecosystems. These organisms are known for their significant metabolic diversity and are likely to be important in lakes in the degradation of various biopolymers and humic materials. The abundant *Betaproteobacteria* tend to be fast-growing species that can respond quickly to pulses of organic nutrients, whereas the *Alphaproteobacteria* are more competitive under conditions of low availability of organic nutrients; this likely accounts for their prevalence in the oligotrophic open ocean (see Figure 20.29).

Taken as a whole, high prokaryotic diversity in freshwater lakes (Figure 20.18) reflects the dynamic character of these habitats. Lakes typically receive seasonally variable inputs of endogenous and exogenous nutrients, a pattern that sustains a phylogenetically and metabolically complex community of *Bacteria* and *Archaea*.

**MINIQUIZ**

- What is a primary producer? In a freshwater lake, would primary producers more likely reside in the epilimnion or the hypolimnion, and why?
- Will addition of organic matter to a water sample increase or decrease its BOD?
- What factors might account for the prokaryotic diversity of freshwater lakes?

## 20.9 The Marine Environment: Phototrophs and Oxygen Relationships

With the exception of oxygen, nutrient levels in the open ocean (the *pelagic zone*) are often very low compared with many freshwater environments. This is especially true of key inorganic nutrients for phototrophic organisms, such as nitrogen, phosphorus, and iron. In addition, water temperatures in the oceans are cooler and more constant seasonally than those of most freshwater lakes. The activity of marine phototrophs is limited by these factors, and thus total microbial cell numbers are typically about 10-fold lower in the oceans than in freshwater environments (~$10^6$/ml versus $10^7$/ml, respectively). These are average numbers, and studies of marine prokaryotic diversity are just beginning to reveal recurrent temporal patterns of diversity and abundance.

The Bermuda Atlantic Time-Series Study (BATS) has a history of continuous biogeochemical monitoring of ocean waters since the mid-1950s, and is now incorporating molecular analyses of microbial population structure. BATS has revealed three seasonal microbial communities in ocean waters: (1) the community corresponding to the spring surface water bloom (featuring small eukaryotic algae, marine actinobacteria, and two groups of *Alphaproteobacteria*); (2) the summertime community in the upper water column associated with water column stratification (featuring *Pelagibacter, Puniceispirillum*, and two groups of *Gammaproteobacteria*); and (3) the deeper, more stable community (featuring *Nitrosopumilus*, representatives of the SAR11 group [see Figure 20.29] with which the genus *Pelagibacter* affiliates, a group of *Deltaproteobacteria*, and species of two additional groups related to the *Chloroflexi* and *Fibrobacter*). A complex and as yet poorly understood interplay of seasonal changes in physicochemical and biotic conditions likely controls these microbial communities that wax and wane in these recurring annual cycles.

Most microbes in marine waters have very small cells, a typical characteristic of organisms living in nutrient-poor environments. Smallness is an adaptive feature for nutrient-limited microorganisms in that it requires less energy for cellular maintenance. The trade-off is that a greater number of transport enzymes relative to cell volume are needed for organisms to acquire nutrients from very dilute (oligotrophic) than from nutrient-rich (eutrophic) aquatic environments. For example, ammonia-oxidizing *Archaea* (*Nitrosopumilus*, Section 17.5) are the dominant chemolithotrophs in pelagic waters and have very high-affinity transport systems for acquiring the ammonia they need as an electron donor in energy metabolism.

In pelagic waters there is a lower return of nutrients from the bottom waters than in freshwater lakes, and thus lower average primary productivity. However, because the oceans are so large, the collective carbon dioxide sequestration and oxygen production from oxygenic photosynthesis in the oceans are major factors in Earth's carbon balance. Salinity is more or less constant in the pelagic zone but is more variable in coastal areas. Terrestrial inputs, retention of nutrients, and upwelling of nutrient-rich waters combine to support higher populations of phototrophic microorganisms in

near-shore waters than in pelagic waters (**Figure 20.19**); the more productive near-shore waters in turn support higher densities of chemoorganotrophic bacteria and aquatic animals, such as fish and shellfish.

In shallow marine waters such as marine bays and inlets, eutrophication resulting from nutrient inputs can actually lead to the waters becoming intermittently anoxic from the removal of $O_2$ by respiration and the production of $H_2S$ by sulfate-reducing bacteria (**Figure 20.20**). An extensive region (6000–7000 square miles) of oxygen depletion in the Gulf of Mexico is associated with high loads of nitrogen and phosphorus carried in by the Mississippi River from agricultural runoff in the Mississippi Valley. This region, called the *Gulf of Mexico Dead Zone*, contributes to the loss and impairment of fish and benthic sea life that sustain major seafood industries in this region. The Gulf of Mexico experiences other ecological problems as well, as we examine now.

## The Deepwater Horizon Catastrophe

In addition to the chronic degradation of the Gulf of Mexico ecology through agricultural runoff, increased offshore oil drilling also poses significant environmental risk. A major catastrophe for the Gulf of Mexico was the April 2010 explosion and sinking of the Deepwater Horizon offshore drilling platform; failure to control well pressure resulted in the rupture of the wellhead at a depth of 1.5 km and the release of over 4 million barrels of oil before the well was capped three months later (**Figure 20.21**). This largest marine oil spill ever was unique in

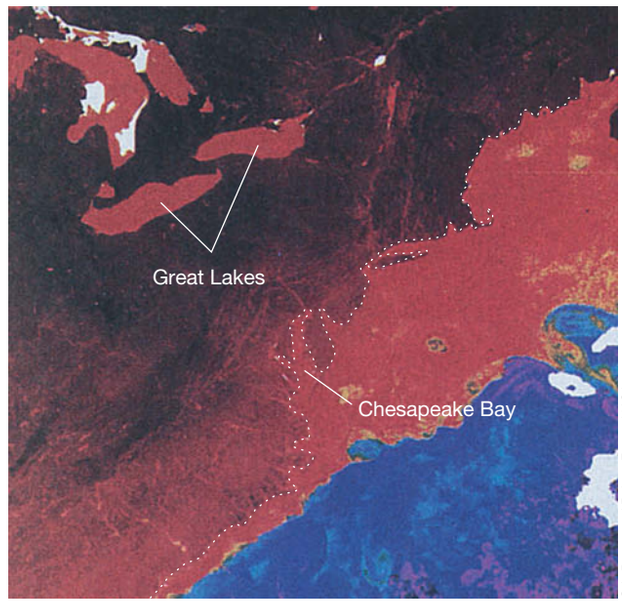



**Figure 20.19 Distribution of chlorophyll in the western North Atlantic Ocean as recorded by satellite.** The east coast of the United States from the Carolinas to northern Maine is shown in dotted outline. Areas rich in phototrophic plankton are shown in red (>1 mg chlorophyll/$m^3$); blue and purple areas have lower chlorophyll concentrations (<0.01 mg/$m^3$). Note the high primary productivity of coastal areas and the Great Lakes.

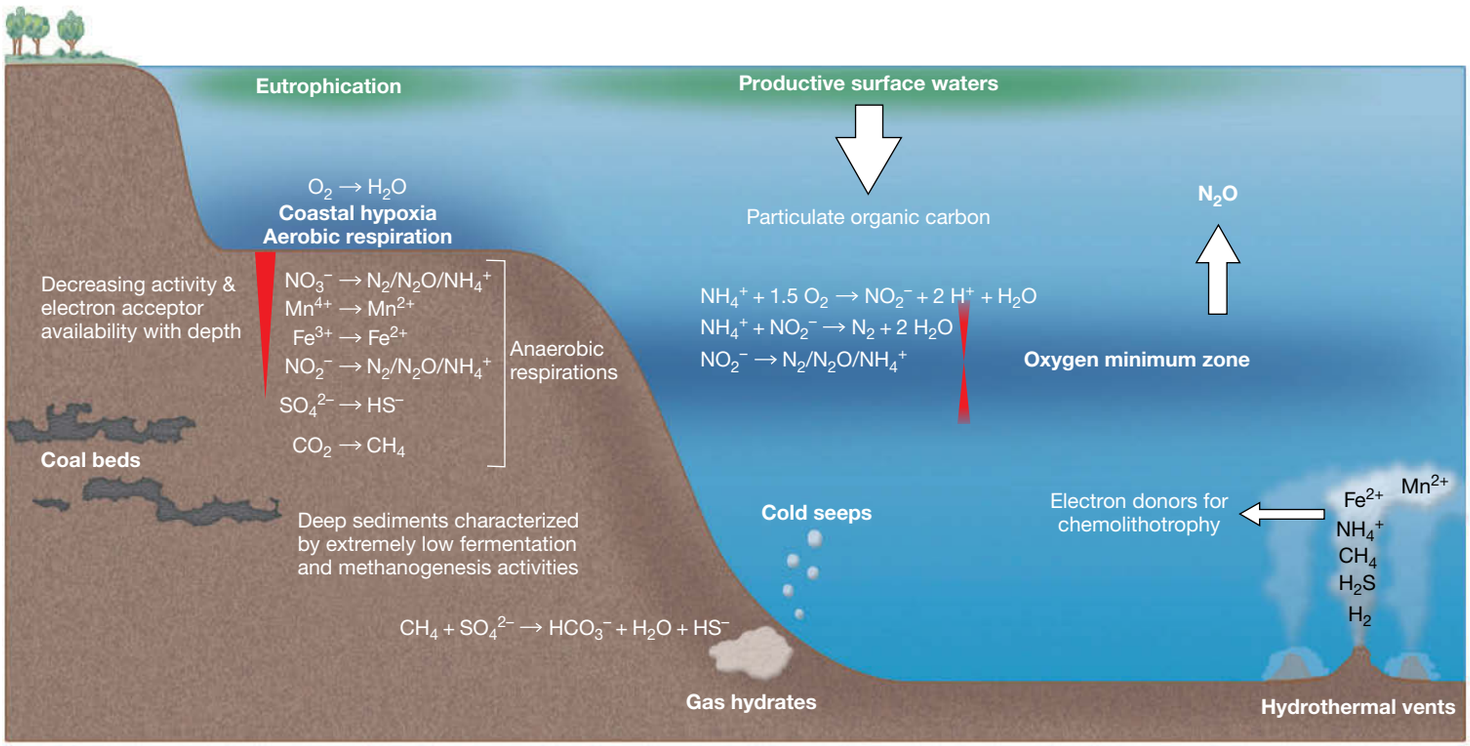



**Figure 20.20 Diversity of marine systems and associated microbial metabolic processes.** Decreasing electron acceptor availability with depth into the sediment or with increasing distance into an oxygen minimum zone is indicated by red wedges. Sulfate becomes limiting only at greater depths in marine sediments. The indicated metabolic diversity is covered in Chapter 14.


(a) (b)

**Figure 20.21 The Deepwater Horizon oil spill in the Gulf of Mexico.** *(a)* Inferno resulting from the wellhead blowout. *(b)* NASA Terra satellite image taken on May 24, 2010, of the Gulf of Mexico near New Orleans, Louisiana. A large plume of oil was released at about 1500 m depth, some of which reached the surface where sunlight reflects off of the oil slick (arrows).

that most of the oil was released as a plume at great depths in the water column. Typically, marine oil spills contaminate primarily the surface waters, resulting in rapid volatilization and loss to the atmosphere of low-molecular-weight oil components such as naphthalene, ethylbenzene, toluene, and xylene. By contrast, the Deepwater Horizon spill released both low-molecular-weight components *and* natural gas (methane, ethane, propane) deep into the water column. These components comprised about 35% of the hydrocarbon plume that extended across many miles of the Gulf from the surface to depths greater than 800 m (Figure 20.21*b*).

The microbial response to hydrocarbon contamination was tracked over several months using both culture-based and molecular methods, including 16S ribosomal RNA gene and metagenomic sequencing, and PhyloChip and GeoChip microarray analyses (Sections 19.6–19.8). These methods showed that the initial microbial response to the spill (May and June 2010) was a bloom of hydrocarbon-degrading *Gammaproteobacteria* species related to genera in the *Oceanospirillales* group, and of the genera *Colwellia* and *Cycloclasticus*. Increased numbers of *Colwellia* and *Oceanospirillales* species were attributed to their use of gaseous hydrocarbons, since both grew rapidly when ethane or propane was added to enrichment cultures (Section 19.1). *Colwellia* species also contributed to the degradation of a variety of other hydrocarbons, as indicated by their growth in crude oil enrichment cultures lacking natural gas and by stable isotope probing experiments (Section 19.10) showing their incorporation of $^{13}C$ benzene. Although there remains considerable uncertainty about the fate of all the hydrocarbons released during the Deepwater Horizon spill, it appears that the early stimulation of a bloom of hydrocarbon-degrading bacteria by the more easily degraded, soluble, low-molecular-weight components helped reduce the environmental impact of this immense oil spill.

## Oxygen Minimum Zones

Another feature of the marine water column is **oxygen minimum zones (OMZs)**, regions of oxygen-depleted waters at intermediate depths, typically in waters between 100 and 1000 m, that extend over wide expanses of the open and coastal ocean (Figure 20.20). These oxygen-depleted regions arise when the respiratory demand for oxygen exceeds oxygen availability, and they are associated with nutrient-rich, highly productive regions. In this way they are similar to the depletion of oxygen caused by agricultural runoff in coastal zones, such as that contributing to the Gulf of Mexico Dead Zone. However, OMZs predate human activity and originate naturally in regions of high surface production and little mixing with oxygen-rich water.

The oxygen saturation values of the largest of the OMZs in the eastern Pacific off the coast of Peru are less than 10% of that at the surface. Oxygen levels at certain depth intervals in the OMZs of the Bay of Bengal and Arabian Sea approach or reach zero. Because of this, OMZs have been recognized as significant sinks for the loss of fixed nitrogen through denitrification (Figure 20.20, Section 14.13) and anammox processes (Figure 20.20, Section 14.12). In addition to contributing to a significant fraction of the 50% loss of fixed nitrogen from the oceans, these regions are also a source of nitrous oxide ($N_2O$), a potent greenhouse gas (Section 21.8) of which approximately one-third is emitted from the oceans. Reduced sulfur can also be an important electron donor for denitrification by sulfide-oxidizing bacteria in the OMZ, the sulfide coming from microbial sulfate reduction (Sections 14.14 and 15.9). At times of exceptionally high surface water productivity, the accumulation and release of sulfide from OMZ regions has been implicated in massive fish kills.

Ongoing studies of OMZs have shown that these regions of oxygen depletion are expanding, and that their recent expansion is almost certainly associated with global warming. As the oceans

absorb more heat, warming of the surface waters increases stratification of near-surface waters and reduces oxygen transfer through mixing to deeper regions. Expansion of the OMZs will favor anaerobic microbial processes at the expense of the aerobic processes that sustain critical oceanic food webs. These changes may further affect atmospheric chemistry by increasing the release of $N_2O$ and will negatively impact marine food webs by reducing levels of fixed nitrogen. Expansion of OMZs might also increase the frequency of toxic sulfidic waters. Ultimately, these changes are expected to impact commercial fisheries.

**MINIQUIZ**

- What did the Deepwater Horizon spill tell us about how mixed hydrocarbons are degraded in nature?
- What is an oxygen minimum zone and why is expansion of these zones a problem for marine and global ecology?

## 20.10 Major Marine Phototrophs

The oceans contain large numbers of phototrophic microorganisms, including both prokaryotic and eukaryotic oxygenic phototrophs as well as significant numbers of a special group of purple (anoxygenic) phototrophs. We consider these organisms here as a prelude to exploring the marine prokaryotic world in general in Section 20.11.

### Primary Productivity: *Prochlorococcus*

Much of the primary productivity in the open oceans, even at significant depths, comes from photosynthesis by prochlorophytes, tiny bacterial phototrophs that phylogenetically affiliate with the cyanobacteria (↔ Section 15.3); **prochlorophytes** contain chlorophylls *a* and *b* but do not contain phycobilins. The organism *Prochlorococcus* is a particularly important primary producer in the marine environment (**Figure 20.22**). Because *Prochlorococcus* lacks phycobilins, the accessory pigments of the cyanobacteria (↔ Section 14.2), dense suspensions of *Prochlorococcus* cells are olive green (as are green algae) rather than the blue-green color of cyanobacteria (compare Figures 20.1*c* and 20.22).

*Prochlorococcus* accounts for up to half of the photosynthetic biomass and primary production in the tropical and subtropical regions of the world's oceans, reaching cell densities of $10^5$/ml. A number of strains of *Prochlorococcus* have now been identified in culture, and each inhabits its own depth range in pelagic waters. The different *Prochlorococcus* strains are considered distinct *ecotypes*, genetic variants of a species that differ physiologically and therefore occupy slightly different niches. For example, different *Prochlorococcus* ecotypes photosynthesize at different light intensities (high-light versus low-light ecotypes) and use different inorganic and organic nitrogen and phosphorus sources. *Prochlorococcus* is thus distributed in both surface waters and deeper waters to depths of 200 m, and when an oxygen minimum zone (Section 20.9) is present, *Prochlorococcus* extends into the upper regions of this zone (Figure 20.20). This is near the bottom of the photic zone where light intensities are very low (see Figure 20.26). Genome sequences of about a dozen *Prochlorococcus* strains in


**Figure 20.22 *Prochlorococcus*, the most abundant oxygenic phototroph in the oceans.** A bottle of *Prochlorococcus* showing the olive green color of the cells containing chlorophylls *a* and *b*. Inset: FISH-stained cells of *Prochlorococcus* in a marine water sample (↔ Section 19.6 and Figure 19.23).

culture revealed that although each contains about 2000 genes, only about 1100 genes are shared by all strains. Each presumptive ecotype contains approximately 200 unique genes, which likely have adaptive significance for growth in the realized niche of that ecotype. This was illustrated in Chapter 19 where we compared the genome of a single cultured *Prochlorococcus* ecotype to metagenome sequences obtained from pelagic waters (↔ Section 19.8 and Figure 19.23).

Single-cell genomic studies (↔ Sections 9.12 and 19.12) of natural *Prochlorococcus* populations have refined our understanding of genetic diversity and the relationship between environmental conditions and the fitness of individual genotypes. These analyses revealed a high degree of fine-scale genetic diversity within the high-light (HL) and low-light (LL) ecotypes. Each ecotype is actually composed of hundreds of subpopulations, each of which is united by a common set of core genes that encode functions that control the interaction of the organism with its environment (e.g., transport functions, oxidative stress responses, and cell surface structure) (**Figure 20.23*b***). However, each subpopulation also contains a small set of genes termed "flexible" genes (ranging from 4 to 14 genes per characterized genome) that vary within that subpopulation. The flexible genes are packaged as "cassettes" localized to highly variable regions (genomic islands) within the *Prochlorococcus* genome(↔ Sections 9.7, 19.8, and Figure 19.23). Variation in flexible gene content contributes to exceptionally high microdiversity within natural populations of *Prochlorococcus*, pointing to tremendous versatility in their adaptive response to new niche opportunities. The importance of genotypic variability in sustaining large numbers of this major marine phototroph is shown by seasonal shifts in different genotypes in response to changing light intensity, nutrient availability, and predator populations (Figure 20.23*a*).

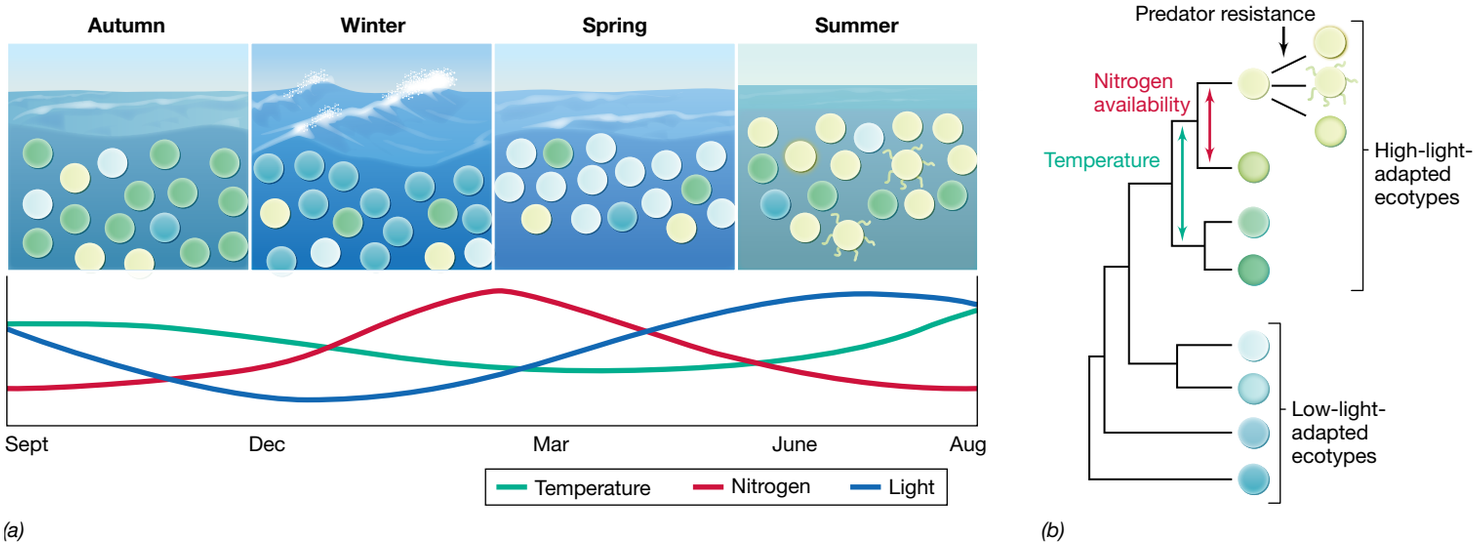


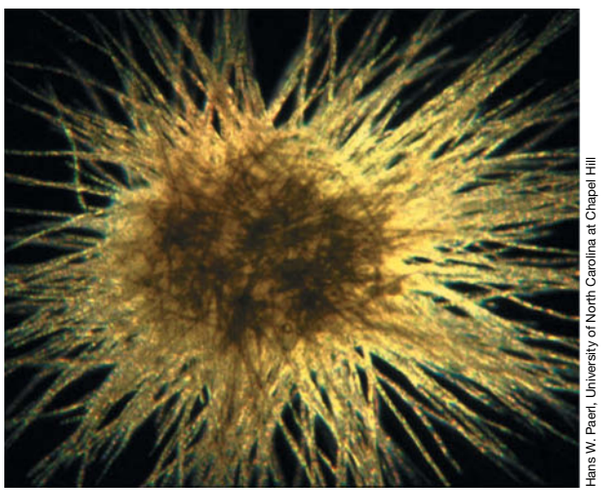

**Figure 20.23 Seasonal variation of *Prochlorococcus* ecotypes in marine surface waters.** *(a)* Single-cell genome sequencing (Sections 9.12 and 19.12) has shown that the abundance of different genotypes, presumptive ecotypes (represented by cell color and surface features), correlate with seasonal changes in temperature, light, nutrients, and predators (grazers and phage). *(b)* Adaptations to different physical conditions (light and temperature) partition at higher taxonomic levels than do adaptations to different nutrient conditions (e.g., nitrogen availability) and resistance to specific predators, which show closer genetic relationships among ecotypes.

## Other Pelagic Oxygenic Phototrophs

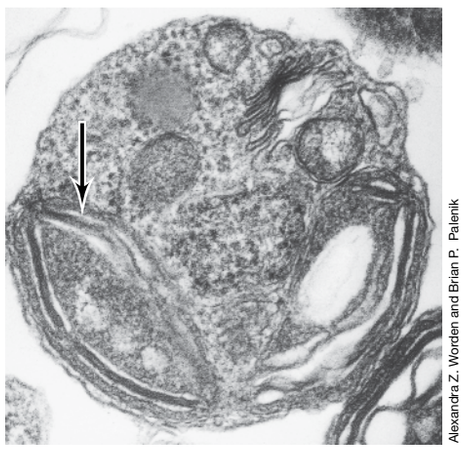
In tropical and subtropical oceans, the planktonic filamentous marine cyanobacterium *Trichodesmium* (**Figure 20.24*a***) is a widespread and occasionally abundant phototroph. Cells of *Trichodesmium* form puffs (colonies) of filaments. Each puff can contain many hundreds of individual filaments, each filament composed of 20–200 cells. In the Caribbean Sea surface waters, colonies of *Trichodesmium* can approach 100/m$^3$. *Trichodesmium* is a nitrogen-fixing cyanobacterium, and the production of fixed nitrogen by this organism is thought to be an important link in the marine nitrogen cycle. *Trichodesmium* contains phycobilins, absent from prochlorophytes, and thus differs from these organisms in its absorption properties (Section 14.2).

Very small phototrophic eukaryotes also inhabit coastal and pelagic waters, and some of these are among the smallest eukaryotic cells known. Three common genera—*Bathycoccus, Micromonas*, and *Ostreococcus*—contain only one mitochondrion and one chloroplast per cell. These genera are now assigned to the *Prasinophyceae*, a family of green algae that diverged early from other lineages of green algae (Section 18.15). Cells of *Ostreococcus* are cocci that measure only about 0.7 µm in diameter (Figure 20.24*b*), which is even smaller than a cell of *Escherichia coli*.

Although cells of *Ostreococcus* and *Prochlorococcus* are of roughly the same dimensions and they are both oxygenic phototrophs, their genomes are distinct. The genome of *Ostreococcus* is 12.6 Mbp (distributed over 20 chromosomes), which is more than seven times the size of the *Prochlorococcus* genome. Even though this is large relative to cyanobacteria, the *Ostreococcus* genome is very gene dense, containing about 8000 genes,




(a)




(b)

**Figure 20.24 *Trichodesmium* and *Ostreococcus*.** *(a)* Light photomicrograph of a puff of cells of the nitrogen-fixing cyanobacterium *Trichodesmium*. The filaments in the puff are chains of cells, each of which is about 6 µm in diameter. *(b)* Transmission electron micrograph of a cell of *Ostreococcus*, a small green alga found primarily in marine coastal waters. The arrow points to the chloroplast. An *Ostreococcus* cell is about 0.7 µm in diameter.

and thought to be near the minimum genome size of a free-living photosynthetic eukaryote. As a reference, the genome of a common plant, Japanese rice (*Oryza sativa* subsp. *japonica*), is 420 Mbp and contains about 50,000 genes.

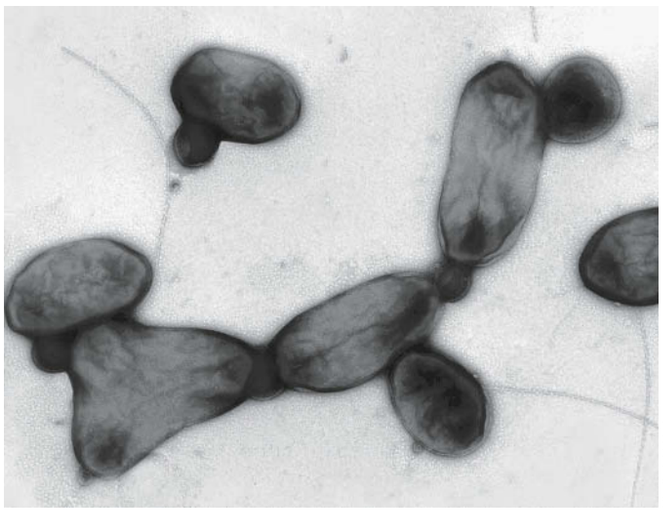
In many marine waters, other small eukaryotic cells are present at about $10^4$/ml. Although many of these are *Ostreococcus* or relatives, some are chemoorganotrophs and some are phototrophs unrelated to *Ostreococcus* that incorporate small amounts of organic matter to supplement their primarily phototrophic lifestyle.

### Aerobic Anoxygenic Phototrophs

Besides *oxygenic* phototrophs, *anoxygenic* phototrophs are also present in coastal and pelagic marine waters. Like purple anoxygenic phototrophs, these organisms contain bacteriochlorophyll *a* (Sections 14.1, 14.3, 15.4, and 15.5). However, unlike classical purple bacteria that carry out photosynthesis only under *anoxic* conditions, these anoxygenic phototrophs carry out photosynthetic light reactions only under *oxic* conditions.

Aerobic anoxygenic phototrophs include bacteria such as *Erythrobacter, Roseobacter*, and *Citromicrobium* (**Figure 20.25**), all genera of *Alphaproteobacteria*. Aerobic anoxygenic phototrophs synthesize ATP by photophosphorylation when oxygen is present (which is all of the time in oxic pelagic waters), but they are unable to grow autotrophically and thus rely on organic carbon for their carbon sources (a nutritional condition called *photoheterotrophy*). These organisms thus use the ATP produced by photophosphorylation to supplement their otherwise chemoorganotrophic metabolism.

Surveys have shown that a great diversity of aerobic anoxygenic phototrophs exist in marine waters, especially near-shore waters. Oligotrophic and highly oxic freshwater lakes are also habitats for these interesting phototrophic bacteria. The physiology of aerobic anoxygenic phototrophs is thus ideal for their illuminated and highly oxic habitats.



Vladimir V. Yurkov

**Figure 20.25 Aerobic anoxygenic phototrophic bacteria.** Transmission electron micrograph of negatively stained cells of *Citromicrobium*. Cells of this marine, aerobic anoxygenic phototroph produce bacteriochlorophyll *a* only under oxic conditions and divide by both budding and binary fission, yielding morphologically unusual and irregular-shaped cells.

**MINIQUIZ**

- How does *Ostreococcus* differ from *Prochlorococcus*? What do they have in common?
- How does the organism *Prochlorococcus* contribute to both the carbon and oxygen cycles in the oceans?
- How does *Roseobacter* differ from *Prochlorococcus*?

## 20.11 Pelagic *Bacteria, Archaea*, and Viruses

Despite vanishingly low nutrient levels, significant numbers of *Bacteria* and *Archaea* live a planktonic existence in pelagic marine waters. Of these, one species in particular has garnered significant attention, a bacterium named *Pelagibacter*.

### Distribution and Activity of *Archaea* and *Bacteria* in Pelagic Waters

The abundance of prokaryotic cells in the open oceans decreases with depth. In surface waters, cell numbers average about $10^6$/ml. Below 1000 m, however, total cell numbers fall to between $10^3$ and $10^5$/ml. The distribution of *Bacteria* and *Archaea* with depth has been tracked in pelagic waters using fluorescence in situ hybridization (FISH) technology (Section 19.5).

Species of *Bacteria* tend to predominate in waters above 1000 m, although cells of *Bacteria* and *Archaea* are found in near-equal abundance in deeper waters (**Figure 20.26**). Deep-water *Archaea* are almost exclusively species of *Thaumarchaeota* (Section 17.5). Many or perhaps even most are ammonia-oxidizing chemolithotrophs (Sections 14.11 and 17.5) that play an important role in coupling the marine carbon and nitrogen cycles (Chapter 21). Extrapolating from the data in Figure 20.26, it is estimated that $1.3 \times 10^{28}$ and $3.1 \times 10^{28}$ cells of *Archaea* and *Bacteria*, respectively, exist in the world's oceans. This means that the oceans contain the largest microbial biomass on Earth's surface.

Pelagic *Bacteria* and *Archaea* are ecologically important because they consume dissolved organic carbon in the oceans, one of the largest pools of usable organic carbon on Earth. These small and free-living planktonic microbes consume about half the total oceanic organic carbon produced from photosynthesis and are responsible for about half of all marine respiration and nutrient regeneration. Planktonic marine microbes thus return organic matter to the marine food web that would otherwise be lost because of the inability of larger marine organisms to take up such diluted organic nutrients. This so-called "secondary production" is balanced by cell losses from grazing protists and from virus attack (see Figure 20.28), leading to a near-steady state in which bacterial abundance in the open ocean remains roughly constant over time. But importantly, secondary production both recycles nutrients and allows some of the dissolved organic carbon in seawater to reach larger organisms, including fish, because protists are passed up the food web by the feeding activities of larger organisms.

### *Pelagibacter*: The Most Abundant Bacterium

Small planktonic chemoorganotrophic bacteria inhabit pelagic marine waters in numbers of $10^5$–$10^6$ cells/ml. The most abundant

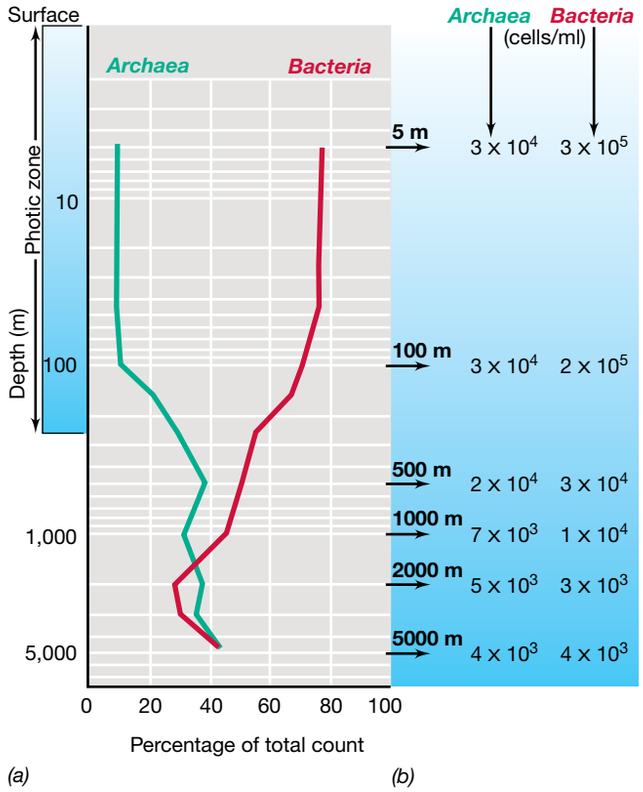



**Figure 20.26 Distribution of *Archaea* and *Bacteria* in North Pacific Ocean water.** *(a)* Percentage of *Archaea* and *Bacteria* with depth. *(b)* Absolute numbers per milliliter of *Archaea* and *Bacteria* with depth in the open ocean.

of these are members of the "SAR11 group" within the *Alphaproteobacteria*, which includes the genus *Pelagibacter*. Environmental metagenomic studies (Sections 9.8 and 19.8) and cell counts done using FISH (Section 19.5) have revealed a great abundance of SAR11 group organisms in pelagic waters. The total oceanic population of this group is estimated to be about $2.4 \times 10^{28}$ cells, making it the most successful microbial group, as reflected by abundance, on the planet. *Pelagibacter* is an oligotroph, an organism that grows best at very low concentrations of nutrients and grows in laboratory culture only up to the densities it is found in nature.

What makes *Pelagibacter* so successful in the open oceans? In part, its success is related to small size. Cells of *Pelagibacter* are small rods with a diameter of only 0.2–0.5 µm, near the limits of resolution of the light microscope (**Figure 20.27**), and a volume of 0.01 $\mu m^3$. The resulting high surface-to-volume ratio (Section 2.2) facilitates nutrient transport, increasing substrate concentration and processing rates within the cell. Proteomic analyses (Section 9.10) have also revealed a high abundance of periplasmic substrate-binding proteins for soluble nutrients such as phosphate, amino acids, and sugars in *Pelagibacter*. Another adaptive feature of *Pelagibacter* is its fairly small genome (1.3 Mbp). Consistent with the proteome analysis, the genome encodes an unusually high number of ABC-type transport systems—transporters that have an extremely high affinity for their substrates (Section 3.2)—and other

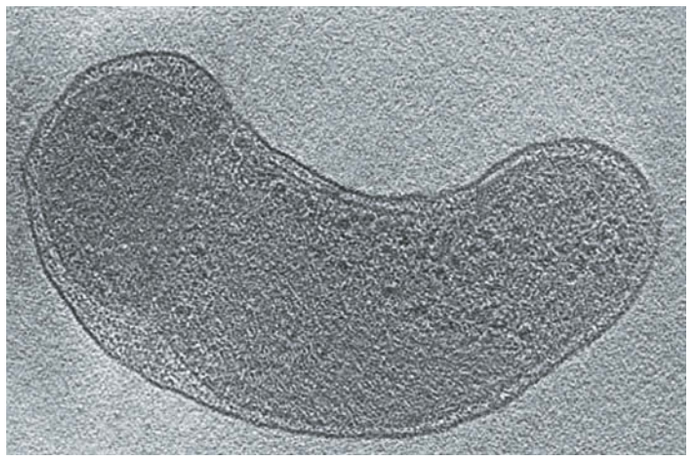



**Figure 20.27 *Pelagibacter*, the most abundant bacterium in the ocean.** Electron micrograph taken by electron tomography, a technique that introduces a three-dimensional effect. A single cell of *Pelagibacter* is about 0.2 µm in diameter.

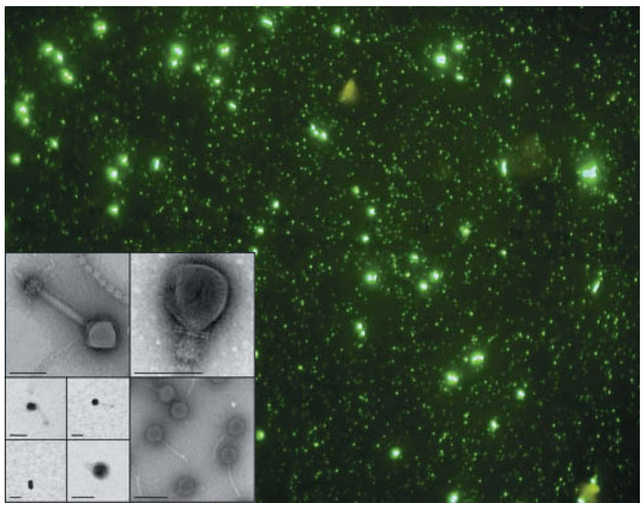
enzymes useful for an oligotrophic lifestyle. The *Pelagibacter* genome is also highly "streamlined," with intergenic spacings averaging only 3 base pairs; such a highly compact genome reduces the cost of replication.

The *Pelagibacter* genome also contains genes encoding a form of the visual pigment rhodopsin that can convert light energy into ATP. In Section 17.1 we discussed the now well-studied molecule *bacteriorhodopsin*, a light-activated protein complex present in the extreme halophile *Halobacterium* (*Archaea*); bacteriorhodopsin functions in ATP synthesis as a simple light-driven proton pump (Figure 17.4). The form of rhodopsin in *Pelagibacter* and other pelagic *Bacteria* is structurally similar to bacteriorhodopsin and has been called **proteorhodopsin** ("proteo" referring to *Proteobacteria*). Although proteorhodopsin was first discovered in species of *Proteobacteria*, it is actually fairly widely distributed in *Bacteria*, including many *Gamma-* and *Alphaproteobacteria, Bacteroidetes*, and *Actinobacteria*, and it has also been found in nonhalophilic species of *Archaea*, such as species of the marine *Euryarchaeota*. The different variants of proteorhodopsins in marine microbes have absorption properties that reflect changing spectral properties of light at increasing depths in the water column, with near-surface variants absorbing green light and those at greater depths absorbing blue light.

Proteorhodopsin-containing marine bacteria survive starvation better in the light than in the dark. This shows that energy-starved cells use light-mediated ATP production to compensate for energy unavailable from carbon respiration when organic carbon levels are low. Proteorhodopsins are thought to exist in ~80% of bacteria in some marine waters and are thus a widespread strategy to supplement the energy metabolism of marine microbes such that they need not rely solely on scarce organic carbon for their energy needs.

## Marine Viruses

In the oceans, viruses are more abundant than cellular microorganisms, often numbering over $10^7$ virions/ml in typical seawater (Section 10.12). In coastal waters, where bacterial cell



Jed Fuhrman, Jennifer R. Brum, Ryan O. Schenck, N. Solonenko, and Matt Sullivan

**Figure 20.28 Viruses in seawater.** A water sample collected on a 0.02-µm filter is stained with SYBR Green and viewed by epifluorescence microscopy. The tiny green dots are viruses while the larger, brighter dots are prokaryotic cells about 0.5 µm in diameter. Viruses are typically 10 times more abundant than the total of prokaryotic cells in seawater. Inset transmission electron micrographs show various marine bacterial viruses (scale bars, 100 nm in all images).

numbers are higher than in the oceans, viral numbers are also higher, as many as $10^8$ virions/ml. Most of these viruses are bacteriophages, which infect species of *Bacteria*, and archaeal viruses, which infect species of *Archaea*. The number of virions in seawater is about 10-fold greater than average prokaryotic cell numbers, suggesting that viruses are actively infecting their hosts, replicating, and being released into seawater (**Figure 20.28**). Only a small fraction of released viruses (an average of one per burst) successfully infects a new host, and most are inactivated or destroyed by sunlight and hydrolytic enzymes. In these ways, the entire viral population is replaced in periods of only a few days or weeks. We considered the diversity of bacterial and archaeal viruses in Chapter 10.

Along with feeding by protists, marine virus infections probably help to maintain prokaryotic cells at the levels that are observed, but viruses may also have other important ecosystem functions. These include facilitating genetic exchange between prokaryotic cells and allowing for lysogeny, the state in which a virus genome integrates within the cellular genome; lysogeny can confer new genetic properties on the cell (↔ Sections 8.7 and 11.7). For example, the discovery that some of the viruses that infect *Prochlorococcus*, the most abundant oxygenic phototroph in the oceans (Figure 20.22 and Section 20.10), contain genes that encode proteins for photosynthesis indicates that even key metabolic properties may be encoded by viruses. Although the genetic diversity of marine viruses is just now being recognized, it is thought that the diversity of marine viral genomes could surpass even that of all prokaryotic cells, making the oceans a hotbed of genetic diversity.

### A Phylogenetic Snapshot of Marine Bacterial and Archaeal Diversity

Several studies have attempted to characterize the diversity of planktonic marine *Bacteria* and *Archaea* by analysis of 16S ribosomal RNA genes obtained from seawater. The existence of abundant alphaproteobacterial populations to which *Pelagibacter* is affiliated was first revealed by such 16S rRNA sequence analysis. Mesophilic *Archaea* related to *Nitrosopumilus maritimus* (↔ Section 17.5) were discovered using similar methods.

Major bacterial groups now recognized as abundant in the open ocean include *Alpha-* and *Gammaproteobacteria*, cyanobacteria, *Bacteroidetes*, and to a lesser extent, *Betaproteobacteria* and *Actinobacteria*; *Firmicutes* are only minor components (**Figure 20.29**). As for soil, a large proportion of unclassified and minor bacterial groups are also present in seawater. A major group of marine *Gammaproteobacteria* is the yet to be cultured "SAR86 group," which accounts for approximately 10% of the total prokaryotic community in the ocean surface layer. Representing the *Archaea* in pelagic waters is a rather restricted diversity of *Euryarchaeota, Crenarchaeota*, and *Thaumarchaeota,* most of which have not yet been brought into laboratory culture.

With the exception of the cyanobacteria, most marine *Bacteria* are thought to be chemoorganotrophs adapted to extremely low nutrient availability, some augmenting energy conservation through proteorhodopsin or aerobic anoxygenic phototrophy (Section 20.10). The discovery of the chemolithotroph *Nitrosopumilus* suggested the possibility that many marine *Archaea* specialize in ammonia oxidation, although heterotrophic species likely exist as well. "Dilution culture" methods employing very dilute culture media have been successful in bringing some pelagic microbes into culture (↔ Section 19.2). It appears that most of these organisms have evolved to grow only at very low nutrient concentrations, so it is either difficult or impossible to culture them to higher cell densities. Cell densities of marine oligotrophs in laboratory cultures are similar to those in their natural environments ($10^5$–$10^6$/ml), which renders many of the common tools for measuring cell growth (turbidity, microscopic counts) useless on samples that are not first concentrated. Nevertheless, there have been notable successes with dilution culturing of marine bacteria, and the aforementioned bacterium *Pelagibacter* is a good example (↔ Figure 19.6).

**MINIQUIZ**

- What is proteorhodopsin and why is it so named? Why might proteorhodopsin make a bacterium such as *Pelagibacter* more competitive in its habitat?
- How do numbers of pelagic prokaryotic cells and viruses compare?
- Which phylum and subgroups of *Bacteria* dominate pelagic marine waters?
- Why are dilute culture media used for isolating pelagic microbes?

## 20.12 The Deep Sea

Light penetrates no farther than about 300 m in pelagic waters; as has been mentioned, this illuminated region is called the *photic zone* (Figure 20.26). Beneath the photic zone, down to a depth

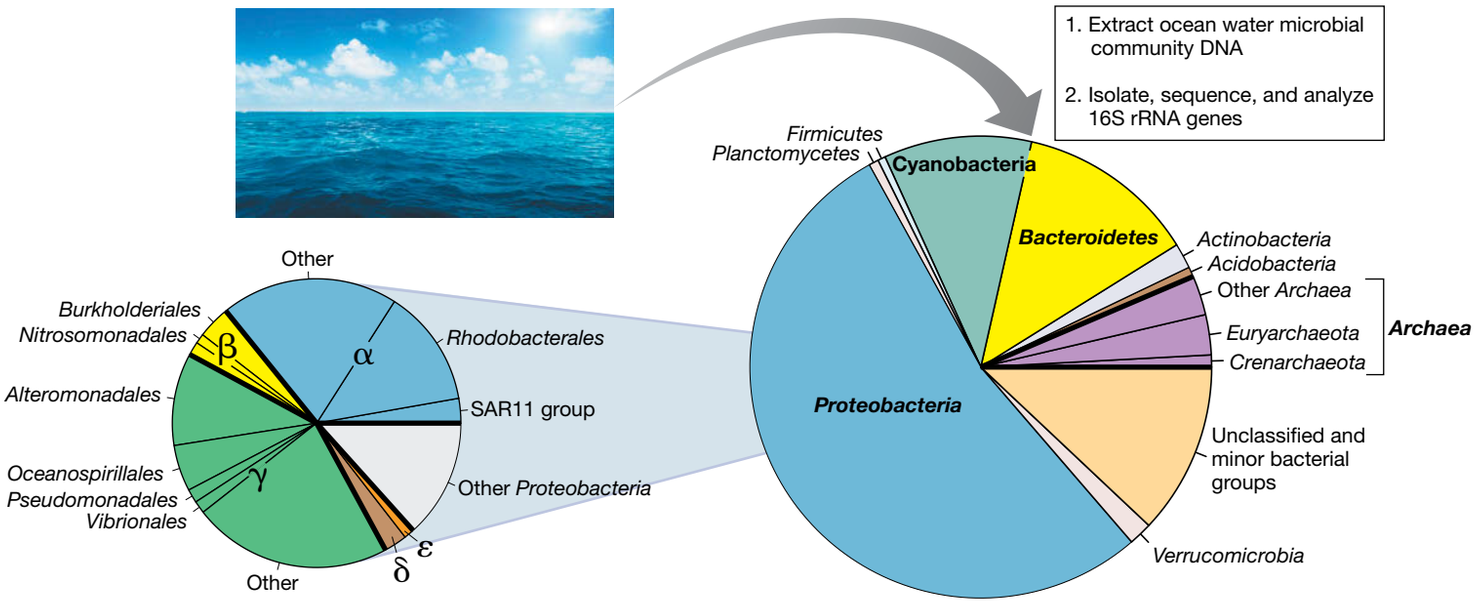



**Figure 20.29 Ocean bacterial and archaeal diversity.** The results are pooled analyses of 25,975 16S ribosomal RNA sequences from several studies of pelagic ocean waters. Many of these groups are covered in Chapters 15 and 16 (*Bacteria*) or 17 (*Archaea*). For *Proteobacteria*, major subgroups are indicated. Note the high proportion of cyanobacterial and *Gammaproteobacteria* sequences. Data assembled and analyzed by Nicolas Pinel. Compare the prokaryotic diversity of seawater with that of freshwater shown in Figure 20.18.

of about 1000 m, there is still considerable biological activity. However, water at depths greater than 1000 m is, by comparison, much less biologically active and is known as the *deep sea*. Greater than 75% of all ocean water is deep-sea water, lying primarily at depths between 1000 and 6000 m. The deepest waters in the oceans lie below 10,000 m. However, because depressions this deep are very rare, the waters in them make up only a very small proportion of all pelagic waters.

## Conditions in the Deep Sea

Organisms that inhabit the deep sea face three major environmental extremes: (1) low temperature, (2) high pressure, and (3) low nutrient levels. In addition, deep-sea waters are completely dark such that photosynthesis is impossible. Thus, microbes that inhabit the deep sea must be chemotrophic and able to grow under high pressure and oligotrophic conditions in the cold.

Below depths of about 100 m, ocean water temperatures stay constant at 2–3°C. We discussed the responses of microorganisms to changes in temperature in Sections 5.9–5.11. As would be expected, bacteria isolated from marine waters below 100 m are psychrophilic (cold-loving) or at least psychrotolerant. Deep-sea microbes must also be able to withstand the enormous hydrostatic pressures associated with great depths. Pressure increases by 1 atm for every 10 m of depth in a water column. Thus, an organism growing at a depth of 5000 m must be able to withstand pressures of 500 atm. We will see that microorganisms in general are remarkably tolerant of high hydrostatic pressures; many species can withstand pressures of 500 atm, and some species can withstand far more than this. Moreover, from studies of deep-sea microbial diversity performed thus far, some unusual microbes call this extreme environment home (see page 653).

## Piezotolerant and Piezophilic *Bacteria* and *Archaea*

Different physiological responses to pressure are observed in different deep-sea microorganisms. Some organisms simply tolerate high hydrostatic pressure, but do not grow optimally under such pressure; these organisms are **piezotolerant** (Figure 20.30). By contrast, others actually *grow best* under elevated hydrostatic pressure; these are called **piezophiles**. Organisms isolated from surface waters down to about 3000 m are typically piezotolerant. In piezotolerant organisms, higher metabolic rates are observed

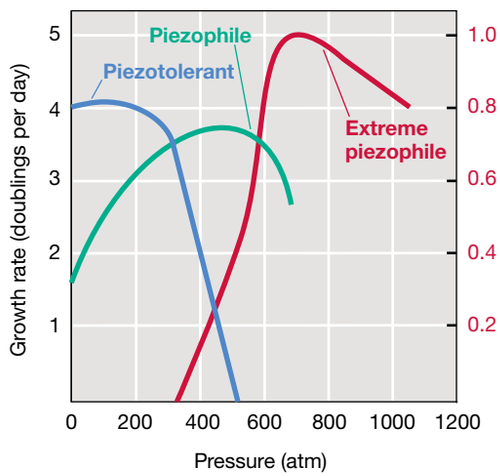



**Figure 20.30 Growth of piezotolerant, piezophilic, and extremely piezophilic bacteria.** Compare the slower growth rate of the extreme piezophile (right ordinate) with the growth rate of the piezotolerant and piezophilic bacteria (left ordinate), and note the inability of the extreme piezophile to grow at low pressures.

at 1 atm than at 300 atm, although growth rates at the two pressures may be similar (Figure 20.30). However, piezotolerant isolates typically do not grow at pressures greater than about 500 atm (Figure 20.30).

By contrast, cultures derived from samples taken at greater depths, 4000–6000 m, are typically piezophilic, growing optimally at pressures of around 300–400 atm. However, although piezophiles grow best under high pressure, they can still grow at 1 atm (Figure 20.30). In even deeper waters (for example, 10,000 m), **extreme piezophiles** are present. These organisms require very high pressure for growth (**Figure 20.31**). For example, the extreme piezophile *Moritella*, isolated from the Mariana Trench (Pacific Ocean, >10,000-m depth) (**Figure 20.32**), grows optimally at a pressure of 700–800 atm and grows nearly as well at 1035 atm (Figure 20.31), the pressure it experiences in its natural habitat.

## Molecular Effects of High Pressure

High pressure affects cellular physiology and biochemistry in many ways. In general, pressure decreases the ability of the subunits of multi-subunit proteins to interact. Thus, large protein complexes in extreme piezophiles must interact in such a way as to minimize pressure-related effects. Protein synthesis, DNA synthesis, and nutrient transport are sensitive to high pressure. Piezophilic bacteria grown under high pressure have a higher proportion of unsaturated fatty acids in their cytoplasmic membranes than when grown at 1 atm. Unsaturated fatty acids allow membranes to remain functional and keep from gelling at high pressures or at low temperatures. The rather slow growth rates of extreme piezophiles such as *Moritella* compared with other marine bacteria (Figure 20.30) are likely due to the combined effects of pressure and low temperature; low temperature slows down the reaction rates of enzymes, directly affecting cell growth (Sections 5.9 and 5.10).

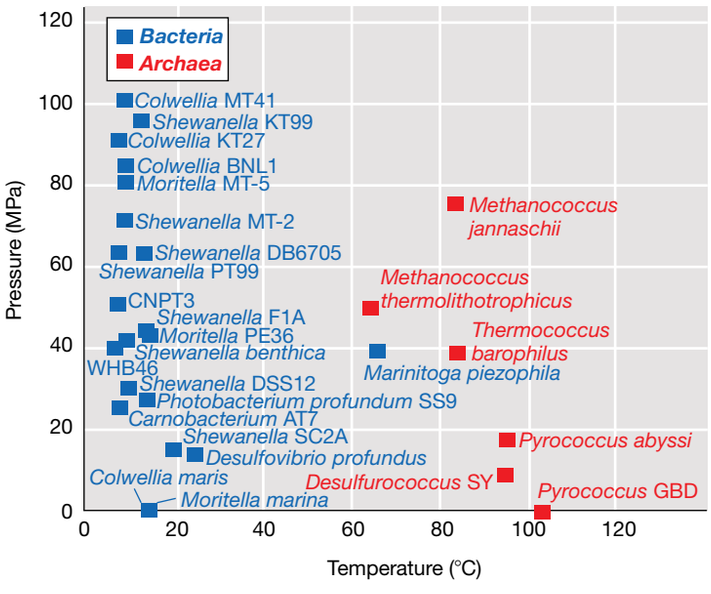



**Figure 20.31 Pressure and temperature optima for cultured bacterial and archaeal piezophiles.** Pressure is in pascals (Pa), the SI unit for pressure. One megapascal (MPa) corresponds to approximately 10 atm. Note that different species of the same genus can have vastly different pressure optima. Data assembled by Doug Bartlett.

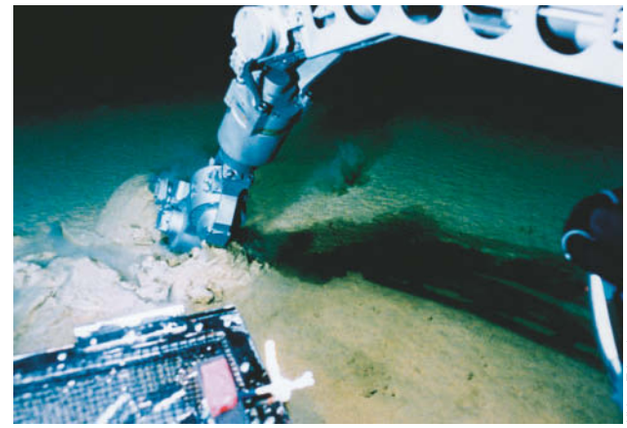



**Figure 20.32 Sampling the deep sea.** The unmanned Japanese submersible *Kaiko* collecting a sediment sample on the seafloor of the Mariana Trench off the Philippines at a depth of 10,897 m. The tubes of sediment are used for enrichment and isolation of piezophilic bacteria, such as the extreme piezophile (*Moritella*) isolated from this seafloor.

Studies of gene expression and adaptive features contributing to growth at high pressure have required special pressurized incubation devices (**Figure 20.33**). These studies have shown that when a gram-negative piezophile is grown under high pressure, a specific outer membrane protein called OmpH (*o*uter *m*embrane *p*rotein H) is present that is absent from cells grown at 1 atm. OmpH is a type of porin. Porins are proteins that form channels through which molecules diffuse into the periplasm (Section 2.5). Presumably, the porin made by cells grown at 1 atm cannot function properly at high pressure and thus a different porin must be synthesized. Interestingly, pressure controls transcription of *ompH*, the gene encoding OmpH. In characterized gram-negative piezophiles, a pressure-sensitive membrane protein complex is present that monitors pressure and triggers transcription of *ompH* only when conditions of high pressure warrant it. Transcriptomic analyses (Section 9.9) indicate that even relatively modest changes in hydrostatic pressure alter the expression of a large number of genes in piezophiles, so it is likely that many other pressure-monitoring proteins exist in these organisms.

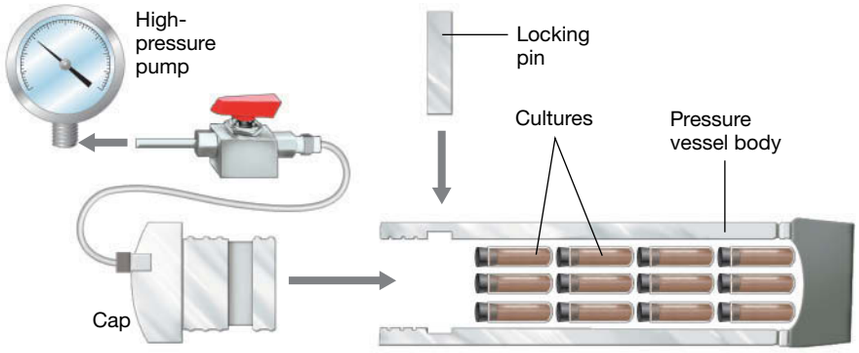
**MINIQUIZ**

- How does pressure change with depth in a water column?
- What molecular adaptations are found in piezophiles that allow them to grow optimally under high pressure?

## 20.13 Deep-Sea Sediments

In addition to deep-sea *waters*, another vast and mostly unexplored microbial biosphere exists below the seafloor in the deep-sea *sediments*. Drilling expeditions to explore far below the ocean seafloor have revealed both archaeal and bacterial populations


(a)




(b)

**Figure 20.33 Pressure cells for growing piezophiles under elevated pressure.** *(a)* Photo of several pressure cells incubating in a cold room (4°C). *(b)* Schematic design of a pressure cell. These vessels are designed to maintain pressures of 1000 atm. Illustration based on drawing by Doug Bartlett.

at depths greater than 2000 m (**Figure 20.34**). Most studies thus far have focused on relatively organic-rich sediments along continental margins. Here, cell numbers typically decrease from about $10^9$ cells/g in surface sediment to about $10^6$ cells/g at depths as great as 1000 m below the seafloor. In these coastal sediments, sulfate-reducing bacteria and other anaerobes deplete sulfate and other electron acceptors within a few meters of the sediment–water interface (Figure 20.20). This depletion of electron acceptors and organic matter with increasing depth constrains energy available to the deep subsurface microbial communities, accounting for the major decrease in cell numbers with depth.

## Cell Numbers in Deep-Sea Sediments

The better-studied continental margins and shelf sediments are not representative of most of the ocean floor, about 90% of which is at greater than 2000 m depth and associated with marine waters of low productivity and therefore of much lower organic matter content (Figure 20.20). In the absence of significant transport of organic material to the sediment surface, sulfate and other electron acceptors may permeate all the way through the sediments to the underlying bedrock. However, because of the dearth of organic matter, cell numbers in these sediments are several orders of magnitude lower than in organic-rich sediments, ranging from about


(a)


(b)

**Figure 20.34 Drilling deep-sea sediments.** *(a)* Deep-sea drilling vessel the JOIDES *Resolution*. Inset: Red dot indicates the location of sediment sampling in the Peru Basin. *(b)* Sediment cores recovered from the Peru Basin at 4800 m depth. Cores were split lengthwise to allow subsampling for molecular characterization. See Section 20.5 and Figure 20.8 for discussion of sulfide-oxidizing microbial mats that grow on the sediment surface off the Chilean and Peruvian coasts.

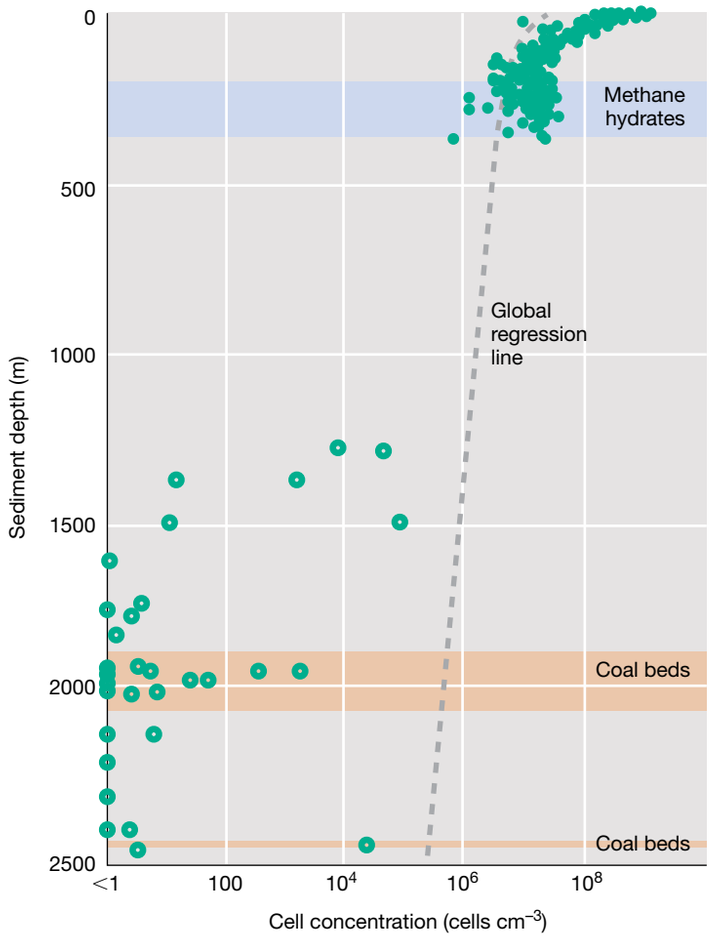



**Figure 20.35 Microbial cell numbers in deep-sea sediments.** Depth-related cell abundance from the analysis of one coastal sediment (filled circles) and the global average for all sampled ocean sediments (dashed regression line) based on cell count data.

$10^6$ cells/gram at the surface to fewer than $10^3$ cells/gram at depths of a few hundred meters (Figure 20.35). Cell counts in all sediments generally decline significantly with depth, reflecting lower organic carbon availability and quality in older deep sediment material. Despite these low numbers, because deep-sea sediments are so vast, it has been estimated that a total of ~$5.4 \times 10^{29}$ prokaryotic cells exist in deep-sea sediments (~$4 \times 10^{15}$ g), a number similar to the sum total in all the oceans.

Figure 20.35 also shows that cell numbers can deviate from the global regression as a function of spotty distributions of resources with depth at any particular drill site. In the example shown in Figure 20.35, cell numbers in coastal plain sediments are elevated near methane seeps (Figure 20.20), a region associated with active anaerobic methane oxidation (Section 14.25), and in coal bed deposits (Figures 20.20 and 20.35), where, in addition to coal, associated organic materials boost electron donor availability.

## A Phylogenetic Snapshot of Marine Sediment Prokaryotic Diversity

Marine sediment communities have been explored only to a limited extent because of the difficulty and expense of obtaining uncontaminated drilling cores from great depths (Figure 20.34). However, analyses of 16S ribosomal RNA gene sequences obtained by using PCR methods (Section 19.6) on deep core samples have clearly established that novel *Archaea* make up a large fraction of the archaeal diversity and that bacterial diversity in both deep and shallow marine sediments is dominated by *Proteobacteria* (Figure 20.36). This abundance of *Proteobacteria* is also true of all the other habitats explored by culture-independent techniques (Figures 20.12, 20.18, and 20.29, and see Figure 20.41).

Within marine sediment *Proteobacteria*, phylotypes associated with sulfate-reducing bacteria such as the *Desulfobacterales* are quite common (Figure 20.36), a fact that is not surprising considering that sulfate reduction is the major form of anaerobic respiration in marine sediments (Sections 14.14 and 15.9). *Bacteroidetes* and the unclassified/minor groups are also well represented in shallow marine sediments. Although major players in marine waters, cyanobacteria compose just a tiny proportion of the total cell population in the permanently dark and anoxic sediments and probably represent cells that have reached the sediments after attaching to a particle or dead animal that eventually sank.

In addition to a variety of *Bacteria*, novel phyla of *Archaea* unrelated to cultured representatives are widespread in the deep subsurface. Genome sequences of uncultured *Bathyarchaeota* recovered from both terrestrial and marine sediments (Figure 20.14) revealed a physiological capacity to degrade and assimilate protein and a capacity for some species to degrade carbohydrates. Other *Bathyarchaeaota* appear to carry out methanogenesis and are autotrophs, likely existing on the small amounts of $H_2$ produced from geochemical reactions (Section 20.7) in deep sediments.

## Energy Limitation and Microbial Life below the Seafloor

How microbes in the deepest marine sediments survive in their nutrient-depleted environment remains unclear, but it is likely that they employ many of the strategies we have seen in marine pelagic microbes including small cell size (Figure 20.15) and small, compact genomes. Sequencing of 16S ribosomal RNA genes selectively amplified by PCR (Section 19.6) using DNA extracted from deep drilling cores, as well as from more limited metagenomic surveys, has identified relatively few sequences related to the classical sulfate-reducing bacteria (Section 15.9) or methanogenic and known methane-oxidizing *Archaea* (Sections 14.17 and 17.2), organisms that by contrast are quite common in surface sediments. Thus, from a nutritional standpoint, how do buried cells thrive under conditions of extreme energy limitation?

In the marine environment, easily degraded organic material is removed by microbial respiration in the water column and anaerobic respiration in surficial sediments (the sediments at and near the sediment surface), leaving behind a dilute pool of less readily degradable organic material that slowly trickles down into deeper sediments. Microbes that inhabit the deep sediments presumably utilize this low-quality organic material along with cell necromass (cellular components released following cell death) as electron donors in energy metabolism. However, since representative organisms have yet to be isolated from deep sediments, our understanding of the supporting energy-conserving metabolisms is sketchy and can only be inferred from a few partial genome

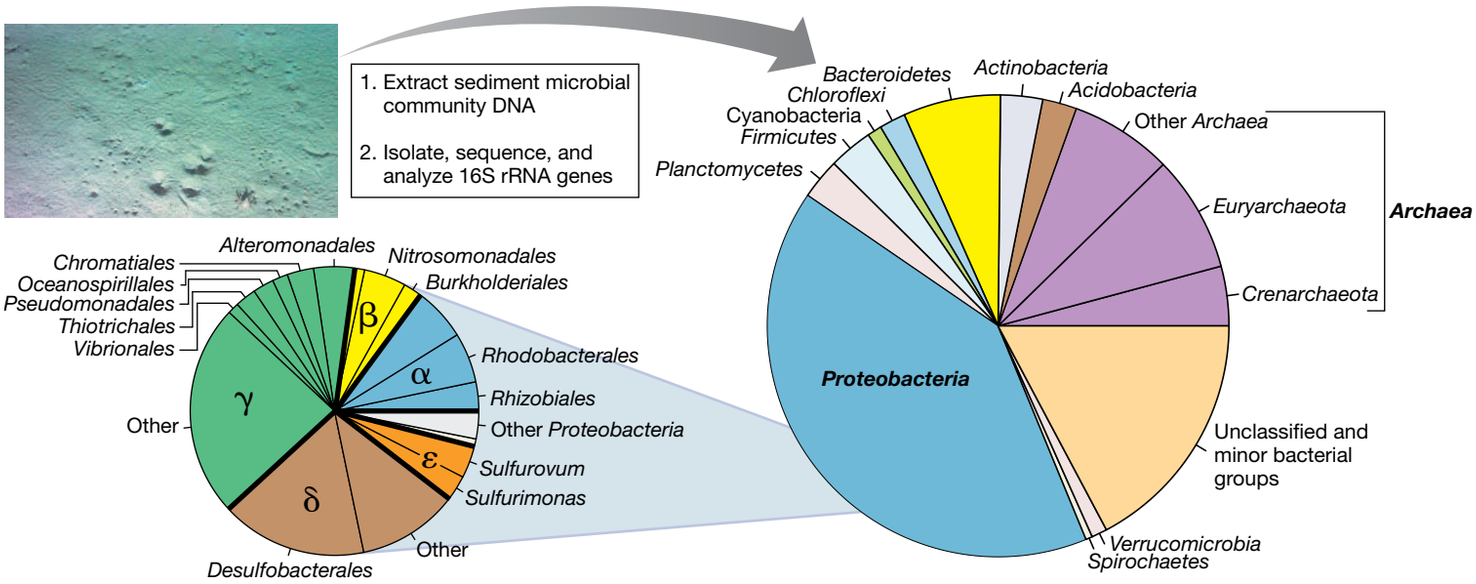



**Figure 20.36 Marine sediment bacterial and archaeal diversity.** The results are pooled analyses of 13,360 16S ribosomal RNA gene sequences from several studies of shallow and deep marine sediments. Many of the groups indicated are covered in Chapters 15 and 16 (*Bacteria*) or 17 (*Archaea*). For *Proteobacteria*, major subgroups are indicated. Note the high proportion of archaeal sequences and of *Gamma-*, *Delta-*, and *Epsilonproteobacteria*. Data assembled and analyzed by Nicolas Pinel. Compare the prokaryotic diversity of marine sediments with that of open ocean water shown in Figure 20.29.

sequences assembled from metagenomes (Sections 9.8 and 19.8) or obtained from single cells isolated directly from sediments, using single-cell genomic technology (Sections 9.12 and 19.12). Various known and yet-to-be-discovered forms of anaerobic respiration and fermentation are the most likely metabolic candidates for deep seafloor metabolisms along with other metabolic options not readily obvious from the conditions known to exist in deep marine sediments.

Since sediment microorganisms largely control the fate of carbon in this vast subsurface reservoir of organic matter, the discovery of novel *Archaea* (Figure 20.14) living at the thermodynamic edge of life has given microbial ecologists a new perspective on carbon cycling in marine sediments. However, the extent to which abiotic processes may also contribute energy sources to these buried microbes is unclear but could be significant. For example, the higher temperatures found at greater depths may promote the alteration of organic material, releasing methane and other hydrocarbons, $H_2$, acetate, and $CO_2$ that may diffuse upwards to nourish microbes inhabiting the deep sediment biosphere. Thus there is clearly much more to learn about the microbial ecology of Earth's deep subsurface, both in terrestrial and marine sediment contexts.

**MINIQUIZ**

- Give two reasons why sulfate-reducing bacteria are common in shallow marine sediments.
- How and why do the numbers of bacterial and archaeal cells vary with depth in marine deep sediments?
- What alternative sources of energy are suggested to nourish microorganisms in extremely deep marine sediments?

## 20.14 Hydrothermal Vents

Although we have thus far described the deep sea and deep-sea sediments as a remote, low-temperature, high-pressure, oligotrophic environment suitable only for slow-growing piezotolerant and piezophilic microorganisms, there are some amazing exceptions. Thriving animal and microbial communities are found clustered in and around deep-sea hot springs throughout the world. These hot springs are located at depths from less than 1000 m to greater than 4000 m in regions of the seafloor where volcanic magma and hot rock have caused the floor to rift apart at crustal spreading centers (Figure 20.20 and **Figure 20.37**), or where iron and magnesium minerals associated with ancient rocks react with seawater and generate heat. Seawater seeping into these dynamic cracking regions of the crust reacts with hot rock, resulting in hot springs saturated with inorganic chemicals and dissolved gases. Collectively, these types of underwater hot springs are called **hydrothermal vents** (Figure 20.20). We discuss several remarkable symbiotic associations between hydrothermal vent–associated animals and microorganisms in Chapter 23. Here we consider the vent environment as a habitat for free-living microbes.

### Types of Vents

Volcanic hydrothermal systems are typically either warm (~5 to >50°C), diffuse vents or very hot vents that emit hydrothermal fluids at 270 to >400°C. The gently flowing, warm, diffuse fluids are emitted from cracks in the seafloor and the exterior walls of hydrothermal chimneys. The fluids originate from the mixing of cold seawater with hot hydrothermal fluids in subsurface regions of the sediments. Hot vents, called *black smokers*, form upright sulfide edifices called *chimneys* that can be less than 1 m to over 30 m in height.

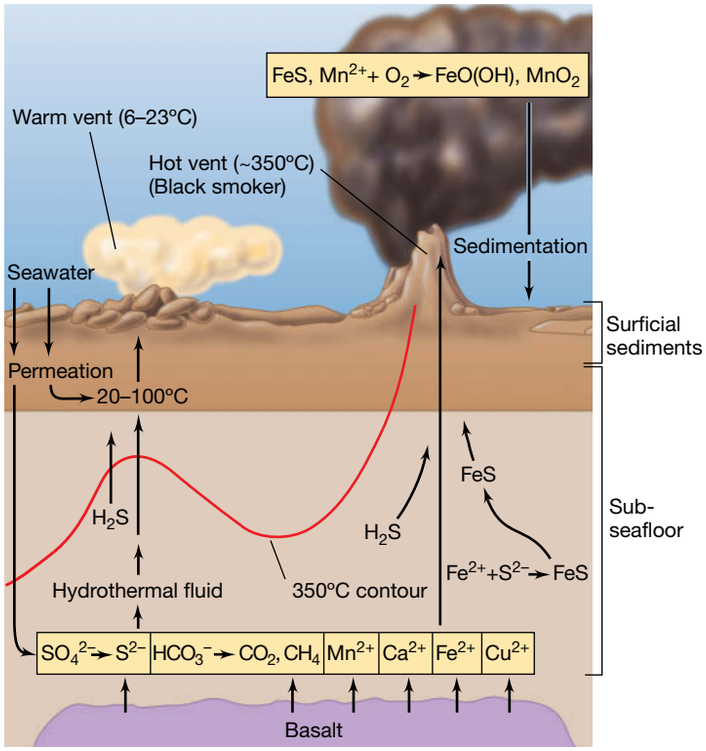



**Figure 20.37 Hydrothermal vents.** Schematic showing geological formations and major inorganic chemicals and minerals that are emitted from warm vents and black smokers. In warm vents, the hot hydrothermal fluid is cooled by cold 2–3°C seawater permeating the sediments. In black smokers, hot hydrothermal fluid near 350°C reaches the seafloor directly. The term "surficial" is a geological term pertaining to Earth's surface.

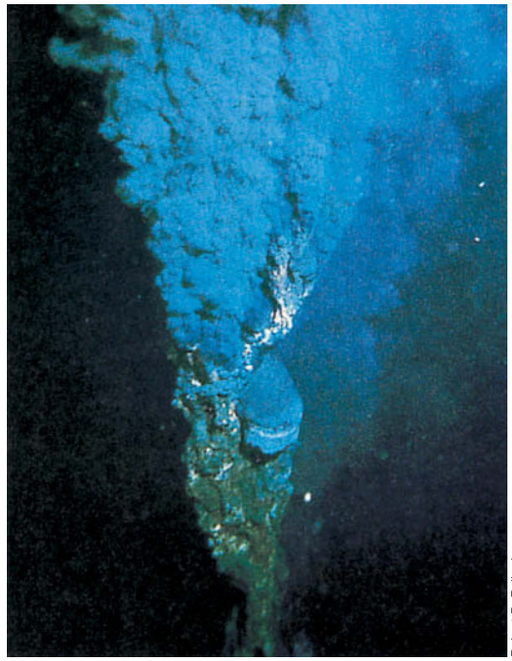



**Figure 20.38 A hydrothermal vent black smoker emitting sulfide- and mineral-rich water at temperatures of 350°C.** The walls of the black smoker chimneys display a steep temperature gradient and contain several types of *Bacteria* and *Archaea*.

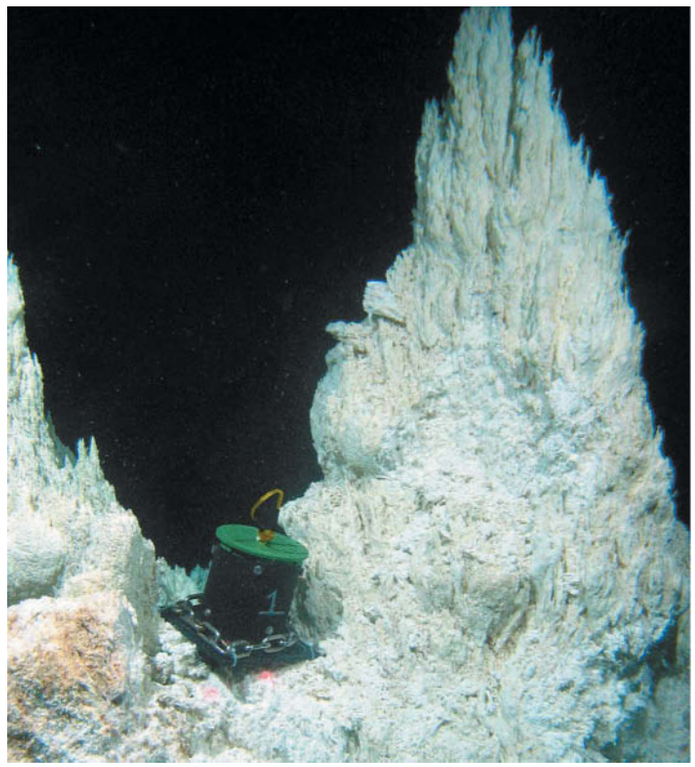



**Figure 20.39 Massive carbonate chimney formation at Lost City peridotite-hosted vent system.** Microbial colonization of freshly exposed mineral surfaces was studied by placing sterile mineral fragments in the green-topped device positioned over an actively venting area of the chimney. The diameter of the cylindrical collection device is approximately 10 cm.

Chimneys form when acidic hydrothermal fluids rich in dissolved metals and magmatic gases are suddenly mixed with cold, oxygenated seawater. The rapid mixing causes fine-grained metal sulfide minerals such as pyrite and sphalerite to precipitate out, forming dark, buoyant plumes that rise above the seafloor (**Figure 20.38**).

A quite different type of hydrothermal vent environment is the "Lost City" formation located in the mid-Atlantic Ocean. Lost City is formed from the exposure of minerals associated with ocean crust 1–2 million years old that was once deep beneath the seafloor. Geological faults in these slow-spreading systems exposed magnesium and iron-rich rocks called *peridotites* at the seafloor. Chemical reactions of seawater and newly exposed peridotite are highly exothermic, generating heat and also driving the pH up to as high as pH 11. Extremely high levels of $H_2$, $CH_4$, and other low-molecular-weight hydrocarbons are also present in the hot (200°C) hydrothermal fluids. In contrast to the acidic volcanic black smoker systems (Figure 20.38), which are relatively transient, mixing of these alkaline fluids with seawater results in the formation of calcium carbonate (limestone) chimneys (**Figure 20.39**) that can reach up to 60 m in height and be active for 100,000 years or more.

## *Bacteria* and *Archaea* in Hydrothermal Vents

Bacteria displaying chemolithotrophic metabolisms dominate hydrothermal vent microbial ecosystems (Figure 20.20).

Sulfidic vents support sulfur bacteria, whereas vents that emit other inorganic electron donors support nitrifying, hydrogen-oxidizing, iron- and manganese-oxidizing, or methylotrophic bacteria, the latter presumably growing on the $CH_4$ and carbon monoxide (CO) emitted from the vents. **Table 20.2** summarizes the inorganic electron donors and electron acceptors that are thought to play a role in chemolithotrophic metabolisms at hydrothermal vents. All of these metabolisms were discussed in Chapter 14.

Although microbes cannot survive in the superheated hydrothermal fluids of black smokers, thermophilic and hyperthermophilic organisms do thrive in the *gradients* that form as the superheated water mixes with cold seawater. For example, the walls of smoker chimneys are teeming with hyperthermophiles such as *Methanopyrus,* a species of *Archaea* that oxidizes $H_2$ and makes $CH_4$ (↔ Section 17.2). Phylogenetic FISH staining (↔ Section 19.5) has detected cells of both *Bacteria* and *Archaea* in smoker chimney walls (**Figure 20.40**). The most thermophilic of all known sulfur-reducing microbes, species of *Pyrolobus* and *Pyrodictium* (Chapter 17), were isolated from black smoker chimney walls. In contrast to the significant microbial diversity in volcanic vent chimney walls, the carbonate chimney walls of the Lost City vents are comprised primarily of methanogens of the genus *Methanosarcina* and are nourished by the $H_2$-rich fluids that permeate the porous chimney walls.

When smokers plug up from mineral debris, hyperthermophiles presumably drift away to colonize active smokers and somehow become integrated into the growing chimney wall. Surprisingly, although they require very high temperatures for growth, hyperthermophiles are remarkably tolerant of cold temperatures and oxygen. Thus, transport of cells from one vent site to another in cold oxic seawater apparently is not a problem.

## A Phylogenetic Snapshot of Hydrothermal Vent Prokaryotic Diversity

Using the powerful tools developed for microbial community sampling (↔ Section 19.6), studies of prokaryotic diversity near volcanic hydrothermal vents have revealed an enormous diversity of *Bacteria*. These 16S rRNA gene sequence surveys include both warm and hot vents. Hydrothermal vent microbial communities are dominated by *Proteobacteria*, in particular *Epsilonproteobacteria* (↔ Section 16.5; **Figure 20.41**). *Alpha-, Delta-,* and *Gammaproteobacteria* are also abundant, whereas *Betaproteobacteria* are much less so. Many *Epsilon-* and *Gammaproteobacteria* oxidize sulfide and sulfur as electron donors with either $O_2$ or nitrate ($NO_3^-$) as electron acceptors.

As shown in the detailed diagram of *Proteobacteria* in Figure 20.41, vent *Epsilonproteobacteria* phylotypes most closely match those of chemolithotrophic sulfur bacteria such as *Sulfurimonas*, *Arcobacter*, *Sulfurovum*, and *Sulfurospirillum*. These bacteria oxidize reduced sulfur compounds as electron donors (↔ Sections 14.9 and 15.11), and such a physiology is consistent with their presence near vent fluids charged with sulfur and sulfide. In addition, most *Deltaproteobacteria* specialize in anaerobic metabolisms using oxidized sulfur compounds as electron acceptors.

In contrast to *Bacteria*, the diversity of volcanic hydrothermal vent *Archaea* is quite limited. Estimates of the number of unique phylotypes indicate that the diversity of *Bacteria* near hydrothermal vents is about 10 times that of *Archaea*. However, *Archaea* are prevalent in samples recovered from the walls of hot vent chimneys (Figure 20.40). Most of the *Archaea* detected near hydrothermal vents are either methanogens (↔ Section 17.2) or species of marine *Crenarchaeota* and *Euryarchaeota* (Chapter 17). With the exception of the ammonia-oxidizing *Nitrosopumilus* (*Thaumarchaeota*, ↔ Section 17.5), organisms in these groups remain uncultured and their physiologies poorly understood.

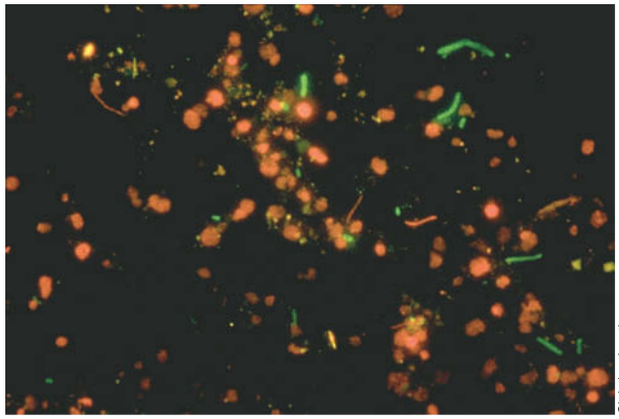

Christian Jeanthon

**Figure 20.40 Phylogenetic FISH staining of black smoker chimney material.** Taken from the Snake Pit vent field in the Mid-Atlantic Ridge, depth of 3500 m. A green fluorescing dye was conjugated to a probe that reacts with the 16S rRNA of all *Bacteria* and a red dye to a 16S rRNA probe for *Archaea*. The hydrothermal fluid going through the center of this chimney was at 300°C.

**TABLE 20.2 Chemolithotrophic *Bacteria* and *Archaea* present near deep-sea hydrothermal vents[a]**

| *Chemolithotroph* | *Electron donor* | *Electron acceptor* | *Product from donor* |
|---|---|---|---|
| Sulfur-oxidizing | $HS^-$, $S^0$, $S_2O_3^{2-}$ | $O_2$, $NO_3^-$ | $S^0$, $SO_4^{2-}$ |
| Nitrifying | $NH_4^+$, $NO_2^-$ | $O_2$ | $NO_2^-$, $NO_3^-$ |
| Sulfate-reducing | $H_2$ | $S^0$, $SO_4^{2-}$ | $H_2S$ |
| Methanogenic | $H_2$ | $CO_2$ | $CH_4$ |
| Hydrogen-oxidizing | $H_2$ | $O_2$, $NO_3^-$ | $H_2O$ |
| Iron- and manganese-oxidizing | $Fe^{2+}$, $Mn^{2+}$ | $O_2$ | $Fe^{3+}$, $Mn^{4+}$ |
| Methylotrophic | $CH_4$, CO | $O_2$ | $CO_2$ |

[a]See Chapter 14 for detailed discussions of these metabolisms and Chapters 15–17 for further coverage of each group of organisms.

### MINIQUIZ

- How does a warm hydrothermal vent differ from a black smoker, both chemically and physically?
- Why is 350°C water emitted from a black smoker not boiling?
- Which phylum of *Bacteria* and which subgroups of this phylum dominate hydrothermal vent ecosystems, and why?

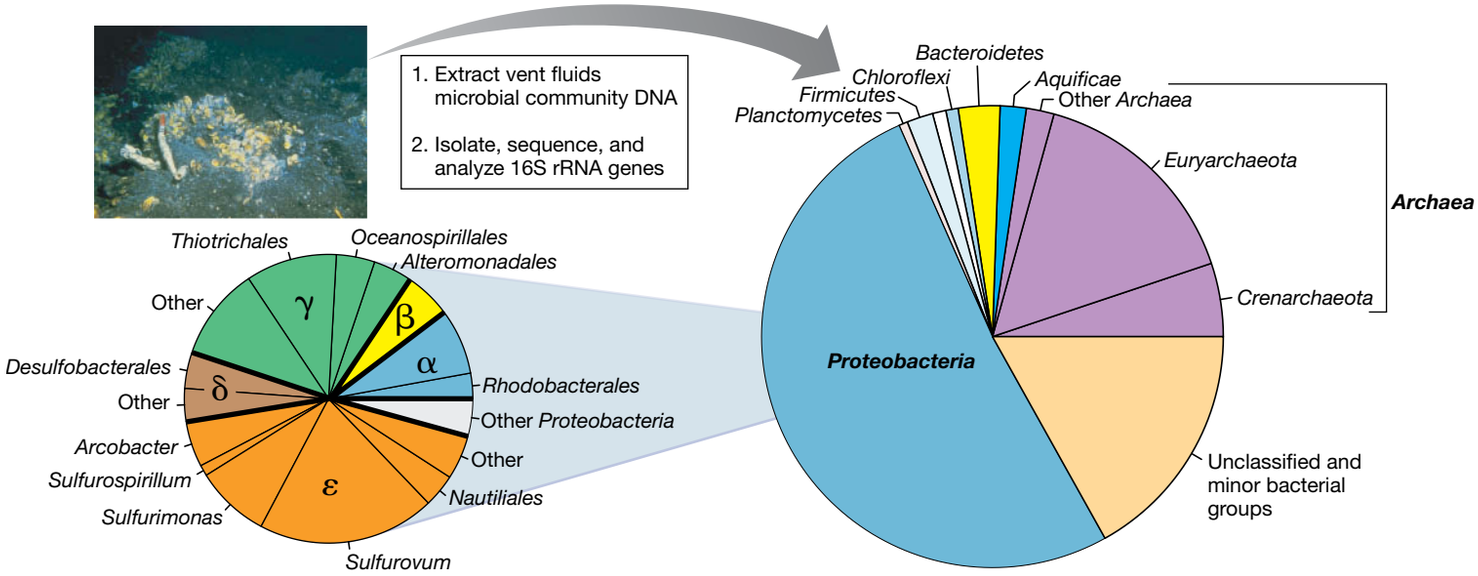



**Figure 20.41 Hydrothermal vent bacterial and archaeal diversity.** The results are pooled analyses from several studies of the 16S rRNA gene content of warm and hot hydrothermal vents. Many of these groups are covered in Chapters 15 and 16 (*Bacteria*) or 17 (*Archaea*). For *Proteobacteria*, major subgroups are indicated. Note the high proportion of *Archaea* and of *Epsilonproteobacteria*. The physiology of many of these organisms is summarized in Table 20.2. Data assembled and analyzed by Nicolas Pinel.

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# Chapter Review

## I • Microbial Ecology

**20.1** Ecosystems consist of organisms, their environments, and all of the interactions among the organisms and environments. The organisms are members of populations and communities and are adapted to habitats. Species richness and abundance are aspects of species diversity in a community and an ecosystem.

**Q Explain the difference between an ecosystem and a habitat. Why are some microbial habitats unsuitable for plant and animal life?**

**20.2** Microbial communities consist of guilds of metabolically similar organisms. Microorganisms play major roles in energy transformations and biogeochemical processes that result in the recycling of elements essential to living systems.

**Q Describe two ways in which the diversity of microbial species in a community can be expressed.**

## II • The Microbial Environment

**20.3** The niche for a microorganism consists of the specific assortment of biotic and abiotic factors within a microenvironment in which that microorganism can be competitive. Microorganisms in nature often live a feast-or-famine existence such that only the best-adapted species reach high population density in a given niche. Cooperation among microorganisms is also important in many microbial interrelationships.

**Q Why are extended periods of exponential microbial growth in nature rare and often slower than rates recorded in laboratory settings?**

**20.4** When surfaces are available, bacteria grow in attached masses of cells called biofilms. Biofilm formation confers several protective advantages on cells. Biofilms can have significant medical and economic effects on humans when unwanted biofilms develop on inert as well as living surfaces.

**Q What property of biofilm microbial communities makes them clinically and industrially relevant? Why do biofilms possess this property? How can this cause problems in many instances?**

**20.5** Microbial mats can be phototrophic or chemolithotrophic. Phototrophic cyanobacterial mats are thick biofilms consisting of microbial cells and trapped particulate materials. They are widespread in hypersaline or thermal

waters where grazing animals are excluded by salt or temperature from feeding on the mat cells. Sulfur-oxidizing chemolithotrophs form extensive mat communities on marine sediment surfaces below the photic zone at the interface between $O_2$ supplied from the overlying water and $H_2S$ produced by sulfate-reducing bacteria.

**Q What is the importance of gliding motility in the formation of marine chemolithotrophic mat systems?**

## III • Terrestrial Environments

**20.6** Soils are complex microbial habitats with numerous microenvironments and niches. Microorganisms are present in the soil primarily attached to soil particles. The most important factors influencing microbial activity in soil are the availability of water and nutrients. However, in very arid soils microorganisms play important roles in stabilizing soil structure.

**Q In what soil horizon are microbial numbers and activities the highest, and why?**

**20.7** The deep subsurface is a significant microbial habitat, most likely sustaining chemolithotrophic populations that can live on a diet of a few minerals, $CO_2$, $SO_4^{2-}$, $N_2$, and $H_2$. Hydrogen is thought to be continually produced by interaction of water with iron minerals or by the radiolysis of water. Novel phyla of small-celled *Archaea* are present in the deep subsurface of both terrestrial and marine sediment environments.

**Q Studies of the deep subsurface have also identified heterotrophic populations. What types and sources of nutrients are available to those organisms?**

## IV • Aquatic Environments

**20.8** In freshwater aquatic ecosystems, phototrophic microorganisms are the main primary producers. Most of the organic matter produced is consumed by bacteria, which can lead to depletion of oxygen in the environment. The BOD of a body of water indicates its relative content of organic matter that can be biologically oxidized.

**Q How and in what way does an input of organic matter, such as sewage, affect the oxygen content of a river or stream?**

**20.9** Pelagic marine waters are more nutrient deficient than most freshwaters, yet substantial numbers of microbes inhabit the oceans. However, in some highly productive and expansive oceanic regions, oxygen can be drawn down to low or unmeasurable levels at depths between 100 and 1000 m; oxygen-depleted waters at these depths are called oxygen minimum zones.

**Q Why is release of sulfide from oxygen minimum zones infrequent, occurring at times of exceptionally high surface water productivity?**

**20.10** The major microbial oxygenic phototrophs in the open oceans include the bacterium *Prochlorococcus* and the alga *Ostreococcus*; both of these phototrophs are small microorganisms. Marine anoxygenic phototrophs include *Roseobacter* and its relatives, the aerobic phototrophic purple bacteria.

**Q How has single-cell genomics contributed to new understanding of factors controlling the diversity of *Prochlorococcus* in the ocean?**

**20.11** Species of *Bacteria* tend to predominate in marine surface waters, whereas in deeper waters *Archaea* make up a larger fraction of the microbial community. Many pelagic *Bacteria* use light to make ATP by rhodopsin-driven proton pumps. Viruses outnumber microbial cells by over an order of magnitude in marine waters.

**Q Many pelagic *Bacteria* can use light energy but are not considered "phototrophs" in the same sense as cyanobacteria or purple bacteria. Explain.**

**20.12** The deep sea is a cold, dark habitat where hydrostatic pressure is high and nutrient levels are low. Piezophiles grow best under pressure but do not require pressure, whereas extreme piezophiles require high pressure, typically several hundred atmospheres, for growth.

**Q What property is shared by piezotolerant, piezophilic, and extremely piezophilic microorganisms?**

**20.13** Deep-sea sediments show decreasing nutrient levels with depth and thus the microbes that reside there experience constant near-starvation conditions. Although not yet cultured, novel phyla of *Archaea* inhabit deep marine sediments (as well as the terrestrial deep subsurface), and these organisms likely survive by scavenging the trace levels of both organic matter and electron acceptors present there.

**Q What are sources of organic matter in deep-sea sediments that could be used to support growth?**

**20.14** Hydrothermal vents are deep-sea hot springs where either volcanic activity or unusual chemistry generates fluids containing large amounts of inorganic electron donors that can be used by chemolithotrophic bacteria.

**Q Would you expect to find the same types of microorganisms associated with black smoker and carbonate vent systems? Explain.**

## Application Questions

1. Imagine a sewage plant that is releasing sewage containing high levels of ammonia and phosphate and very low levels of organic carbon. Which types of microbial blooms might be triggered by this sewage? How would the graphs of oxygen near and beyond the plant's release point differ from the graph shown in Figure 20.17*a*?
2. Keeping in mind that the open-ocean waters are highly oxic, predict the possible metabolic lifestyles of open-ocean *Archaea* and *Bacteria*. Why might rhodopsin-like pigments be more abundant in one group of organisms than in the other?
3. Global warming has been suggested to result in reduced transfer of oxygen to deeper waters in the ocean (Section 20.9). How might global warming also result in reduced nutrient availability to planktonic species in marine surface waters?

## Chapter Glossary

**Biochemical oxygen demand (BOD)** the microbial oxygen-consuming properties of a water sample

**Biofilm** colonies of microbial cells encased in a porous organic matrix and attached to a surface

**Biogeochemistry** the study of biologically mediated chemical transformations in the environment

**Community** two or more cell populations coexisting in a certain area at a given time

**Ecosystem** a dynamic complex of organisms and their physical environment interacting as a functional unit

**Epilimnion** the warmer and less dense surface waters of a stratified lake

**Extreme piezophile** an organism requiring several hundred atmospheres of pressure for growth

**Guild** metabolically similar microbial populations that exploit the same resources in a similar way

**Habitat** part of the ecosystem best suited to one or a few populations

**Hydrothermal vents** warm or hot water–emitting springs associated with crustal spreading centers on the seafloor

**Hypolimnion** the colder, denser, and often anoxic bottom waters of a stratified lake

**Microbial mat** a thick, layered, diverse community nourished either by light in a hypersaline or an extremely hot aquatic environment, in which cyanobacteria are essential; or by chemolithotrophs growing on the surface of sulfide-rich marine sediments

**Microenvironment** a micrometer-scale space surrounding a microbial cell or group of cells

**Niche** in ecological theory, the biotic and abiotic characteristics of the microenvironment that contribute to an organism's competitive success

**Oligotroph** an organism that grows only or grows best at very low levels of nutrients

**Oxygen minimum zone (OMZ)** an oxygen-depleted region of intermediate depth in the marine water column

**Piezophile** an organism that grows best under a hydrostatic pressure greater than 1 atm

**Piezotolerant** able to grow under elevated hydrostatic pressures but growing best at 1 atm

**Population** a group of organisms of the same species in the same place at the same time

**Primary producer** an organism that synthesizes new organic material from $CO_2$ and obtains energy from light or from oxidation of inorganic compounds

**Prochlorophyte** a bacterial oxygenic phototroph that contains chlorophylls *a* and *b* and lacks phycobiliproteins

**Proteorhodopsin** a light-sensitive protein present in some pelagic *Bacteria* that fuels a proton pump that yields ATP

**Rhizosphere** the region immediately adjacent to plant roots

**Species abundance** the proportion of each species in a community

**Species richness** the total number of different species present in a community

**Stratified water column** a body of water separated into layers having distinct physical and chemical characteristics

# Nutrient Cycles

21



## microbiologynow

### The Big Thaw and the Microbiology of Climate Change

As Earth warms in response to the accumulation of greenhouse gases, attention has focused on high-latitude regions where temperatures are rising twice as fast as the global average. As temperatures rise, normally frozen ground (the permafrost subsurface layer in tundra, photo on left) thaws, making vast stores of organic carbon available for microbial decomposition. Accumulated over thousands of years from past plant and animal life, the pool of carbon in the permafrost zone is estimated to be 1300–1600 billion tons. This is twice as much carbon as is currently in the atmosphere, and predicting the rate and extent of microbial conversion of permafrost organic carbon to greenhouse gases (primarily $CO_2$ and $CH_4$) is essential in developing future climate scenarios.

It has been estimated that 5–15% of permafrost carbon will decompose in this century, releasing primarily $CO_2$ at a rate unlikely to cause abrupt climate change in the near future. However, many uncertainties remain with this estimate, including how localized permafrost thawing might accelerate the rate at which carbon is made available for microbial decomposition. Abrupt thawing forms surface depressions that accumulate snow and water (right photo), and this creates saturated and anoxic conditions that favor microbial production of methane, a potent greenhouse gas.

A recent study highlighted the importance of incorporating vegetation change in modeling permafrost thawing. For example, the disappearance of a dominant tundra shrub species (the dwarf birch, which covers the surface in the wide-angle photo on the left and is indicated with an arrow in the close-up photo on the right) left grasses and sedges as the primary ground cover and resulted in localized thawing and formation of saturated soils within 6 years. Increased surface warming from the loss of shade provided by the low-lying shrub triggered a positive feedback loop: Depressions formed that trapped snow and water, thereby accelerating thawing. This transition also shifted the microbial community in the disturbed tundra from that of a methane sink to a methane source.

Thus, although overall permafrost carbon emissions may only rise gradually as temperatures rise, localized pockets of intense microbial activity must also be considered if reliable estimates of total greenhouse gas emissions from the massive store of organic carbon in permafrost regions are to be achieved.


**Source:** Nauta, A.L., et al. 2016. Permafrost collapse after shrub removal shifts tundra ecosystem to a methane source. *Nature Climate Change 5:* 67–70.

# I • Carbon, Nitrogen, and Sulfur Cycles

The key nutrients for life are cycled by both microorganisms and macroorganisms, but for any given nutrient, it is microbial activities that dominate. Understanding how microbial nutrient cycles work is important because the cycles and their many feedback loops are essential for plant agriculture and the overall health of sustainable plant life.

We begin our coverage of nutrient cycles with the carbon cycle. Major areas of interest here are the magnitude of carbon reservoirs on Earth, the rates of carbon cycling within and between reservoirs, and the coupling of the carbon cycle to other nutrient cycles. We emphasize the gases *carbon dioxide* ($CO_2$) and *methane* ($CH_4$) as major components of the carbon cycle and of human impacts on the global ecosystem. We return to the carbon cycle in Section 21.8 to consider how human activities are affecting this critical nutrient cycle.

## 21.1 The Carbon Cycle

On a global basis, carbon (C) cycles as $CO_2$ through all of Earth's major carbon reservoirs: the atmosphere, the land, the oceans, freshwaters, sediments and rocks, and biomass (Figure 21.1). As we have already seen for freshwater environments, the carbon and oxygen cycles are intimately linked (Section 20.8). All nutrient cycles link in some way to the carbon cycle, but the nitrogen (N) cycle links particularly strongly because, other than water ($H_2O$), C and N make up the bulk of living organisms (Section 3.1 and see Figure 21.5).

### Carbon Reservoirs

By far the largest C reservoir on Earth is the sediments and rocks of Earth's crust (Figure 21.1), but the rate at which sediments and rocks decompose and carbon is removed as $CO_2$ is so slow that flux out of this reservoir is insignificant on a human time scale. A large amount of C is found in land plants. This is the organic C of forests, grasslands, and agricultural crops—the major sites of phototrophic $CO_2$ fixation. However, more C is present in dead organic material, called **humus**, than in living organisms. Humus is a complex mixture of organic materials that have resisted rapid decomposition and is derived primarily from dead plants and microorganisms. Some humic substances are quite recalcitrant, with a decomposition time of several decades, but certain other humic components decompose much more rapidly.

The most rapid means of transfer of C is via the atmosphere. Carbon dioxide is removed from the atmosphere primarily by photosynthesis of land plants and marine microorganisms and is returned to the atmosphere by respiration of animals and chemoorganotrophic microorganisms (Figure 21.1). The single most important contribution of $CO_2$ to the atmosphere is by microbial decomposition of dead organic material. However, since the Industrial Revolution, human activities have increased atmospheric $CO_2$ levels by nearly 40%, primarily from the combustion of fossil fuels. This rise in $CO_2$, a major *greenhouse gas*, has triggered a period of steadily increasing global temperatures called **global warming** (see Section 21.8 and Figure 21.19). Although the consequences of global warming on microbial nutrient cycling are currently unpredictable, everything we know about the physiology of microorganisms tells us that microbial activities in nature will change in response to higher temperatures. Whether these responses will be favorable or unfavorable to higher organisms (including humans) is a major area of active research today (Section 21.8).

**Major Carbon Reservoirs on Earth**

| Reservoir | Percent of Total[a] |
|---|---|
| Rocks and sediments | 99.5[b] |
| Oceans | 0.05 |
| Methane hydrates | 0.014 |
| Fossil fuels | 0.006 |
| Terrestrial biosphere | 0.003 |
| Aquatic biosphere | 0.000002 |

[a]Total carbon, 76 × $10^{15}$ tons
[b]80% inorganic

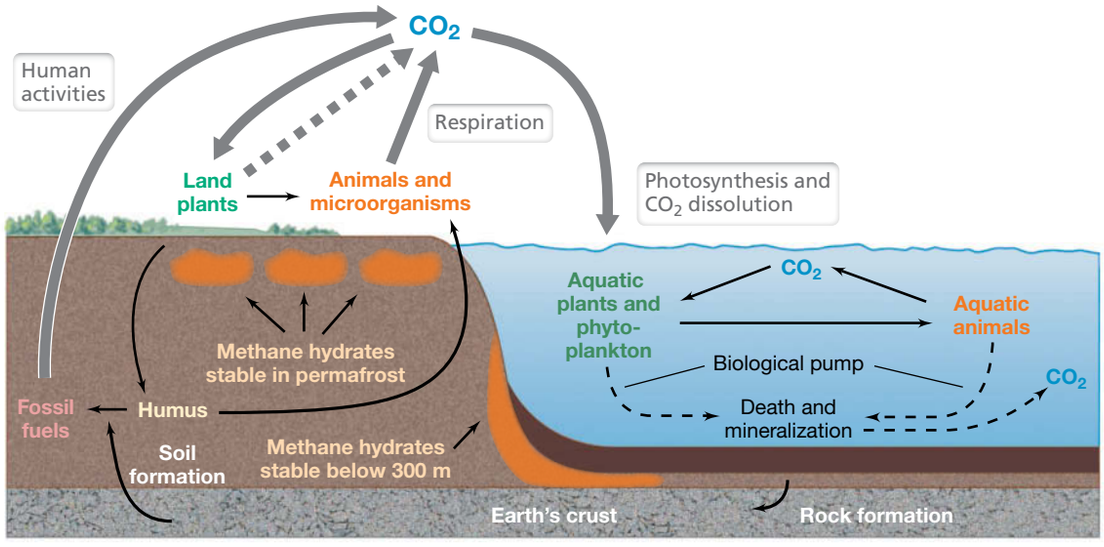



**Figure 21.1 The carbon cycle.** The carbon and oxygen cycles are closely connected, as oxygenic photosynthesis both removes $CO_2$ and produces $O_2$, and respiration both produces $CO_2$ and removes $O_2$. The accompanying table shows that the greatest reservoir of carbon on Earth is in rocks and sediments, and most of this is in inorganic form as carbonates.

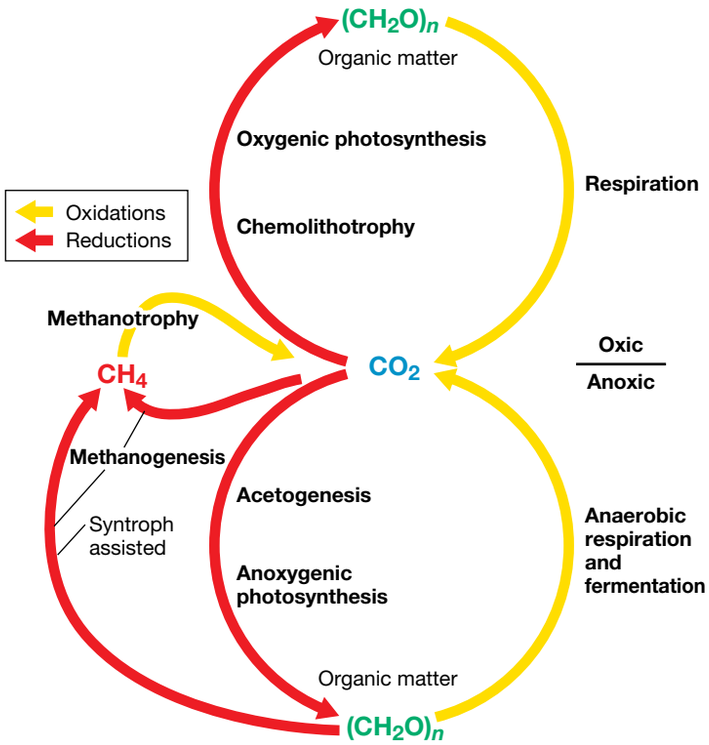



**Figure 21.2 Redox cycle for carbon.** The diagram contrasts autotrophic processes ($CO_2$ → organic compounds) and heterotrophic processes (organic compounds → $CO_2$).

## Photosynthesis and Decomposition

New organic compounds are biologically synthesized on Earth only by $CO_2$ fixation by phototrophs and chemolithotrophs. Most organic compounds originate in photosynthesis and thus phototrophic organisms are the foundation of the carbon cycle (Figure 21.1). However, phototrophic organisms are abundant in nature only in habitats where light is available. The deep sea, deep terrestrial subsurface, and other permanently dark habitats are devoid of indigenous phototrophs. There are two groups of oxygenic phototrophic organisms: *plants* and *microorganisms*. Plants are the dominant phototrophic organisms of terrestrial environments, whereas phototrophic microorganisms dominate in aquatic environments.

The redox cycle for C (**Figure 21.2**) begins with photosynthetic $CO_2$ fixation, driven by the energy of light:

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

$CH_2O$ represents organic matter at the oxidation–reduction level of cell material. Phototrophic organisms also carry out respiration, both in the light and the dark. The overall equation for respiration is the reverse of oxygenic photosynthesis:

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

For organic matter to accumulate, the rate of photosynthesis must exceed the rate of respiration. In this way, autotrophic organisms build biomass from $CO_2$, and then this biomass in one way or another supplies the C heterotrophic organisms need. Anoxygenic phototrophs and chemolithotrophs also produce excess organic compounds, but in most environments the contributions of these organisms to the net accumulation of organic matter are minor compared to the inputs of oxygenic phototrophs. This is because the reductant used by oxygenic phototrophs, $H_2O$ (Section 14.1), is in virtually unlimited supply.

Organic compounds are degraded biologically to $CH_4$ and $CO_2$ (Figure 21.2). Carbon dioxide, most of which is of microbial origin, is produced by aerobic and anaerobic respirations (Section 14.7). Methane is produced in anoxic environments by *methanogens* from the reduction of $CO_2$ with hydrogen ($H_2$) or from the splitting of acetate into $CH_4$ and $CO_2$ (Section 14.17). However, any naturally occurring organic compound can eventually be converted to $CH_4$ from the cooperative activities of methanogens and various fermentative bacteria, as we will see in Section 21.2. Methane produced in anoxic habitats is insoluble and most often diffuses rapidly to oxic environments where it is either released to the atmosphere or oxidized to $CO_2$ by *methanotrophs* (Figure 21.2). Hence, most of the C in organic compounds eventually returns to $CO_2$, and the links in the carbon cycle are closed.

## Methane Hydrates

Although present in the atmosphere at levels lower than even $CO_2$, $CH_4$ is a potent greenhouse gas that is over 20 times more effective in trapping heat than is $CO_2$. Some $CH_4$ enters the atmosphere from methanogenic production, but not all biologically produced $CH_4$ is immediately consumed or released to the atmosphere. Huge amounts of $CH_4$ derived primarily from past microbial activities are trapped underground or under marine sediments as *methane hydrates* (Figure 21.1 and **Figure 21.3**), molecules of frozen $CH_4$. Methane hydrates form when sufficient $CH_4$ is present in environments of high pressure and low temperature such as beneath the permafrost in the Arctic and in marine sediments (Figure 21.1). These deposits can be up to several hundred meters thick and are estimated to contain 700–10,000 petagrams (1 petagram = $10^{15}$ g) of $CH_4$. This exceeds other known $CH_4$ reserves on Earth by several orders of magnitude.


**Figure 21.3 Burning methane hydrate.** Frozen methane ice retrieved from marine sediments is ignited.

Methane hydrates are highly dynamic, absorbing and releasing $CH_4$ in response to changes in pressure, temperature (Figure 21.4), and fluid movement. Methane hydrates also fuel deep-water ecosystems, called *cold seeps* (Figure 20.20). Here, the slow release of $CH_4$ from seafloor hydrates nourishes not only anaerobic methane-oxidizing *Archaea* (Section 14.25), but also animal communities that contain aerobic methane-oxidizing endosymbionts that oxidize $CH_4$ and release organic matter to the animals (Section 23.9). Anaerobic oxidation of $CH_4$ is coupled to the reduction of sulfate ($SO_4^{2-}$), nitrate ($NO_3^-$), and oxides of iron and manganese [e.g., FeO(OH)], dampening release of free methane.

Although deep oceanic methane hydrates are stabilized by high pressure, hydrates in shallower coastal sediments are much more sensitive to small changes in temperature (Figure 21.4). Hydrates in shallow sediments are at the margin of what is called the *gas hydrate stability zone* (GHSZ). In these marginal regions, relatively small changes in temperature can destabilize the hydrates. For example, on-site monitoring of hydrate deposits in a marine coastal plain showed them to be sensitive to 1–2°C seasonal changes in bottom-water temperature. During periods of seasonally elevated water temperature, methane was observed to bubble freely (a phenomenon called methane flares) from the sediments (Figure 21.4). In addition to the release of methane from marine hydrates, as permafrost melts, its huge reserve of organic matter could be catabolized by microbes, leading to the formation of yet more methane (Section 21.8).

## Carbon Balances and Coupled Cycles

Although it is convenient to consider carbon cycling as a series of reactions separate from those in other nutrient cycles, in reality, all nutrient cycles are *coupled cycles*; major changes in one cycle affect the functioning of others. But certain cycles, such as the carbon and nitrogen cycles (Figure 21.5), are extremely closely coupled because of the large amount of C and N in living organisms. The rate of primary productivity ($CO_2$ fixation) is controlled by several factors, in particular by the magnitude of photosynthetic biomass and by available N, often a limiting nutrient. Thus, large-scale reductions in biomass, for instance by widespread deforestation, reduce rates of primary productivity and increase levels of $CO_2$. High levels of organic C stimulate microbial nitrogen fixation ($N_2 \rightarrow NH_3$) and this in turn adds more fixed N to the pool for primary producers; low levels of organic C have just the opposite effect (Figure 21.5). High levels of ammonia ($NH_3$) stimulate primary production and nitrification, but inhibit nitrogen fixation. High levels of nitrate ($NO_3^-$), an excellent N source for plants and aquatic phototrophs, stimulate primary production but also increase the rate of denitrification; the latter removes fixed forms of N from the environment and feeds back in a negative way on primary production (Figure 21.5).

This simple example illustrates how nutrient cycles are anything but isolated entities. Instead, they are coupled systems that must maintain a delicate balance of inputs and outputs. Thus, one could expect the C and N cycles to respond to large inputs in specific components (for example, through inputs of $CO_2$ or nitrogen fertilizers) in ways that are not always beneficial to the biosphere and that can have unintended consequences (Section 21.8).

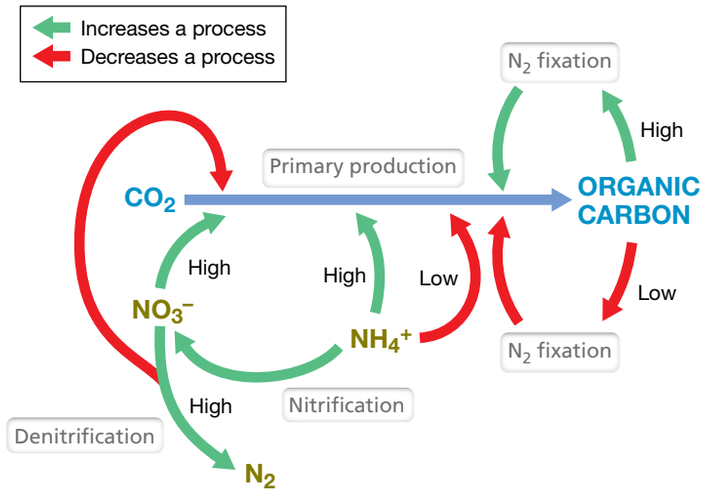



**Figure 21.5 Coupled cycles.** All nutrient cycles are interconnected, but the carbon and nitrogen cycles are intimately coupled. In the carbon cycle, $CO_2$ supplies the C for carbon compounds. The N cycle, shown in more detail in Figure 21.8, supplies N for many biological compounds.

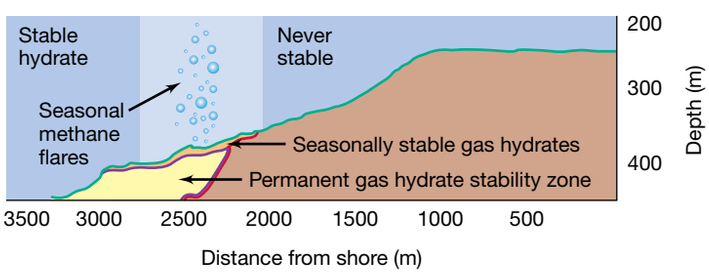



**Figure 21.4 Seasonal flares of methane bubbling from methane hydrates.** Methane hydrates in shallow coastal sediments are sensitive to seasonal changes in bottom water temperature. Flares of methane bubbles are observed when water temperatures warm by as little as 1–2°C.

**MINIQUIZ**

- How is new organic matter made in nature?
- In what ways are oxygenic photosynthesis and respiration related?
- What is a methane hydrate?

# 21.2 Syntrophy and Methanogenesis

Most organic compounds are oxidized in nature by *aerobic* microbial processes. However, because oxygen ($O_2$) is a poorly soluble gas and is actively consumed when available, much organic carbon still ends up in anoxic environments. Methanogenesis, the biological production of $CH_4$, is a major process in anoxic habitats and is catalyzed by a large group of *Archaea*, the *methanogens*, which are strict anaerobes. We discussed the biochemistry of methanogenesis in Section 14.17 and other aspects of methanogens in Section 17.2.

Most methanogens can use $CO_2$ as a terminal electron acceptor in anaerobic respiration, reducing it to $CH_4$ with $H_2$ as electron

donor. Only a very few other substrates, chiefly acetate, are directly converted to $CH_4$ by methanogens. To convert most organic compounds to $CH_4$, methanogens must team up with partner organisms called *syntrophs* that function to supply them with precursors for methanogenesis.

## Anoxic Decomposition and Syntrophy

In Section 14.23 we discussed the biochemistry of **syntrophy**, a process in which two or more organisms cooperate in the anaerobic degradation of organic compounds. Here we consider the interactions of syntrophic bacteria with their partner organisms and their significance for the carbon cycle. Our focus will be anoxic freshwater sediments and anoxic wastewater treatment, both of which are major sources of $CH_4$.

Polysaccharides, proteins, lipids, and nucleic acids from dead organisms find their way into anoxic habitats, where they are catabolized. The monomers released by hydrolysis of these polymers become major electron donors for energy metabolism. For the breakdown of a typical polysaccharide such as cellulose, the process begins with *cellulolytic* bacteria (Figure 21.6). These organisms hydrolyze cellulose into glucose, which is catabolized by fermentative organisms to short-chain fatty acids (acetate, propionate, and butyrate), alcohols such as ethanol and butanol, and the gases $H_2$ and $CO_2$. Hydrogen ($H_2$) and acetate are consumed by methanogens directly, but the bulk of the carbon remains in the form of fatty acids and alcohols; these cannot be directly catabolized by methanogens and require the activities of syntrophic bacteria (Section 14.23; Figure 21.6).

Syntrophic bacteria are *secondary* fermenters because they ferment the products of the primary fermenters, yielding $H_2$, $CO_2$, and acetate as products. For example, *Syntrophomonas wolfei* oxidizes $C_4$ to $C_8$ fatty acids, yielding acetate, $CO_2$ (if the fatty acid was $C_5$ or $C_7$), and $H_2$ (Table 21.1 and Figure 21.6). Other species of *Syntrophomonas* use fatty acids up to $C_{18}$ in length, including some unsaturated fatty acids. *Syntrophobacter wolinii* specializes in propionate ($C_3$) fermentation, generating acetate, $CO_2$, and $H_2$,

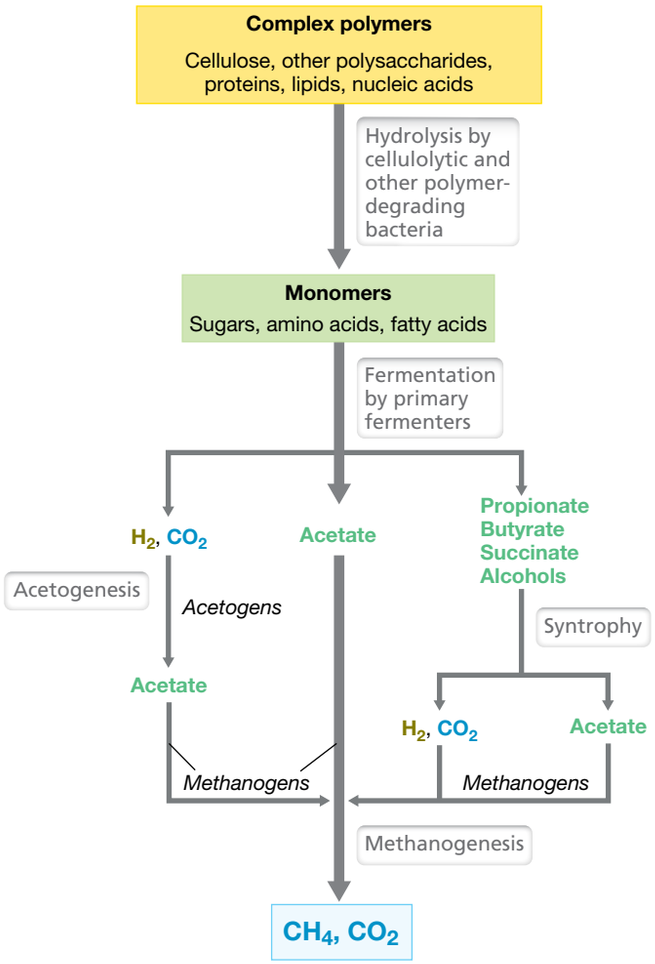



**Figure 21.6 Anoxic decomposition.** In anoxic decomposition, various groups of fermentative anaerobes cooperate in the conversion of complex organic materials to $CH_4$ and $CO_2$. This pattern holds for environments in which sulfate-reducing bacteria play only a minor role; for example, in freshwater lake sediments, sewage sludge bioreactors, or the rumen.

**TABLE 21.1 Major reactions in the anoxic conversion of organic compounds to methane[a]**

| | | Free energy change (kJ/reaction) | |
|---|---|---|---|
| *Reaction type* | *Reaction* | $\Delta G^{0\prime}$ [b] | $\Delta G$ [c] |
| Fermentation of glucose to acetate, $H_2$, and $CO_2$ | Glucose + 4 $H_2O$ → 2 acetate$^-$ + 2 $HCO_3^-$ + 4 $H^+$ + 4 $H_2$ | −207 | −319 |
| Fermentation of glucose to butyrate, $CO_2$, and $H_2$ | Glucose + 2 $H_2O$ → butyrate$^-$ + 2 $HCO_3^-$ + 2 $H_2$ + 3 $H^+$ | −135 | −284 |
| Fermentation of butyrate to acetate and $H_2$ | Butyrate$^-$ + 2 $H_2O$ → 2 acetate$^-$ + $H^+$ + 2 $H_2$ | +48.2 | −17.6 |
| Fermentation of propionate to acetate, $CO_2$, and $H_2$ | Propionate$^-$ + 3 $H_2O$ → acetate$^-$ + $HCO_3^-$ + $H^+$ + $H_2$ | +76.2 | −5.5 |
| Fermentation of ethanol to acetate and $H_2$ | 2 Ethanol + 2 $H_2O$ → 2 acetate$^-$ + 4 $H_2$ + 2 $H^+$ | +19.4 | −37 |
| Fermentation of benzoate to acetate, $CO_2$, and $H_2$ | Benzoate$^-$ + 7 $H_2O$ → 3 acetate$^-$ + 3 $H^+$ + $HCO_3^-$ + 3 $H_2$ | +70.1 | −18 |
| Methanogenesis from $H_2$ + $CO_2$ | 4 $H_2$ + $HCO_3^-$ + $H^+$ → $CH_4$ + 3 $H_2O$ | −136 | −3.2 |
| Methanogenesis from acetate | Acetate$^-$ + $H_2O$ → $CH_4$ + $HCO_3^-$ | −31 | −24.7 |
| Acetogenesis from $H_2$ + $CO_2$ | 4 $H_2$ + 2 $HCO_3^-$ + $H^+$ → acetate$^-$ + 4 $H_2O$ | −105 | −7.1 |

[a] Data adapted from Zinder, S. 1984. Microbiology of anaerobic conversion of organic wastes to methane: Recent developments. *Am. Soc. Microbiol. 50:* 294–298.
[b] Standard conditions: solutes, 1 M; gases, 1 atm; 25°C.
[c] Concentrations of reactants in typical anoxic freshwater ecosystems: fatty acids, 1 mM; $HCO_3^-$, 20 mM; glucose, 10 μM; $CH_4$, 0.6 atm; $H_2$, $10^{-4}$ atm. Data from $G_f^0$ values in Table 3.2 (or references therein) and Figure 3.10, and bioenergetics calculations as described in Sections 3.4 and 3.6.

and *Syntrophus gentianae* degrades aromatic compounds such as benzoate to acetate, $H_2$, and $CO_2$ (Table 21.1).

Despite rather extensive metabolic diversity, syntrophs are unable to carry out any of these reactions in pure culture. Instead, they depend on a $H_2$-consuming partner organism because of the unusual bioenergetics linked to the syntrophic process. As described in Section 14.23, $H_2$ consumption by a partner organism is absolutely essential for growth of syntrophic bacteria (in the absence of other electron acceptors), and the association of $H_2$ producer and $H_2$ consumer can be very intimate. In fact, it is thought that $H_2$ transfer in some syntrophic associations may not be directly via $H_2$ transfer but through *direct conduction*, where electrons are transferred between species using electrically conductive wire-like structures (see Explore the Microbial World, "Microbially Wired," later in this chapter). But no matter the mechanisms, it is the transfer of $H_2$ (or electrons) that makes the syntrophic association work. How is this so?

When the reactions listed in Table 21.1 for the fermentation of butyrate, propionate, ethanol, or benzoate are written with all reactants at standard conditions (solutes, 1 M; gases, 1 atm; 25°C), the reactions yield free-energy changes ($\Delta G^{0\prime}$, Section 3.4) that are positive in arithmetic sign; that is, the reactions *require* rather than *release* energy. But the consumption of $H_2$ dramatically affects the energetics, making the reaction favorable and allowing energy to be conserved. This can be seen in Table 21.1, where the $\Delta G$ values (free-energy change measured under actual conditions in the habitat) are negative in arithmetic sign if $H_2$ concentrations are kept near zero by a $H_2$-consuming partner organism. This allows the syntrophic bacterium to conserve a small amount of energy that is used to produce ATP.

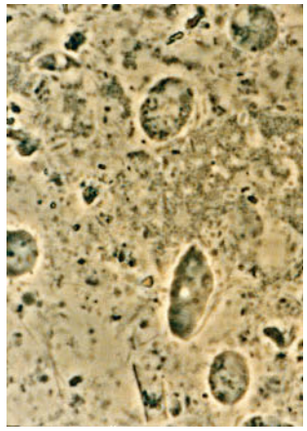
The final products of the syntrophic partnership are $CO_2$ and $CH_4$ (Figure 21.6), and any naturally occurring organic compound that enters a methanogenic habitat will eventually be converted to these products. This includes even complex aromatic and aliphatic hydrocarbons. Additional organisms other than those shown in Figure 21.6 may participate in such degradations, but eventually fatty acids and alcohols will be generated and they will be converted to methanogenic substrates by syntrophs. Acetate produced by syntrophs (as well as by the activities of acetogenic bacteria, Figure 21.6 and Section 14.16) is a direct methanogenic substrate and is converted to $CO_2$ and $CH_4$ by various methanogens.

### Methanogenic Symbionts and Acetogens in Termites

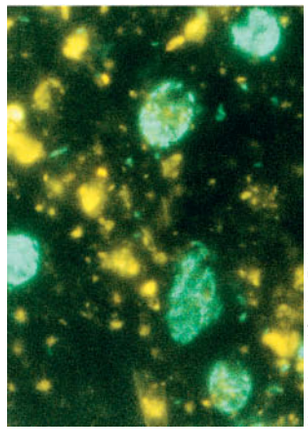
A variety of anaerobic protists that thrive under strictly anoxic conditions, including ciliates and flagellates, play a major role in the carbon cycle. Methanogenic *Archaea* live within some of these protist cells as $H_2$-consuming endosymbionts. For example, methanogens are present *within* cells of trichomonal protists inhabiting the termite hindgut (**Figure 21.7**) where methanogenesis and acetogenesis are major metabolic processes. Methanogenic symbionts of protists are species of the genera *Methanobacterium* or *Methanobrevibacter* (Section 17.2).

In the termite hindgut, endosymbiotic methanogens along with acetogenic bacteria are thought to benefit their protist hosts by consuming $H_2$ generated from glucose fermentation by cellulolytic protists. The acetogens are not endosymbionts but instead reside in the termite hindgut itself, consuming $H_2$ from primary fermenters and reducing $CO_2$ to make acetate. Unlike methanogens, acetogens can ferment glucose directly to acetate. Acetogens can also ferment methoxylated aromatic compounds to acetate. This is especially important in the termite hindgut because termites live on wood, which contains lignin, a complex polymer of methoxylated aromatic compounds. The acetate produced by acetogens in the termite hindgut is consumed by the insect as its primary carbon and energy source. Microbial symbioses in the termite hindgut are discussed in more detail in Section 23.7.


(a)




(b)




(c)

**Figure 21.7 Termites and their carbon metabolism.** *(a)* A subterranean termite worker larva shown beneath a hindgut dissected from another worker. The animal is about 0.5 cm long. Two views of the same microscopic field show termite hindgut protists photographed by *(b)* phase-contrast and *(c)* epifluorescence. Endosymbiotic methanogens in the protist cells fluoresce blue-green because of the methanogenic coenzyme $F_{420}$ (compare with Figure 14.46). The average diameter of the protist cells is 15–20 μm.

**MINIQUIZ**

- Why does *Syntrophomonas* need a partner organism in order to ferment fatty acids or alcohols?
- What kinds of organisms are used in coculture with *Syntrophomonas*?
- What is the final product of acetogenesis?

## 21.3 The Nitrogen Cycle

Nitrogen is an essential element for life (Section 3.1) and exists in a number of oxidation states. We have discussed four major microbial N transformations thus far: nitrification, denitrification,

**Key Processes and Microbes in the Nitrogen Cycle**

| Processes | Example organisms |
|---|---|
| **Nitrification** ($NH_4^+ \rightarrow NO_3^-$) | |
| $NH_4^+ \rightarrow NO_3^-$ | Comammox (*Nitrospira* species) |
| $NH_4^+ \rightarrow NO_2^-$ | *Nitrosomonas, Nitrosopumilus* (*Archaea*) |
| $NO_2^- \rightarrow NO_3^-$ | *Nitrobacter* |
| **Denitrification** ($NO_3^- \rightarrow N_2$) | *Bacillus, Paracoccus, Pseudomonas* |
| **$N_2$ Fixation** ($N_2 + 8\ H \rightarrow NH_3 + H_2$) | |
| Free-living | |
| Aerobic | *Azotobacter* |
| | Cyanobacteria |
| Anaerobic | *Clostridium*, purple and green phototrophic bacteria |
| | *Methanobacterium (Archaea)* |
| Symbiotic | *Rhizobium* |
| | *Bradyrhizobium* |
| | *Frankia* |
| **Ammonification** (organic-N $\rightarrow NH_4^+$) | |
| | Many organisms can do this |
| **Anammox** ($NO_2^- + NH_3 \rightarrow 2\ N_2$) | *Brocadia* |

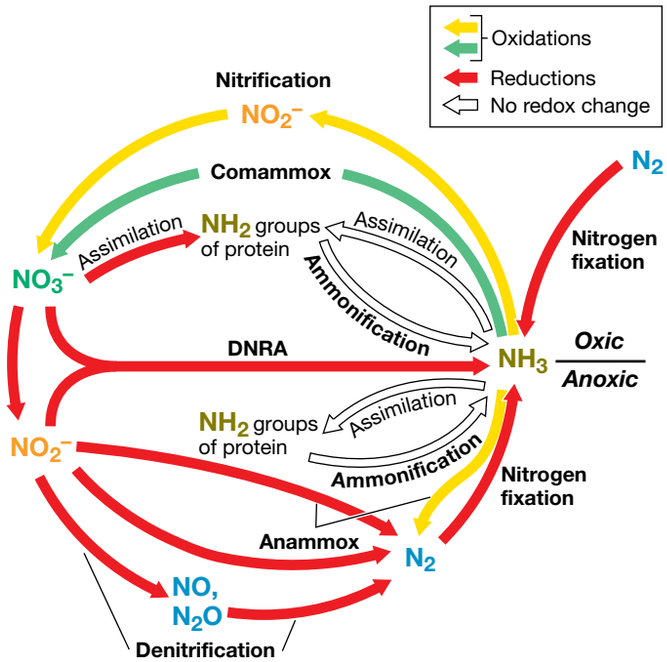



**Figure 21.8 Redox cycle for nitrogen.** The actual anammox reaction is $NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$ (Figure 14.34). DNRA, dissimilative reduction of nitrate to ammonia.

anammox, and nitrogen fixation (Chapter 14). These and other key N transformations are summarized in the redox cycle shown in Figure 21.8.

## Nitrogen Fixation and Denitrification

Nitrogen gas ($N_2$) is the most stable form of N and is a major reservoir for N on Earth. However, only a relatively small number of *Bacteria* and *Archaea* are able to use $N_2$ as a cellular N source by the process of *nitrogen fixation* ($N_2 + 8\ H \rightarrow 2\ NH_3 + H_2$) (Section 14.6). The N recycled on Earth is mostly already "fixed N"; that is, N in combination with other elements, such as in ammonia ($NH_3$) or nitrate ($NO_3^-$). In many environments, however, the short supply of fixed N puts a premium on biological nitrogen fixation, and in these habitats, nitrogen-fixing bacteria flourish.

We discussed the role of $NO_3^-$ as an alternative electron acceptor in anaerobic respiration in Section 14.13. Under most conditions, the end product of $NO_3^-$ reduction is $N_2$, nitric oxide (NO), or nitrous oxide ($N_2O$). The reduction of $NO_3^-$ to these gaseous N compounds, called **denitrification** (Figure 21.8), is the main means by which $N_2$ and $N_2O$ are formed biologically. On the one hand, denitrification is a detrimental process. For example, if agricultural fields fertilized with $NO_3^-$ fertilizer become waterlogged following heavy rains, anoxic conditions can develop and denitrification can be extensive; this removes fixed N from the soil. On the other hand, denitrification can aid in wastewater treatment (Section 22.7). By converting $NO_3^-$ in wastewater to volatile forms of N, denitrification minimizes fixed N in discharge waters and subsequent algal growth triggered by the nitrate influx.

The production of $N_2O$ and NO by denitrification can have other environmental consequences. Nitrous oxide can be photochemically oxidized to NO in the atmosphere. Nitric oxide reacts with ozone ($O_3$) in the upper atmosphere to form nitrite ($NO_2^-$), and this returns to Earth as nitric acid ($HNO_2$). In addition, $N_2O$ is a very potent greenhouse gas. Although $N_2O$ molecules persist on average only about 100 years because of their reactivity, on a per weight basis, the contribution of $N_2O$ to warming is about 300 times that of $CO_2$. Thus, denitrification contributes to global warming; to $O_3$ destruction, which increases passage of ultraviolet radiation to the surface of Earth; and to acid rain, which increases acidity of soils. Increases in soil acidity can change microbial community structure and function and, ultimately, soil fertility, impacting both plant diversity and agricultural yields of crop plants.

## Ammonification and Ammonia Fluxes

Ammonia is released during the decomposition of organic N compounds such as amino acids and nucleotides, a process called *ammonification* (Figure 21.8). Another process contributing to the generation of $NH_3$ is the respiratory reduction of $NO_3^-$ to $NH_3$, called *dissimilative nitrate reduction to ammonia* (DNRA, Figure 21.8). DNRA dominates $NO_3^-$ and nitrite ($NO_2^-$) reduction in reductant-rich anoxic environments, such as highly organic marine sediments and the human gastrointestinal tract. It is thought that nitrate-reducing bacteria exploit this pathway primarily when $NO_3^-$ is limiting because DNRA consumes eight electrons compared with the four and five electrons consumed when $NO_3^-$ is reduced to $N_2O$ or $N_2$, respectively.

At neutral pH, $NH_3$ exists as ammonium ($NH_4^+$). Much of the $NH_4^+$ released by aerobic decomposition in soils is rapidly recycled and converted to amino acids in plants and microorganisms. However, because $NH_3$ is volatile, some of it can be lost from alkaline soils by vaporization, and there are major losses of $NH_3$ to the atmosphere in areas with dense animal populations (for example, cattle feedlots). On a global basis, however, $NH_3$ constitutes only about 15% of the N released to the atmosphere, the rest being primarily $N_2$ or $N_2O$ from denitrification.

### Nitrification and Anammox

*Nitrification*, the oxidation of $NH_3$ to $NO_3^-$, is a major process in well-drained oxic soils at neutral pH, and is carried out by the nitrifying *Bacteria* and *Archaea* (Figure 21.8). Whereas denitrification *consumes* $NO_3^-$, nitrification *produces* $NO_3^-$. If materials high in $NH_3$, such as manure or sewage, are added to soils, the rate of nitrification increases.

Nitrification was long considered an obligatory two-step process in which some species oxidize $NH_3$ to $NO_2^-$ and then other species oxidize $NO_2^-$ to $NO_3^-$. Many species of both *Bacteria* and *Archaea* can oxidize $NH_3$ (↻ Sections 14.11, 15.13, 17.5), whereas thus far, only species of *Bacteria* are known that oxidize $NO_2^-$. Archaeal nitrifiers generally greatly outnumber their bacterial counterparts in marine and most terrestrial systems and likely control rates of $NH_3$ oxidation in nature. However, novel *Nitrospira* species were recently discovered that have the capacity to oxidize ammonia completely to nitrate. Although the environmental significance of these organisms (termed *comammox* bacteria) has yet to be established, their discovery has overturned a century of conventional wisdom.

Although $NO_3^-$ is readily assimilated by plants, it is very soluble and therefore is rapidly leached or denitrified from waterlogged soils; consequently, nitrification is not beneficial for plant agriculture. Ammonium, on the other hand, is positively charged and strongly adsorbed to negatively charged soils. Anhydrous $NH_3$ is therefore used extensively as an agricultural fertilizer, but to prevent its conversion to $NO_3^-$, chemicals are added to the $NH_3$ to inhibit nitrification. One common inhibitor is a pyridine compound called *nitrapyrin* (2-chloro-6-trichloromethylpyridine). Nitrapyrin specifically inhibits the *first* step in nitrification, the oxidation of $NH_3$ to $NO_2^-$. However, this effectively inhibits both steps in nitrification because the second step, $NO_2^- \rightarrow NO_3^-$, depends on the first (↻ Section 14.11). The addition of nitrapyrin to anhydrous $NH_3$ has greatly increased the efficiency of crop fertilization and has helped prevent pollution of waterways by $NO_3^-$ leached from nitrified soils.

Ammonia can be oxidized under anoxic conditions in a process called *anammox*. The anammox bacteria affiliate with five genera (*Brocadia, Kuenenia, Scalindua, Anammoxoglobus*, and *Jettenia*) in a single phylogenetically cohesive family (*Brocadiaceae*) within the *Planctomycetes* (↻ Section 16.16). Since anammox bacteria have yet to be isolated in pure culture, the genus and species names are prefaced by the term "Candidatus" to indicate this tentative taxonomic status (↻ Section 13.10). In the anammox reaction, $NH_3$ is oxidized anaerobically with $NO_2^-$ as the electron acceptor, forming $N_2$ as the final product (Figure 21.8), which is released to the atmosphere. Although a major process in sewage and in anoxic marine basins and sediments, anammox is not a significant process in well-drained (oxic) soils. The microbiology and biochemistry of anammox was discussed in Section 14.12.

**MINIQUIZ**

- What is nitrogen fixation and why is it important to the nitrogen cycle?
- How do the processes of nitrification and denitrification differ? How do nitrification and anammox differ?
- How does the compound nitrapyrin benefit both agriculture and the environment?

## 21.4 The Sulfur Cycle

Microbial transformations of sulfur (S) are even more complex than those of N because of the large number of oxidation states of S and the fact that several transformations of S also occur spontaneously (abiotically). Chemolithotrophic S oxidation and sulfate ($SO_4^{2-}$) reduction were covered in Sections 14.9, 14.14, 15.9, and 15.11. The redox cycle for microbial S transformations is shown in **Figure 21.9**.

Although a number of oxidation states of S are possible, only three are significant in nature, −2 (sulfhydryl, R–SH, and sulfide, $HS^-$), 0 (elemental sulfur, $S^0$), and +6 (sulfate, $SO_4^{2-}$). The bulk of S on Earth resides in sediments and rocks in the form of sulfate minerals, primarily gypsum ($CaSO_4$), and sulfide minerals (pyrite, $FeS_2$), but the oceans constitute the most significant reservoir of $SO_4^{2-}$ in the biosphere. A significant amount of S, in particular sulfur dioxide ($SO_2$, a gas), enters the S cycle from human activities, primarily the burning of fossil fuels.

### Hydrogen Sulfide and Sulfate Reduction

A major volatile S gas is hydrogen sulfide ($H_2S$). Hydrogen sulfide is produced from bacterial sulfate reduction ($SO_4^{2-} + 4\ H_2 \rightarrow H_2S + 2\ H_2O + 2\ OH^-$) (Figure 21.9) or is geochemically produced and emitted from sulfide springs and volcanoes. Although $H_2S$ is volatile, different forms exist depending on pH: $H_2S$ predominates below pH 7 and the nonvolatile $HS^-$ and $S^{2-}$ predominate above pH 7. Collectively, $H_2S$, $HS^-$, and $S^{2-}$ are referred to as "sulfide."

Sulfate-reducing bacteria are a large and highly diverse group (↻ Sections 14.14 and 15.9) and are widespread in nature. However, in anoxic habitats such as freshwater sediments and many soils, sulfate reduction is limited by $SO_4^{2-}$ availability. Moreover, because organic electron donors (or $H_2$, which is a product of the fermentation of organic compounds) are needed to support sulfate reduction, it only occurs where significant amounts of organic material are present.

In marine sediments, the rate of sulfate reduction is typically carbon-limited and can be greatly increased by an influx of organic matter. This is important because the disposal of sewage or garbage in the oceans or coastal regions can trigger sulfate reduction. Hydrogen sulfide is toxic to many plants and animals and therefore its formation is potentially detrimental; sulfide is toxic because it combines with the iron of cytochromes and blocks respiration. Sulfide is commonly detoxified in nature by combination with iron, forming the insoluble minerals FeS (pyrrhotite) and $FeS_2$ (pyrite). The black color of sulfidic sediments or sulfate-reducing bacterial cultures is due to these metal sulfide minerals (↻ Figure 15.23*g*).

### Sulfide and Elemental Sulfur Oxidation–Reduction

Under oxic conditions, sulfide rapidly oxidizes spontaneously at neutral pH. Sulfur-oxidizing chemolithotrophic bacteria, most of which are aerobes (↻ Sections 14.9 and 15.11), can catalyze the oxidation of sulfide. However, because of the rather rapid spontaneous reaction, microbial sulfide oxidation is significant only in areas where $H_2S$ emerging from anoxic environments meets air. Where light is available, there can be anoxic oxidation of sulfide,

**Key Processes and Microbes in the Sulfur Cycle**

| Process | Example organisms |
|---|---|
| **Sulfide/sulfur oxidation** ($H_2S \rightarrow S^0 \rightarrow SO_4^{2-}$) | |
| Aerobic | Sulfur chemolithotrophs (*Thiobacillus*, *Beggiatoa*, many others) |
| Anaerobic | Purple and green phototrophic bacteria, some chemolithotrophs |
| **Sulfate reduction** (anaerobic) ( $SO_4^{2-} \rightarrow H_2S$) | *Desulfovibrio*, *Desulfobacter* *Archaeoglobus (Archaea)* |
| **Sulfur reduction** (anaerobic) ( $S^0 \rightarrow H_2S$) | *Desulfuromonas*, many hyperthermophilic *Archaea* |
| **Sulfur disproportionation** ($S_2O_3^{2-} \rightarrow H_2S + SO_4^{2-}$) | *Desulfovibrio*, and others |
| **Organic sulfur compound oxidation or reduction** ($CH_3SH \rightarrow CO_2 + H_2S$) ($DMSO \rightarrow DMS$) | Many organisms can do this |
| **Desulfurylation** (organic–S $\rightarrow H_2S$) | Many organisms can do this |

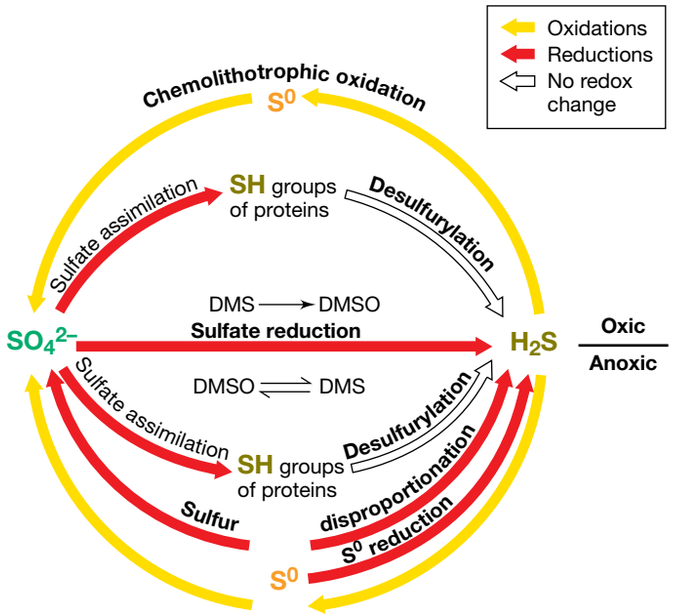



**Figure 21.9 Redox cycle for sulfur.** DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide.

catalyzed by the phototrophic purple and green sulfur bacteria (Sections 14.3, 15.4, and 15.6).

$S^0$ is chemically stable but is readily oxidized by sulfur-oxidizing chemolithotrophic bacteria such as *Thiobacillus* and *Acidithiobacillus*. Because $S^0$ is insoluble, the bacteria that oxidize it must attach to the $S^0$ crystals to obtain their substrate (Figure 14.27). The oxidation of $S^0$ forms sulfuric acid ($H_2SO_4$), and thus $S^0$ oxidation characteristically lowers the pH in the environment, sometimes drastically. For this reason, small amounts of $S^0$ can be added to alkaline soils as an inexpensive and natural way to lower the pH, relying on the ubiquitous sulfur chemolithotrophs to carry out the acidification process.

$S^0$ can be reduced as well as oxidized. The reduction of $S^0$ to sulfide (a form of anaerobic respiration) is a major ecological process of some *Bacteria* and hyperthermophilic *Archaea* (Section 15.10 and Chapter 17). Although sulfate-reducing bacteria can also reduce $S^0$, most $S^0$ reduction occurs by the activities of the physiologically specialized sulfur reducers, organisms that are incapable of $SO_4^{2-}$ reduction (Section 15.10). The habitats of the sulfur reducers are generally those of the sulfate reducers, so from an ecological standpoint, the two groups form a metabolic guild unified by their formation of $H_2S$.

### Organic Sulfur Compounds

In addition to *inorganic* forms of S, several *organic* S compounds are also cycled in nature. Many of these foul-smelling compounds are highly volatile and can thus enter the atmosphere. The most abundant organic S compound in nature is *dimethyl sulfide* ($CH_3—S—CH_3$); it is produced primarily in marine environments as a degradation product of dimethylsulfoniopropionate [$(CH_3)_2S^+CH_2CH_2COO^-$], a major osmoregulatory solute in marine algae (Section 5.13). This compound can be used as a carbon source and electron donor by microorganisms and is catabolized to $CH_3—S—CH_3$ and acrylate ($CH_2{=}CHCOO^-$). The latter, a derivative of the fatty acid propionate, is used to support growth.

Dimethyl sulfide released to the atmosphere undergoes photochemical oxidation to methanesulfonate ($CH_3SO_3$), $SO_2$, and $SO_4^{2-}$. By contrast, $CH_3—S—CH_3$ produced in anoxic habitats can be microbially transformed in at least three ways: (1) by methanogenesis (yielding $CH_4$ and $H_2S$), (2) as an electron donor for photosynthetic $CO_2$ fixation in phototrophic purple bacteria (yielding dimethyl sulfoxide, DMSO), and (3) as an electron donor in energy metabolism in certain chemoorganotrophs and chemolithotrophs (also yielding DMSO). DMSO can be an electron acceptor for anaerobic respiration (Section 14.15), producing $CH_3—S—CH_3$. Many other organic S compounds affect the global sulfur cycle, including methanethiol ($CH_3SH$), dimethyl disulfide ($H_3C—S—S—CH_3$), and carbon disulfide ($CS_2$), but on a global basis, $CH_3—S—CH_3$ is the most significant.

**MINIQUIZ**

- Is $H_2S$ a substrate or a product of the sulfate-reducing bacteria? Of the chemolithotrophic sulfur bacteria?
- Why does the bacterial oxidation of sulfur result in a pH drop?
- What organic sulfur compound is most abundant in nature?

# II • Other Nutrient Cycles

In Part II of this chapter we explore the interactions of microorganisms with metals—in particular iron and manganese—and with some nonmetals whose microbial transformations are of major global significance.

## 21.5 The Iron and Manganese Cycles

Iron (Fe) is one of the most abundant elements in Earth's crust. On the surface of Earth, Fe exists naturally in two oxidation states, ferrous [$Fe^{2+}$, also Fe(II)] and ferric [$Fe^{3+}$, also Fe(III)]. A third oxidation state, $Fe^0$, is abundant in Earth's core and is also a major product of human activities from the smelting of iron ores to form cast iron.

In nature, iron cycles primarily between the $Fe^{2+}$ and $Fe^{3+}$ forms, and these redox transitions are one-electron oxidations and reductions. Ferric iron is reduced both chemically and as a form of anaerobic respiration, and $Fe^{2+}$ is oxidized both chemically and as a form of chemolithotrophic metabolism (**Figure 21.10**). Manganese (Mn), although present at 5- to 10-fold lesser abundance than Fe in the near-surface environment, is another redox-active metal of microbiological significance, existing primarily in two oxidation states ($Mn^{2+}$ and $Mn^{4+}$, see Figure 21.11).

A key feature of the iron and manganese cycles is the different solubilities of these metals in their oxidized versus reduced forms. Reduced iron ($Fe^{2+}$) and manganese ($Mn^{2+}$) are soluble. In contrast, oxidized minerals of iron such as iron oxide-hydroxides [e.g., $Fe(OH)_3$, FeOOH, and $Fe_2O_3$] and manganese oxide ($MnO_2$) are insoluble and tend to settle out in aquatic environments. As a consequence, these strong oxidants can comprise several percent by weight of marine and freshwater sediments, making them among the most abundant of potential electron acceptors in many anoxic systems (see Figure 21.11).

### Bacterial Reduction of Iron and Manganese Oxides

Some *Bacteria* and *Archaea* can use $Fe^{3+}$ as an electron acceptor in anaerobic respiration (Section 14.15). These organisms also commonly have the capacity to use $Mn^{4+}$ as an electron acceptor, and some have the capacity to reduce oxidized uranium (Section 22.3).

Ferric iron and manganese oxide reduction is common in waterlogged soils, bogs, and anoxic lake sediments (**Figure 21.11**). When soluble reduced iron and manganese reach oxic regions, for example, through diffusion from anoxic regions of sediments, they are oxidized chemically [e.g., $Fe^{2+} + \frac{1}{4}\ O_2 + 2\frac{1}{2}\ H_2O \rightarrow Fe(OH)_3 + 2\ H^+$] or microbiologically (Figure 21.10). The chemical oxidation of $Fe^{2+}$ is very rapid at near-neutral pH. Although the spontaneous oxidation of $Mn^{2+}$ is very slow at neutral pH, the rate of oxidation can be increased up to five orders of magnitude by a variety of manganese-oxidizing bacteria and even fungi. The oxidized metal oxides and hydroxides then precipitate, returning the oxidized metals to the sediments where they can again serve as electron acceptors, completing the cycle.

Oxidized iron ($Fe^{3+}$) and manganese ($Mn^{4+}$) are chemically very reactive. Phosphate is trapped as insoluble ferric phosphate precipitates. Chemical oxidation of refractory organic compounds by $Mn^{4+}$ oxide may yield more available sources of carbon for microbial growth. Other metals [e.g., copper (Cu), cadmium (Cd), cobalt (Co), lead (Pb), and arsenic (As)] form insoluble complexes with the iron and manganese oxides. When these oxides are subsequently reduced, the bound phosphate is liberated along with the soluble forms of these metals.

In recent years it has been recognized that the surfaces and appendages of cells of bacteria that interact with iron and manganese oxides, such as *Geobacter*, are electrically conductive, functioning as "nanowires" to move electrons around in microbial habitats. This movement of electrons is a form of electricity, and the process may eventually have commercial applications for power generation (see Explore the Microbial World, "Microbially Wired"). Humic substances (Section 21.1) can also facilitate microbial metal reduction. Since some constituents of humics

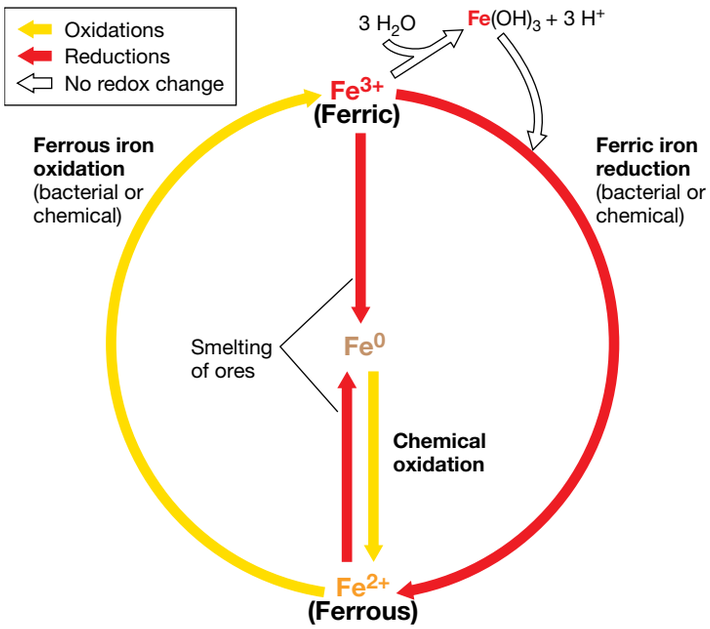



**Figure 21.10 Redox cycle for iron.** The major forms of iron in nature are $Fe^{2+}$ and $Fe^{3+}$. $Fe^0$ is primarily a product of smelting of iron ores. $Fe^{3+}$ forms various minerals such as ferric hydroxide, $Fe(OH)_3$.

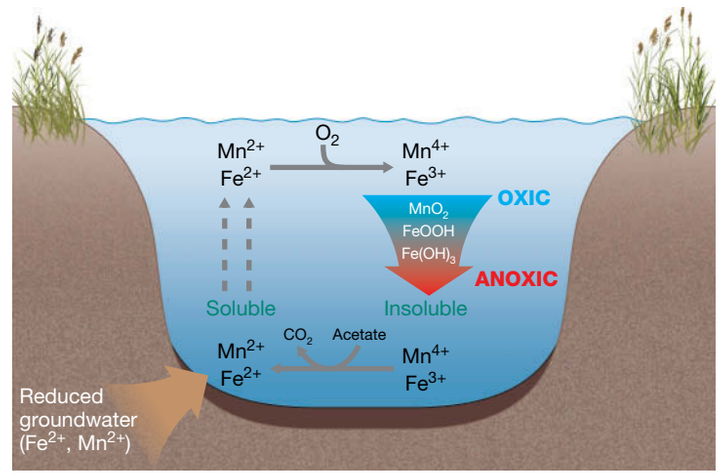



**Figure 21.11 Iron and manganese redox cycling in a typical freshwater system.** Iron and manganese oxides in sediments are used as electron acceptors by metal-reducing bacteria. The resulting reduced forms are soluble and diffuse into the oxic regions of the sediment or water column, where they are oxidized microbially or chemically. Precipitation of the insoluble oxidized metals then returns the metals to the sediments, completing the redox cycle.

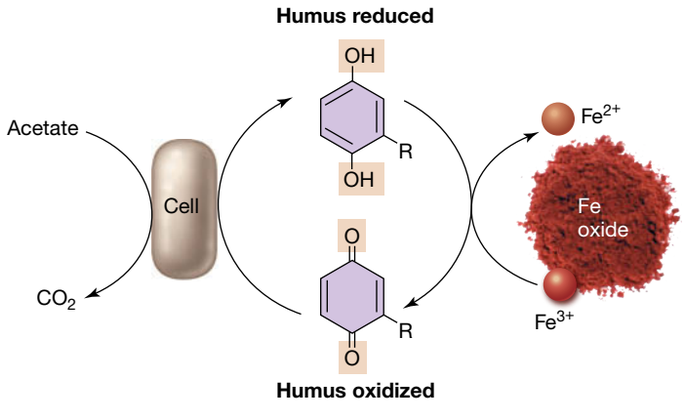



**Figure 21.12 Role of humic substances in humus as an electron shuttle in microbial metal reduction.** Quinone-like functional groups in humus are reduced by acetate-oxidizing bacteria. The reduced humus then donates electrons to metal oxides, releasing reduced soluble iron ($Fe^{2+}$) and oxidized humus. The cycle continues as oxidized humus is again reduced by the bacteria.


**Figure 21.13 Oxidation of ferrous iron ($Fe^{2+}$).** A microbial mat growing in the Rio Tinto, Spain. The mat consists of acidophilic green algae (eukaryotes) and various iron-oxidizing chemolithotrophic bacteria. The Rio Tinto has a pH of about 2 and contains high levels of dissolved metals, in particular $Fe^{2+}$. The red-brown precipitates consist of $Fe(OH)_3$ and other ferric minerals.

can alternate between oxidized and reduced forms, they can function to shuttle electrons from the bacterium to the reduction of the iron or manganese oxides (**Figure 21.12**).

## Microbial Oxidation of Reduced Iron and Manganese

At neutral pH, ferrous iron ($Fe^{2+}$) is rapidly oxidized abiotically in oxic environments. In contrast, at *acidic* pH (pH < 4), $Fe^{2+}$ is not oxidized spontaneously. Thus, much of the research on microbial oxidation of iron is focused on acidic, ferrous-iron-rich habitats, where acidophilic chemolithotrophs such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* oxidize $Fe^{2+}$ to $Fe^{3+}$ (**Figure 21.13**).

The oxidation of $Fe^{2+}$ to $Fe^{3+}$ yields a single electron; consequently very little energy can be conserved (↔ Sections 14.10 and 15.15) and so these bacteria must oxidize large amounts of $Fe^{2+}$ in order to grow. In such environments, even a relatively small population of cells can precipitate a large amount of iron minerals. Although $O_2$ is the most environmentally significant electron acceptor, $Fe^{2+}$ oxidation can also be coupled to $NO_3^-$ reduction by some anaerobic microbes (↔ Sections 14.10 and 15.15) and $Fe^{2+}$ functions as an electron donor in photosynthesis for some anoxygenic phototrophs (↔ Sections 14.10, 15.2, and 15.5). Even though the oxidation of $Mn^{2+}$ to $Mn^{4+}$ is also potentially energetically favorable for growth, and many microorganisms catalyze $Mn^{2+}$ oxidation, as yet no organism has been conclusively shown to derive energy from the oxidation of reduced manganese.

Since the abiotic oxidation of reduced iron is rapid at near-neutral pH, iron-oxidizing bacteria that inhabit nonacidic environments are restricted to a very narrow redox region in which ferrous iron–rich water impinges on oxygenated water (Figure 21.11). These microoxic habitats include freshwater and coastal sediments, slow-moving streams, ferrous iron–rich waters from springs, and hydrothermal vents (**Figure 21.14**). For example, when ferrous iron–rich groundwaters are exposed to air, $Fe^{2+}$ is oxidized at the interface of these two zones by iron-oxidizing bacteria such as *Leptothrix* and *Gallionella* (Figure 21.14*b*, *c*, *d*; ↔ Sections 14.10 and 15.15). Thus, their physiology dictates that they maintain a position within a narrow environment of low levels of $O_2$ and high levels of reduced metals. How these organisms secure and maintain a position within such a narrow range of abiotic conditions is not well understood, but the sheath and stalk structures typically found in iron oxidizers may assist in their proper positioning or attachment (Figure 21.14*b*, *d*, *f*; ↔ Figures 15.36 and 15.58).

As we have seen, organisms that reduce insoluble metal oxides can use extracellular conductors for electron transfer, such as electrically conductive pili or cell-surface-associated cytochromes. However, a similar problem exists for organisms that oxidize metals: Insoluble metal oxides are the product of metal oxidation, and the organism must ensure that these insoluble oxides are deposited external to the cell. Thus, organisms that oxidize $Fe^{2+}$ or $Mn^{2+}$ use surface-associated electron transfer proteins to ensure that metals are oxidized *outside* the cytoplasm. Cytochromes participate in both iron reduction and iron oxidation, and genomes of metal-oxidizing *Gallionella* and *Sideroxydans* species contain genes encoding periplasmic *c*-type cytochromes that resemble those encoding proteins known to reduce metal oxides in *Shewanella*, suggesting that mechanistically similar electron transfer pathways are likely used for both the reduction and oxidation of extracellular metals.

Although possibly sharing similar electron transfer mechanisms, metal-oxidizing bacteria are confronted with another problem—their metabolism could soon encase the cell in an iron oxide shell. To prevent this, metal oxidizers produce extracellular organic material that captures metal oxides and deposits it some distance away from the cell. Some metal oxidizers, such as *Gallionella*, produce extended organic stalks that become encrusted with metal oxides away from the cell (Figure 21.14*d*; see also Figure 15.36). An alternative strategy is used by *Leptothrix* species. These bacteria produce an organic sheath surrounding the cells that becomes encrusted with metal oxides (Figure 21.14*b* and

## MICROBIALLY WIRED

Regardless of the electron acceptor they use, when bacteria respire, they carry out oxidations and reductions that generate electricity. They do this when they oxidize an organic or inorganic electron donor and separate electrons from protons during electron transport. The electrons eventually reduce some electron acceptor and the protons generate the proton motive force.

In any form of respiration, electron disposal is necessary for energy conservation. When the electron acceptor is oxygen ($O_2$), nitrate ($NO_3^-$), or many of the other soluble substances used by bacteria as electron acceptors (↻ Sections 14.13–14.17), the final product diffuses away from the cell. Many bacteria reduce ferric iron ($Fe^{3+}$) as an electron acceptor under anoxic conditions, including the bacterium *Geobacter sulfurreducens* (**Figure 1*a***). However, in contrast to soluble electron acceptors, $Fe^{3+}$ is typically present in nature as an insoluble mineral, such as an iron oxide (Figure 21.12), and thus the reduction of $Fe^{3+}$ occurs *outside* the cell. Under such conditions, the ferric iron functions as an electrical anode, and the bacterial cell facilitates transfer of electrons from the electron donor to the anode.[1]

Research has shown that *Geobacter* forms direct electrical connections with insoluble materials that can either accept or donate electrons. Electron transfer involves cytochromes localized along the length of pili that are generally 10–20 micrometers long (↻ Figure 15.35*c*). These electrically conductive structures function as electrical nanowires, much as copper wire does in a household electrical circuit. Being conductive structures, nanowires can form direct electrical connections with insoluble materials that either accept or donate electrons, or alternatively, nanowires can form connections between cells. In this way, electrons obtained by *Geobacter* from the oxidation of organic electron donors or from $H_2$ can be shuttled to a suitable electron acceptor.

Surprisingly, bacterial electron shuttling can occur over rather large spatial distances, much larger than the cell itself. For example, in microsensor studies (↻ Section 19.9) of hydrogen sulfide ($H_2S$) oxidation in anoxic marine sediments (sulfide is the product of sulfate-reducing bacteria), the oxidation of $H_2S$ deep in the sediments released electrons that reduced $O_2$ at the sediment water interface, some 2.5 cm away (**Figure 1*c***). The electrical conductors in the sediment are filamentous bacteria (**Figure 1*b***) affiliated with the *Desulfobulbaceae* family of sulfate-reducing bacteria (↻ Section 15.9). Although phylogenetically affiliated with *sulfate reducers*, the filamentous bacteria actually function as *sulfide oxidizers*, using $O_2$ as the terminal electron acceptor.[2]

The surface of the filamentous bacterial cells has ridges running along its entire length. Microscopically these ridges appear much like cables, each microbial filament surrounded by 15 to 17 structures 400–700 nm in width that run continuously along the length of the filament (**Figure 1*b***). These structures are implicated in electron transfer from the sulfide oxidized at one end of the filament to the reduction of $O_2$ near the sediment surface at the other end of the filament. Although reminiscent of *Geobacter* nanowires, the mechanism for electron transfer over such large distances is unknown.

In nature, electrical communication between bacterial cells may be a major way by which electrons generated from microbial metabolism in anoxic habitats are shuttled to oxic regions. Moreover, research on the microbiology of the process indicates that this microbial electricity could be harnessed in the form of microbial "fuel cells" that could oxidize toxic and waste carbon compounds in anoxic environments, with the resulting electrons coupled to power generation. In such a scheme, bacteria would be exploited to function as catalysts for diverting electrons from electron donors directly to artificial anodes, with the resulting electrical current being siphoned off to supply a portion of human power needs.

[1] Lovley, D.R. 2006. Bug juice: Harvesting electricity with microorganisms. *Nat. Rev. Microbiol. 4:* 497–508.
[2] Pfeffer, C., et al. 2012. Filamentous bacteria transport electrons over centimetre distances. *Nature 491:* 218–221.



↻ Figure 15.58). In this case the cells can move out of the sheath, leaving the metal oxide crust behind.

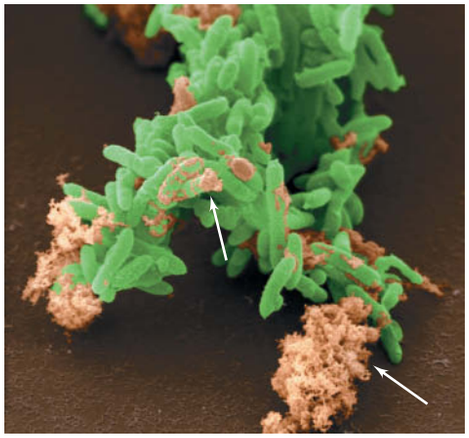
Although not all metal oxidizers produce such morphologically conspicuous structures, it is thought that most, if not all, metal oxidizers are forced to produce some form of extracellular organic material in order to sequester the insoluble product of their energy metabolism. In addition, the incorporation of this organic matter into the metal oxides likely alters the physical and chemical properties of the minerals themselves.

**MINIQUIZ**

- In what oxidation state is Fe in $Fe(OH)_3$? In FeS? How is $Fe(OH)_3$ formed?
- Why does biological $Fe^{2+}$ oxidation under oxic conditions occur mainly at acidic pH?
- Why is excreted organic matter important to many iron oxidizers?

## 21.6 The Phosphorus, Calcium, and Silica Cycles

Many other chemical elements undergo microbial cycling and we focus on three key ones here—phosphorus (P), calcium (Ca), and silica (Si). The cycling of these elements is important in aquatic environments, particularly in the oceans, which are major reservoirs of Ca and Si and where large amounts of Ca and Si are incorporated into the exoskeletons of certain microbes. However, unlike the C, N, and S cycles, in the Ca and Si cycles there are no redox changes or gaseous forms that can escape and alter Earth's atmospheric chemistry, and only recently have different redox states of phosphorus been discovered to be biogeochemically significant. However, as we will see, keeping these cycles in balance—especially that of Ca—is important for maintaining sustainable life on Earth.



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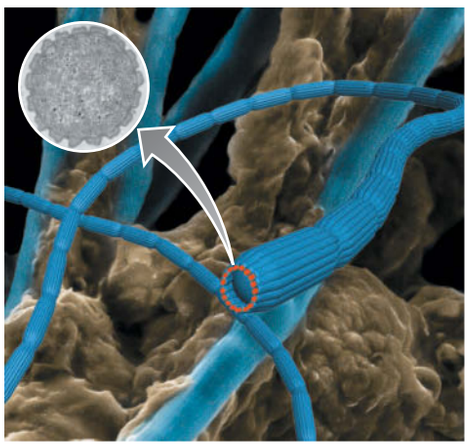

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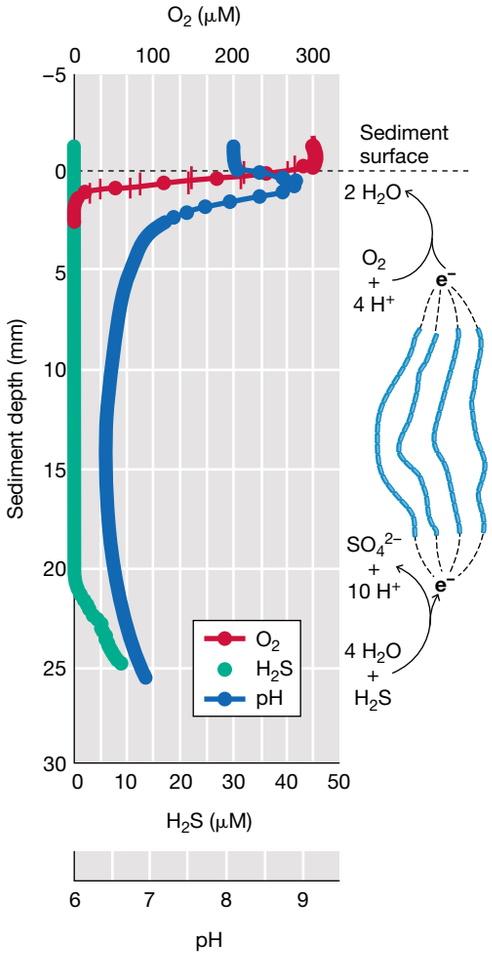


(c)

**Figure 1** *(a)* Cells of *Geobacter* attached to ferric iron precipitates (arrows) reduce $Fe^{3+}$ to $Fe^{2+}$. *(b)* Three-dimensional rendering of cabled filamentous sulfide-oxidizers, with inset transmission electron microscopic cross-section displaying presumptive electrically conductive filaments surrounding the cell perimeter. *(c)* Microsensor profiles of sediments colonized by cabled filamentous bacteria, showing wide separation of depths where sulfide oxidation and oxygen reduction occur. The profiles are explained as follows: Oxygen ($O_2$) is quickly consumed by bacterial respiration in the upper regions of the sediment, whereas $H_2S$, produced in anoxic regions, only accumulates deeper in the sediments. The pH is more acidic lower in the sediments because sulfide oxidation yields protons. As $O_2$ is consumed near the sediment surface, protons are also consumed and the pH rises.



## Phosphorus

Phosphorus exists in nature primarily as organic and inorganic phosphates. Phosphorus reservoirs include phosphate-containing minerals in rocks, dissolved phosphates in freshwaters and marine waters, and the nucleic acids and phospholipids of living organisms. Although P has multiple oxidation states, most environmental phosphates are at the +5 oxidation state (for example, inorganic phosphate, $HPO_4^{2-}$). In nature P cycles through living organisms (as cellular P), waters and soils (as inorganic and organic P), and Earth's crust (as inorganic P). P is typically the limiting nutrient for photosynthesis in freshwaters, which receive it from the weathering of rocks.

In the oceans, a fraction of dissolved P is organic, in the form of phosphate esters and *phosphonates*. Phosphonates are organophosphate compounds that contain a direct bond between the P and C atoms. In this form the P atom is more reduced (+3 oxidation state) than in phosphate (+5 oxidation state). Phosphonates are produced by certain microorganisms and comprise about a quarter of the organic P pool in nature. For many organisms, phosphonates are a less available source of P than is $HPO_4^{2-}$ because the organisms lack the enzymes required to degrade phosphonates. Such organisms can be phosphorus-limited even when sufficient P is present as phosphonates.

Phosphonates and reduced inorganic forms of P—phosphite ($H_2PO_3^-$, +3 oxidation state) and hypophosphite ($H_2PO_2^-$, +1 oxidation state)—are rapidly cycled in the marine environment by producers and consumers. This previously unrecognized P cycle is now thought to be important for organisms that live in P-depleted environments and have the capacity to make or consume these alternative forms of phosphorus. Why marine organisms produce so much of these reduced phosphorus species is unknown. However, the fact that some marine microorganisms produce methylphosphonate may have solved another metabolic conundrum. The degradation of methylphosphonate

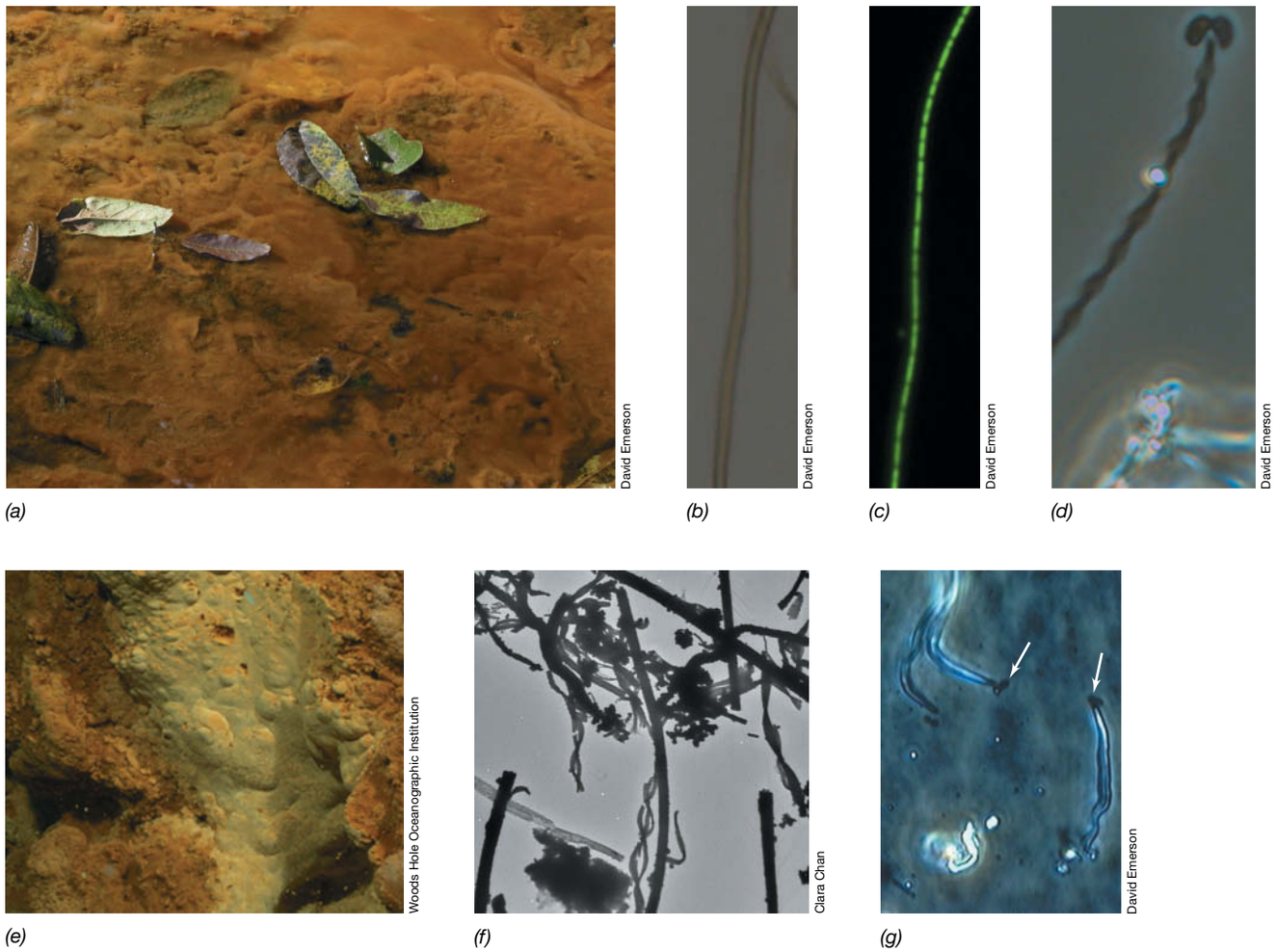


**Figure 21.14 Fe-oxidizing microbial mats.** *(a)* Freshwater microbial mat in a slow-moving stream where $Fe^{2+}$-enriched groundwater is mixing with oxygenated surface water, triggering growth of $Fe^{2+}$-oxidizing bacteria and precipitation of iron oxides. *(b–d)* Fe-oxidizing *Bacteria*. *(b, c)* Phase-contrast and epifluoresence photomicrographs of the sheath-forming Fe-oxidizer *Leptothrix ochracea* (the sheath is approximately 2 μm wide). *(d)* The stalk-forming $Fe^{2+}$-oxidizer *Gallionella ferruginea* showing bean-shaped cells in the process of cell division at the end of the iron oxide–encrusted stalk (each bean-shaped cell is about 2 μm long). *(e)* An iron-oxidizing mat at a deep-sea hydrothermal vent (1000-meter depth) at Lō'ihi Seamount. *(f)* TEM image of biogenic oxides produced at Lō'ihi; note the variety of helical stalks and tubular sheathlike filaments (the filaments vary from 2 to 4 μm wide). *(g)* Phase-contrast photomicrograph of marine $Fe^{2+}$-oxidizers growing at the ends of iron oxide filaments (cells denoted by arrows) from an experimental incubation at Lō'ihi (the filaments are approximately 2 μm wide).



($CH_4O_3P^-$) by some marine microorganisms—a process that liberates $CH_4$—may explain the previously puzzling observation that relatively high levels of $CH_4$ are present in the oxygenated surface waters of the ocean (methanogenic *Archaea* are strict anaerobes; Section 17.2).

## Calcium

The major global reservoirs of calcium (Ca) are calcareous rocks and the oceans. In the oceans, where dissolved Ca exists as $Ca^{2+}$, calcium cycling is a highly dynamic process although the concentration of $Ca^{2+}$ in seawater remains constant at about 10 mM. Several marine eukaryotic phototrophic microorganisms take up $Ca^{2+}$ to form their calcareous exoskeletons; these include in particular the *coccolithophores* and *foraminifera* (**Figure 21.15**; Section 18.6). The calcium-cycling activities of these planktonic phototrophs are also tightly coupled with inorganic components of the carbon cycle.

The precipitation of calcium carbonate ($CaCO_3$) to form the shells of calcareous phytoplankton controls both $CO_2$ flux into ocean surface water and inorganic C transport into deep ocean water and the sediments. Moreover, the formation of $CaCO_3$ both depletes surface dissolved bicarbonate ($HCO_3^-$) and increases the level of dissolved $CO_2$ (Figure 21.15*c*). The latter reduces the influx of atmospheric $CO_2$ into surface ocean waters and this helps maintain the slightly alkaline pH of the oceans. When these calcareous organisms die and sink toward the sediments, $Ca^{2+}$ and inorganic and organic C are transported to the deep ocean from which they are only slowly released over long periods.

The formation of $CaCO_3$ exoskeletons brings into play a delicate balance between $Ca^{2+}$ and C and is a process sensitive to changes in atmospheric $CO_2$ levels. This is because increased levels of atmospheric $CO_2$ increase the formation of carbonic acid ($H_2CO_3$), and as this dissociates to form $HCO_3^-$ and $H^+$, $CaCO_3$ dissolves and


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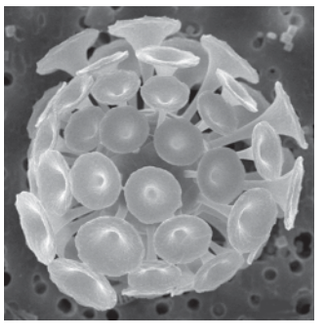


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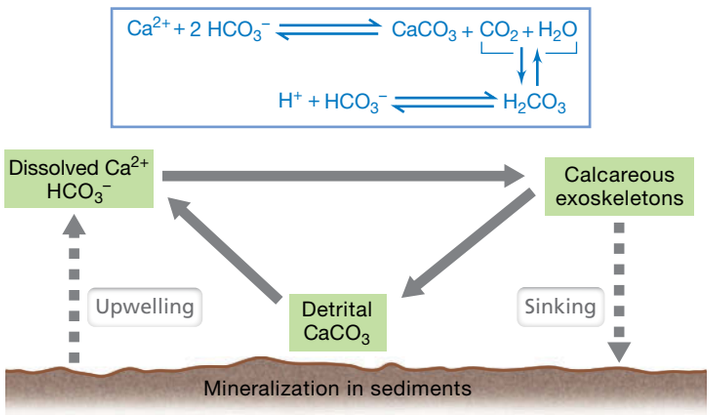
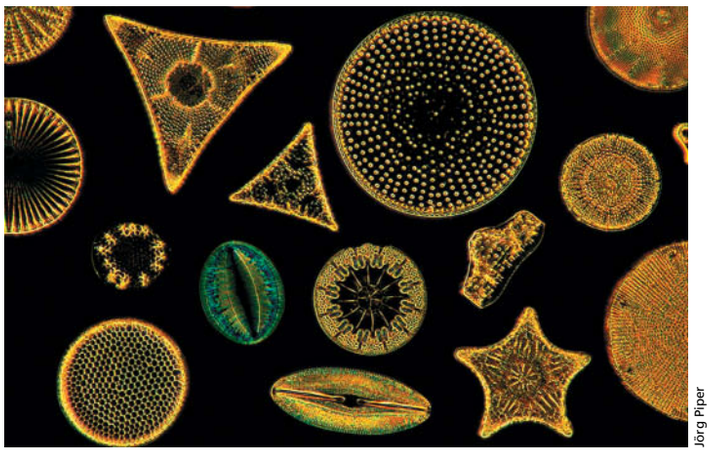



(c)

**Figure 21.15 The marine calcium cycle.** Scanning electron micrographs of cells of the calcareous phytoplankton *(a) Emiliania huxleyi* and *(b) Discosphaera tubifera.* The exoskeletons of these coccolithophores are made of calcium carbonate ($CaCO_3$). A cell of *Emiliana* is about 8 µm wide and a cell of *Discosphaera* is about 12 µm wide. *(c)* The marine calcium cycle; dynamic pools of $Ca^{2+}$ are shaded in green. Detrital $CaCO_3$ is that in fecal pellets and other organic matter from dead organisms. Note how $H_2CO_3$ formation lowers ocean pH when it dissolves to form $H^+$ and $HCO_3^-$.

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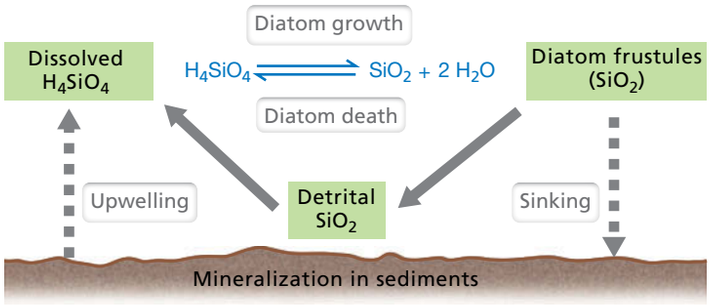



(b)

**Figure 21.16 The marine silica cycle.** *(a)* Dark-field photomicrograph of a collection of diatom shells (frustules). The frustules are made of $SiO_2$. *(b)* The marine silica cycle; dynamic pools of Si are shaded in green.

seawater pH decreases (Figure 21.15*c*). Greater ocean acidity resulting from rising atmospheric $CO_2$ is predicted to reduce the rate of formation of calcareous shells, which will likely have effects on other microbial nutrient cycles and plant and animal communities (Section 21.8).

## Silica

The marine Si cycle is controlled primarily by unicellular eukaryotes (diatoms, silicoflagellates, and radiolarians) that build ornate external cell skeletons called *frustules* (**Figure 21.16*a***) (↩ Sections 18.5 and 18.6). These structures are not constructed of $CaCO_3$ as in the coccolithophores, but of opal ($SiO_2$), whose formation begins with the uptake by the cell of dissolved silicic acid (Figure 21.16*b*).

Diatoms are rapidly growing phototrophic eukaryotes and often dominate blooms of phytoplankton in coastal and open ocean waters. However, unlike other major phytoplankton groups, diatoms require Si and can become silica-limited when blooms develop. Also, because of their large size, diatom cells tend to sink faster than other organic particles, and in this way, they contribute significantly to the return of Si and C to deeper ocean waters. The transport of organic material produced through primary production in near-surface waters to deeper ocean waters, primarily by sinking particles, is called the *biological pump* and is an important aspect of the carbon cycle in terms of carbon burial and mineralization in marine environments (Figure 21.1).

In addition to the major nutrient requirements of any phototrophic organism ($CO_2$, N, P, Fe), diatoms require sufficient dissolved Si, and in nature this originates primarily from Si released from the skeletons of dead diatoms (Figure 21.16*b*). Although Si is released fairly rapidly following cell death, during periods of high diatom production in relatively shallow waters, a significant fraction of dissolved Si can be buried in sediments and remain there for millions of years. This has consequences for continued diatom growth and their phototrophic consumption of dissolved $CO_2$ from ocean waters. The flux of $CO_2$ into and out of ocean water affects its pH (Figure 21.15*c*), and through this link, the Si and C cycles are coupled in similar fashion to what we have seen with the Ca and C cycles.

### MINIQUIZ

- How does the formation of $CaCO_3$ skeletons by calcareous phytoplankton retard $CO_2$ uptake and help maintain ocean water pH?
- How might Si depletion in the photic zone influence the biological pump?

# III • Humans and Nutrient Cycling

Humans have a profound impact on microbial nutrient cycles by adding and removing specific components of the cycles in large amounts. Here we consider human inputs of three major species: mercury (Hg), $CO_2$ and other atmospheric gases, and various fixed N compounds. These compounds either cause toxicity problems (Hg) or affect planet Earth in globally significant ways (gases and N compounds). We begin with the very toxic metal Hg, which is transformed by bacteria in many different ways.

## 21.7 Mercury Transformations

Mercury is not a biological nutrient (Figure 3.1), but microbial transformations of various mercuric compounds help to detoxify some of the most toxic forms. Mercury is a widely used industrial product, especially in the electronics industry. Mercury is also an active ingredient in many pesticides, a pollutant from the chemical and mining industries and from the combustion of coal and municipal wastes, and a common contaminant of aquatic ecosystems and wetlands. Because of its propensity to concentrate in living tissues, Hg is of considerable environmental importance. The major form of Hg in the atmosphere is elemental mercury ($Hg^0$), which is volatile and is oxidized to mercuric ion ($Hg^{2+}$) photochemically. Most Hg thus enters aquatic environments as $Hg^{2+}$ (**Figure 21.17**).

### Microbial Redox Cycle for Mercury

Mercuric ion readily adsorbs to particulate matter, and when deposited in anoxic environments, such as lake or marine sediments, $Hg^{2+}$ can be metabolized from there by anaerobic microbes. Microbial activity methylates Hg, yielding *methylmercury* ($CH_3Hg^+$) (Figure 21.17), and this can be further methylated to form *dimethylmercury* ($CH_3—Hg—CH_3$). Methylmercury and dimethylmercury are extremely toxic to animals because they are readily absorbed through the skin and are potent neurotoxins. But in addition, $CH_3Hg^+$ is soluble and can be concentrated in the food chain, primarily in the muscle tissues of fish, and its concentration increases with each trophic level (a process called *biomagnification*), causing a threat to humans whose diets rely on fish. Methylmercury is about 100 times more toxic than $Hg^0$ or $Hg^{2+}$, and its accumulation in the aquatic food chain seems to be particularly acute in freshwater lakes and marine coastal waters where enhanced levels of $CH_3Hg^+$ have been detected in fish caught for human consumption. Mercuric compounds can cause liver and kidney damage in humans and other animals.

Methylation was long associated with sulfate-reducing and iron-reducing bacteria, but only recently have the contributing enzyme systems been identified. Methylation by sulfate-reducing bacteria requires two genes, *hgcA* (encoding a putative methyltransferase corrinoid protein) and *hgcB*, encoding a putative [4Fe-4S] ferredoxin (Figure 21.17). In the proposed reaction sequence (Figure 21.17), a methyl group is transferred from the methylated HgcA protein to

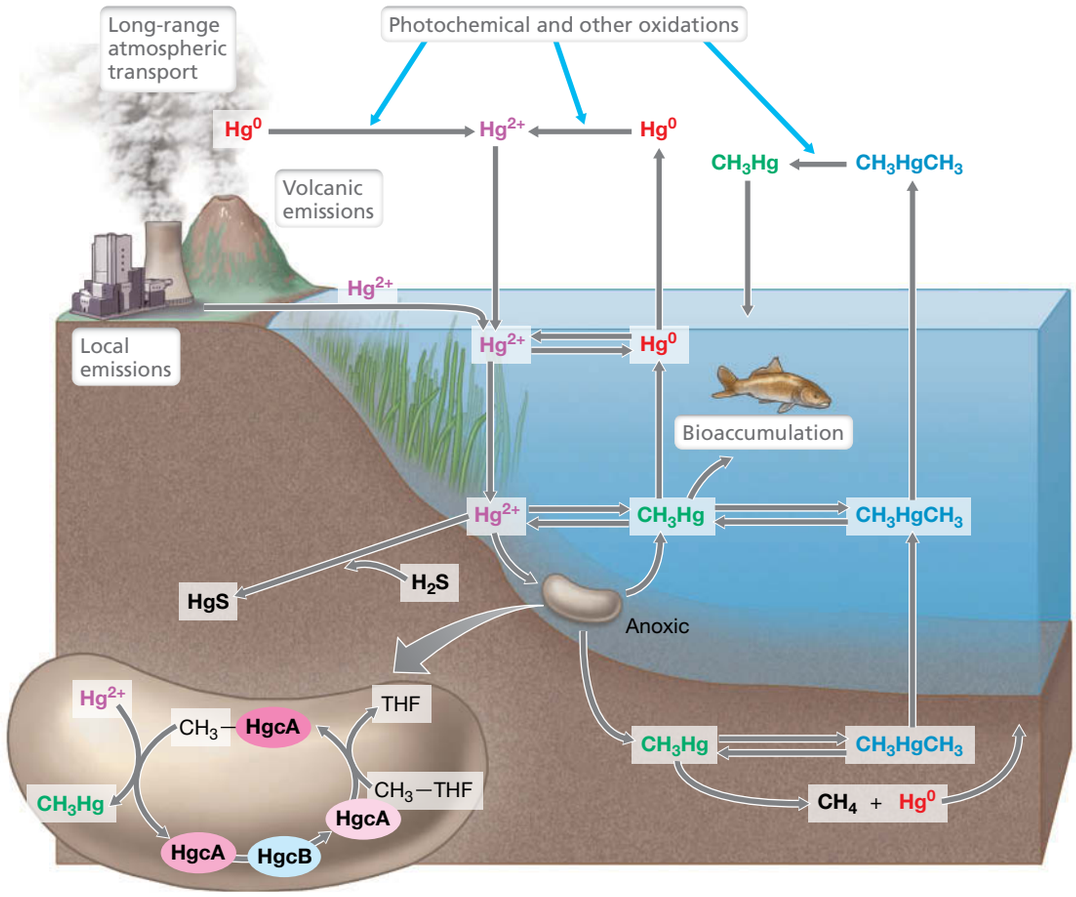



**Figure 21.17 Biogeochemical cycling of mercury.** The major reservoirs of mercury are water and sediments. Mercury in water can be concentrated in animal tissues; it can be precipitated as HgS from sediments. The volatile forms of mercury are $Hg^0$ and $CH_3HgCH_3$. The enlarged bacterial cell shows the enzyme system responsible for mercury methylation by sulfate-reducing bacteria (Section 21.7). Common forms of mercury are shown by different colors. THF, tetrahydrofolate. HgcA and HgcB are proteins that function to methylate mercury.

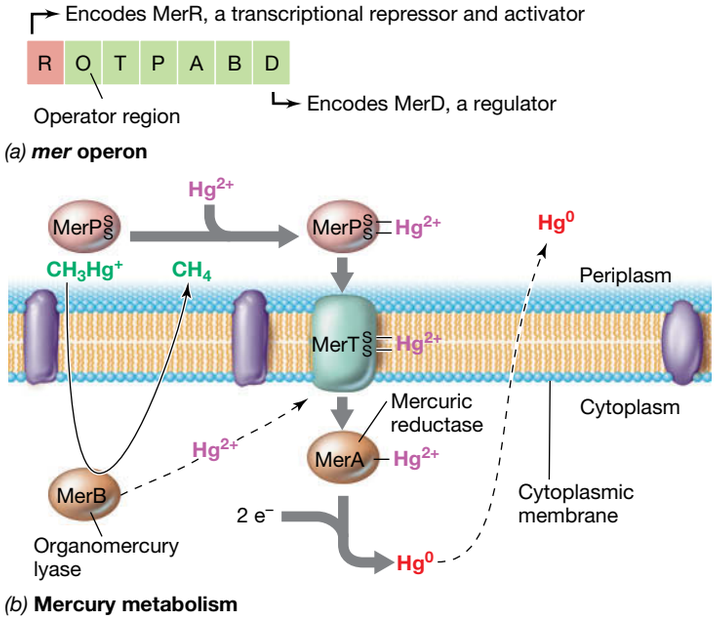



**Figure 21.18 Mechanism of mercury transformations and resistance.** *(a)* The *mer* operon. MerR can function as either a repressor (in the absence of $Hg^{2+}$) or a transcriptional activator (in the presence of $Hg^{2+}$). *(b)* Transport and reduction of $Hg^{2+}$ and $CH_3Hg^+$; the $Hg^{2+}$ is bound by cysteine residues in the MerP and MerT proteins. MerA is the enzyme mercuric reductase and MerB is organomercury lyase.

inorganic $Hg^{2+}$. The HgcB protein then regenerates the reduced form of HgcA required for accepting the methyl group from methyltetrahydrofolate (THF). Identification of the genes encoding these enzymes provided the opportunity to survey their distribution in available genomic and metagenomic sequences, and this has revealed that genes for mercury methylation are widely distributed in both *Bacteria* and methanogenic *Archaea*. The discovery of mercury methylation genes not only paves the way for developing genetic tools for surveying environments for the potential to methylate mercury, but will contribute to our understanding of the physiological significance of methylation (hypothesized to be used for detoxification). It also allows for the identification of environmental variables that control gene expression and could possibly provide insight into the observation that mercury methylation also occurs in oxic marine surface waters.

Several other microbial Hg transformations occur in anoxic sediments, including reactions catalyzed by sulfate-reducing bacteria ($H_2S + Hg^{2+} \rightarrow HgS$) and methanogens ($CH_3Hg^+ \rightarrow CH_4 + Hg^0$) (Figure 21.17). The solubility of mercuric sulfide (HgS) is very low, so in sulfidic sediments, most Hg exists as HgS. But upon aeration, HgS can be oxidized to $Hg^{2+}$ and $SO_4^{2-}$ by metal-oxidizing bacteria (Section 21.5), and the $Hg^{2+}$ is eventually converted to $CH_3Hg^+$. However, it is not the Hg in HgS that is oxidized here, but instead the *sulfide*, probably by organisms related to *Acidithiobacillus* (Section 15.11).

### Mercury Resistance

At sufficiently high concentrations, $Hg^{2+}$ and $CH_3Hg^+$ can be toxic to microorganisms as well as to macroorganisms. However, several gram-positive and gram-negative bacteria convert toxic forms of Hg to nontoxic or less toxic forms. These mercury-resistant bacteria employ the enzyme *organomercury lyase* to degrade the highly toxic $CH_3Hg^+$ to $Hg^{2+}$ and methane ($CH_4$), and the NADPH (or NADH)-linked enzyme *mercuric reductase* to reduce $Hg^{2+}$ to $Hg^0$, which is volatile and thus mobile (Figure 21.18).

In many mercury-resistant bacteria, genes encoding Hg resistance reside on plasmids or transposons (Sections 4.2 and 11.11). These *mer* genes are arranged in an operon under control of the regulatory protein MerR, which can function as either a repressor or an activator of transcription (Sections 6.2 and 6.3), depending on Hg availability. In the absence of $Hg^{2+}$, MerR functions as a *repressor* and binds to the operator region of the *mer* operon, thus preventing transcription of the structural genes, *merTPABD*. However, when $Hg^{2+}$ is present, it forms a complex with MerR, which then binds to the *mer* operon and functions as an *activator* of transcription of *mer* structural genes (Figure 21.18).

MerP is a periplasmic mercuric ion–binding protein; it binds $Hg^{2+}$ and transfers it to the membrane transport protein MerT, which interacts with mercuric reductase (MerA) to reduce $Hg^{2+}$ to $Hg^0$ (Figure 21.18*b*). Thus, $Hg^{2+}$ is not released into the cytoplasm and the final result is the release of $Hg^0$ from the cell. Mercuric ion produced from the activity of MerB is trapped by MerT and reduced by MerA, again releasing $Hg^0$ (Figure 21.18*b*). In this way, $Hg^{2+}$ and $CH_3Hg^+$ are converted to the relatively nontoxic $Hg^0$.

**MINIQUIZ**

- What forms of mercury are most toxic to organisms?
- How is mercury methylated by microbes?
- How is mercury detoxified by bacteria?

## 21.8 Human Impacts on the Carbon and Nitrogen Cycles

Human activities have major effects on the carbon and nitrogen cycles, and these effects have significance for the health of our planet in general. The period of marked human influence on these nutrient cycles began with the Industrial Revolution and is informally termed the *Anthropocene*, a new geological epoch. Although the greatest human impacts have been on the release of $CO_2$ through the burning of fossil fuels (oil, gas, and coal) and from extensive and ongoing deforestation, human activity has also profoundly affected the nitrogen cycle. We discussed earlier the close coupling of the carbon and nitrogen cycles (Section 21.1), and here we consider some of the projected biogeochemical consequences of human alteration of these two critical nutrient cycles.

### $CO_2$, Other Trace Gases, and Global Warming

Atmospheric $CO_2$ levels have increased approximately 40% since the beginning of the Industrial Revolution in the 1800s, and are now higher than at any time in the last 800,000 years. Carbon dioxide is one of several *trace gases* (primarily water vapor, $CO_2$, $CH_4$, and $N_2O$) which comprise less than 0.5% of the atmosphere but contribute significantly to terrestrial and atmospheric warming due to the *greenhouse effect*, the ability of these gases to trap the infrared radiation emitted by the Earth. Atmospheric concentrations of all

of these trace gases are rapidly increasing as a consequence of human activities (**Figure 21.19**). The change in global warming potential resulting from the addition of these gases to the atmosphere is expressed as **radiative forcing**, defined as the difference between sunlight energy absorbed by Earth and energy radiated back to space, measured in watts per square meter of Earth's surface ($W/m^{-2}$) (Figure 21.19*d*). Radiative forcing is used to calculate an Annual Greenhouse Gas Index (AGGI), defined as the ratio of the total direct radiative forcing due to long-lived greenhouse gases for a given year to that which was present in 1990 (the baseline year for the Kyoto Protocol for controlling greenhouse gas emissions).

In 2014 the AGGI was 1.36 (an increase in radiative forcing of 36% since 1990). Climate models incorporate both the radiative forcing values and the atmospheric lifetimes of the major greenhouse gases. Although methane and $N_2O$ have greater radiative forcing per unit molecule than $CO_2$ (58- and 206-fold, respectively), they are at much lower concentrations and therefore contribute less to warming (Figure 21.19*d*). $CO_2$ is the major contributor to the AGGI in terms of both amount and rate of increase. The atmospheric lifetime of a molecule of $N_2O$ is around 150 years. In contrast, there is more uncertainty about the atmospheric lifetimes of methane and $CO_2$, now estimated at about 10 and 120 years, respectively. The long lifetimes of $N_2O$ and $CO_2$ mean that once these gases are added to the atmosphere they will impact Earth's climate for centuries.

Also shown in Figure 21.19*d* is radiative forcing attributed to chlorofluorocarbons (CFCs). There is no natural source of CFCs. These compounds were chemically synthesized for use as refrigerants, aerosol propellants, and cleaning solvents. Following the discovery that they were destroying stratospheric ozone, their manufacture was effectively suspended through an international agreement, the Montreal Protocol on substances that deplete the ozone layer, which entered into force on January 1, 1989. As a result, levels of major CFCs are now constant or declining. Although their long atmospheric lifetimes mean that some CFCs will remain in the atmosphere for over 100 years, the Montreal Protocol offers some hope that nations can come together to address shared environmental threats.

## $CO_2$ and Its Effects on Aquatic Microbial Ecosystems

The increase in atmospheric $CO_2$ concentration, measured across a global network of sampling stations (Figure 21.19*a*), is currently about 2 parts per million per year. This increase would be much more rapid were it not for the high solubility of $CO_2$ in water, which produces carbonic acid; much anthropogenic $CO_2$ thus dissolves in the oceans (Figures 21.1 and 21.15*c*). The surface waters of the oceans have taken up an estimated 500 billion tons of $CO_2$ from the atmosphere out of a total of 1300 billion tons of total anthropogenic emissions, thus modulating the greenhouse effect somewhat. The increase in average Earth air temperature, estimated to have increased 0.75°C in the twentieth century and projected to increase by anywhere from 1.1 to 6.4°C in the twenty-first century, would also have been more rapid without the buffering influence of the oceans. Since three orders of magnitude more energy is required to raise the temperature of a cubic meter of water than a cubic meter of air, it can be calculated that over 80% of the heat retained on Earth as a result of the greenhouse effect thus far has actually entered the ocean.

Although there is considerable uncertainty about the consequences of ocean warming and $CO_2$ consumption on Earth's biological systems, there is agreement on how these changes will affect biogeochemistry. Warmer ocean surface waters are more buoyant (because of their lower density) than are deeper waters. Thus, as occurs seasonally in lakes (Section 20.8), the oceans will become more stratified with future global warming. Stratification tends to slow the transfer of nutrients from deeper waters that are needed to nourish phototrophic microbes at the base of the food web in surface waters. This in turn reduces ocean productivity and export of a portion of that production to the deeper ocean through sedimentation (the biological pump, Figure 21.1).

Ocean warming is also contributing to the expansion of oxygen minimum zones (OMZs), regions of naturally occurring low $O_2$ concentration in subsurface waters between 100 and 1000 m in depth (Section 20.9). OMZs are a consequence of both the reduced solubility of $O_2$ in warmer water and the increasing stratification associated with surface warming, which reduces mixing of

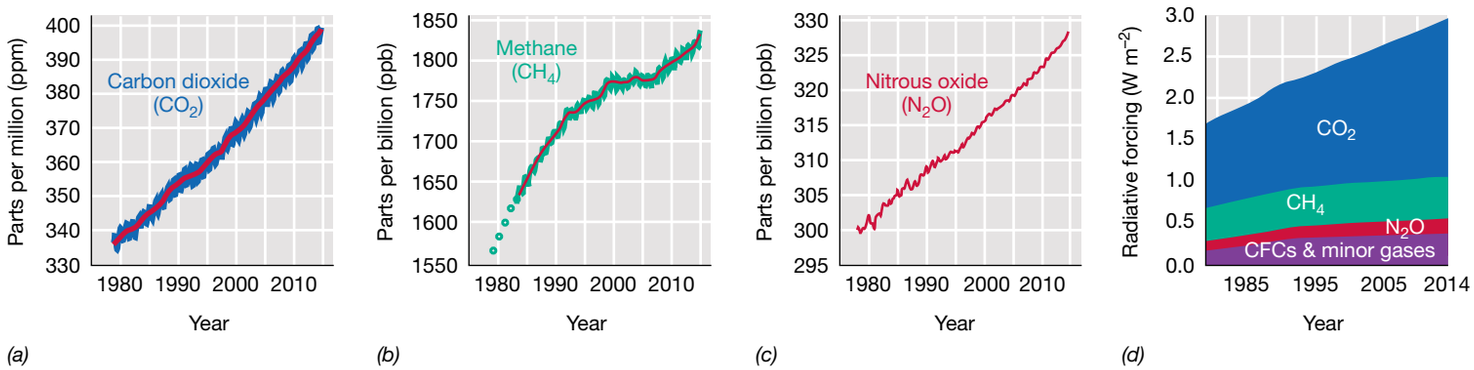



**Figure 21.19 Past 35-year increases in greenhouse gases and associated radiative forcing.** Global average values for *(a)* $CO_2$, *(b)* $CH_4$, *(c)* $N_2O$. Increases in $CO_2$ have averaged 1.4 ppm per year before 1995 and 2 ppm per year thereafter. *(d)* Radiative forcing (see text for definition) from chlorofluorocarbons (CFCs) is now stable or declining as a result of the Montreal Protocol banning production of substances that deplete the ozone layer. These data are continuously collected by the Global Monitoring Division of the National Oceanic and Atmospheric Association/Earth System Research Laboratory.

surface and subsurface waters. Animals will be excluded from the expanding OMZs whereas anaerobic microbial processes, such as denitrification and anammox, that directly influence the nitrogen cycle and production of the greenhouse gas $N_2O$, will be enhanced.

Acidification of the ocean resulting from the ongoing dissolution of anthropogenic $CO_2$ has reduced ocean pH by 0.1 pH units since the beginning of the Industrial Revolution and may further reduce the pH by 0.3–0.4 units by the year 2100. The ongoing reduction in carbonate ($CO_3^{2-}$) concentration, a consequence of increasing acidification, is expected to be detrimental to marine calcifiers (organisms synthesizing $CaCO_3$ shells or skeletons, Figure 21.15). Since the concentration of Ca in seawater is relatively constant, continued reduction in $CO_3^{2-}$ will ultimately reach a point where the dissolution of existing $CaCO_3$ is chemically favored, ultimately releasing more dissolved $CO_2$ (Figure 21.15), which reduces the capacity of the oceans to absorb more atmospheric $CO_2$.

Although the biological response to ocean acidification is unknown, it is likely that coral reef ecosystems, a major component of the marine biosphere (Section 23.11), will cease to occur naturally on Earth if $CO_2$ emissions continue at their present rate (Figure 21.19*a*). Calcification in foraminifera (Section 18.6) will likely be impaired significantly by ocean acidification, as will calcification in coccolithophores (Figure 21.15). Over periods of a century or so, the invasion of anthropogenic $CO_2$ into the deep ocean will ultimately result in a significant reduction in the levels of $CaCO_3$ sequestered there, and this will likely affect the carbon cycle in major but as yet unpredictable ways.

## Methane and Global Warming

Increases in atmospheric methane have contributed to about one-fifth of the increase in radiative forcing since 1750. About two-thirds of this increase is related to industrial activities (e.g., coal mining, natural gas wells, pipelines, and fracking). Major natural sources of methane are wetlands, ruminants, thawing permafrost, and methane hydrates (Figures 21.1, 21.3, and 21.4). Although increases in atmospheric methane slowed during the 1990s, and were nearly constant between 1999 and 2006, strong growth resumed in 2007 (Figure 21.19*b*). Analysis of the stable isotopic composition of new atmospheric methane (Section 19.10) suggests that recent growth is from increased release from tropical wetlands. Wetlands are now the world's largest natural source of atmospheric methane, and increased methane release has been attributed to recent changes in tropical climate.

Ongoing warming will also increase atmospheric inputs of methane by destabilizing coastal methane hydrates (Figure 21.4) and by the melting of permafrost (see page 687 for more on this problem). That is, there is *positive climate feedback* linked to methane release. Permafrost contains about 50% of the global soil carbon, and thawing will lead to a loss of soil carbon in the form of methane and carbon dioxide. The positive climate feedback of these new greenhouse gas emissions is unknown and may depend on the microbial community composition controlling organic matter decomposition. For example, recent studies in the Arctic have documented changes in methanogenic community structure (from $H_2$-oxidizing to partly acetate-oxidizing methanogens) as a function of changes in vegetation patterns and permafrost melting. Thus, a refined understanding of the microbial sources of methane in a warming Arctic will likely be essential to developing predictive models of further climate change.

An additional positive climate feedback is associated with the rapid loss of summer sea ice in the Arctic Ocean, reduced by more than 11% since 1979. Instead of reflecting back most sunlight as ice does, the sea ice melt has opened a new expanse of dark open water to absorb the sun's energy. This extra energy input, and associated flux of moisture and heat to the Arctic atmosphere, is contributing to a strong local positive feedback called the *Arctic amplification*. Surface temperatures in the Arctic are rising twice as fast as at lower latitudes, and will influence methane release from hydrates and permafrost. Reduction of the temperature differential between the Arctic and global midlatitudes may also be contributing to recent changes in weather patterns. The reduced temperature differential is associated with weakening of west-to-east winds and a slowing of the jet stream, causing the jet stream to "meander" deeper south. In turn, these meanders have been linked to the increasing occurrence of extreme weather events in midlatitude regions.

## Anthropogenic Effects on the Nitrogen Cycle

Anthropogenic impacts on the microbial ecology of the nitrogen cycle are as profound as those on the carbon cycle. The yearly industrial production of nitrogenous fertilizers through the Haber–Bosch process, which combines $N_2 + H_2$ to form $NH_3$ under high temperature and pressure, is now comparable to the amount of fixed nitrogen entering the biosphere through biological nitrogen fixation, a key link in the nitrogen cycle (Section 21.3). Most of the industrially produced N is applied to farmland, but a significant fraction runs off to the oceans and contributes to coastal eutrophication (Section 20.9). Large amounts are also lost as gaseous nitrogen compounds ($N_2$, $N_2O$, and NO), primarily from nitrification of $NH_3$ and denitrification of $NO_3^-$ (Section 21.3). $N_2O$ emission is now increasing at a rate of 0.2–0.3% per year (Figure 21.19*c*). Agriculture—including the microbial breakdown of manure and urine—contributes about 80% of $N_2O$ emissions in the United States. Lesser amounts come from motor vehicle emissions, and industrial production of fertilizers and nitrogen-based polymers.

Transport of N from industrial and agricultural centers through the atmosphere fertilizes both terrestrial and marine systems. Atmospheric deposition of industrially sourced fixed nitrogen to the oceans is now about the same as that which enters through biological nitrogen fixation. The ecological consequences of this fertilization are a major unknown. On the one hand, if deposition suppresses microbial nitrogen fixation, this would to some degree mitigate the fertilization effect. On the other hand, a greater supply of both $CO_2$ and iron (caused by greater deposition of dust from areas of increasing desertification, Section 20.6) along with increased N depositions could enhance primary production, since iron is also often a limiting nutrient. Either way, major effects on the carbon cycle should be expected from human inputs in the nitrogen cycle.

Although changes in Earth's biosphere from human intervention in microbial nutrient cycles are a certainty, precisely what these changes will be is less clear. However, because major nutrient cycles are closely coupled (Section 21.1 and Figure 21.5), it is likely

that the significant changes already in play in the carbon and nitrogen cycles will have feedback effects on other nutrient cycles as well. Collectively, these events could upset the balance and interrelationships of Earth's nutrient cycles in general—cycles driven by the activities of microbial communities keenly attuned to their environments—and have significant (and likely negative) consequences for plants, animals, and humans.

**MINIQUIZ**

- What is the greenhouse effect and what causes it?
- What is the fate of most nitrogen used in agricultural applications?
- Why are the OMZs expanding and what are the likely impacts on nutrient cycles?



# Chapter Review

## I • Carbon, Nitrogen, and Sulfur Cycles

**21.1** The oxygen and carbon cycles are interconnected through the complementary activities of autotrophic and heterotrophic organisms. Microbial decomposition is the single largest source of $CO_2$ released to the atmosphere.

**Q What metabolic class of microorganisms are considered primary producers and where are they typically found in nature?**

**21.2** Under anoxic conditions, organic matter is degraded to $CH_4$ and $CO_2$. Methane is formed primarily from the reduction of $CO_2$ by $H_2$ and from acetate, both supplied by syntrophic bacteria; these organisms depend on $H_2$ consumption as the basis of their energetics.

**Q How can organisms such as *Syntrophobacter* and *Syntrophomonas* grow when their metabolism is based on thermodynamically unfavorable reactions? How does coculture of these syntrophs with certain other bacteria allow them to grow?**

**21.3** The principal form of nitrogen on Earth is $N_2$, which can be used as a N source only by nitrogen-fixing bacteria. Ammonia produced by nitrogen fixation or by ammonification can be assimilated into organic matter or oxidized to $NO_3^-$. Denitrification and anammox cause major losses of fixed nitrogen from the biosphere.

**Q Compare and contrast the processes of nitrification and denitrification in terms of the organisms involved, the environmental conditions that favor each process, and the changes in nutrient availability that accompany each process.**

**21.4** Bacteria play major roles in both the oxidative and reductive sides of the sulfur cycle. Sulfur- and sulfide-oxidizing bacteria produce $SO_4^{2-}$, whereas sulfate-reducing bacteria consume $SO_4^{2-}$, producing $H_2S$. Because sulfide is toxic and reacts with various metals, $SO_4^{2-}$ reduction is an important biogeochemical process. Dimethyl sulfide is the major organic sulfur compound of ecological significance in nature.

**Q If sulfur chemolithotrophs had never evolved, would there be a problem in the microbial cycling of sulfur compounds? What impact would coastal marine eutrophication have on microbial sulfur transformations?**

## II • Other Nutrient Cycles

**21.5** Iron and manganese exist naturally in two oxidation states: $Fe^{2+}/Fe^{3+}$ and $Mn^{2+}/Mn^{4+}$. Bacteria reduce the oxidized metals in anoxic environments and oxidize the reduced forms primarily in oxic environments. At neutral pH, bacteria compete with abiotic oxidation in the presence of $O_2$.

**Q Why are most iron-oxidizing chemolithotrophs obligate aerobes, and why are the better-studied iron oxidizers acidophilic?**

**21.6** P, Ca, and Si are elements cycled by microbial activities, primarily in aquatic environments. Calcium and silica play important roles in the biogeochemistry of the oceans as components of the exoskeletons of coccolithophores and diatoms, respectively.

**Q In what ways are Ca and Si cycling in ocean waters similar, and in what ways do they differ? How do the calcium and silica cycles couple to the carbon cycle?**

## III • Humans and Nutrient Cycling

**21.7** A major toxic form of Hg in nature is $CH_3Hg^+$, which can yield $Hg^{2+}$ and later reduced by bacteria to $Hg^0$. Genes conferring resistance to the toxicity of Hg include those encoding enzymes that detoxify or pump out the metal.

**Q Where do Hg resistant genes reside? How are they controlled?**

**21.8** Anthropogenic inputs of $CO_2$ and reactive nitrogen are impacting major nutrient cycles. Although some consequences are reasonably well understood, including expansion of OMZs and impaired growth of calcareous organisms, the long-term changes to the nutrient cycles that sustain Earth's biosphere are not well understood.

**Q Describe the Haber-Bosch process to make fertilizers and the relative amounts of fixed nitrogen it produces.**

## Application Questions

1. Compare and contrast the carbon, sulfur, and nitrogen cycles in terms of the physiologies of the organisms that participate in the cycle. Which physiologies are part of one cycle but not another?
2. $^{14}C$-labeled cellulose is added to a vial containing some anoxic freshwater lake sediments and sealed under anoxic conditions. A few hours later, $^{14}CH_4$ appears in the vial. Discuss what has happened to yield such a result.
3. Carbon can be sequestered in the ocean in a variety of forms. Discuss the different forms, their biological sources, and how global warming will influence them.

## Chapter Glossary

**Denitrification** the biological reduction of nitrate ($NO_3^-$) to gaseous N compounds

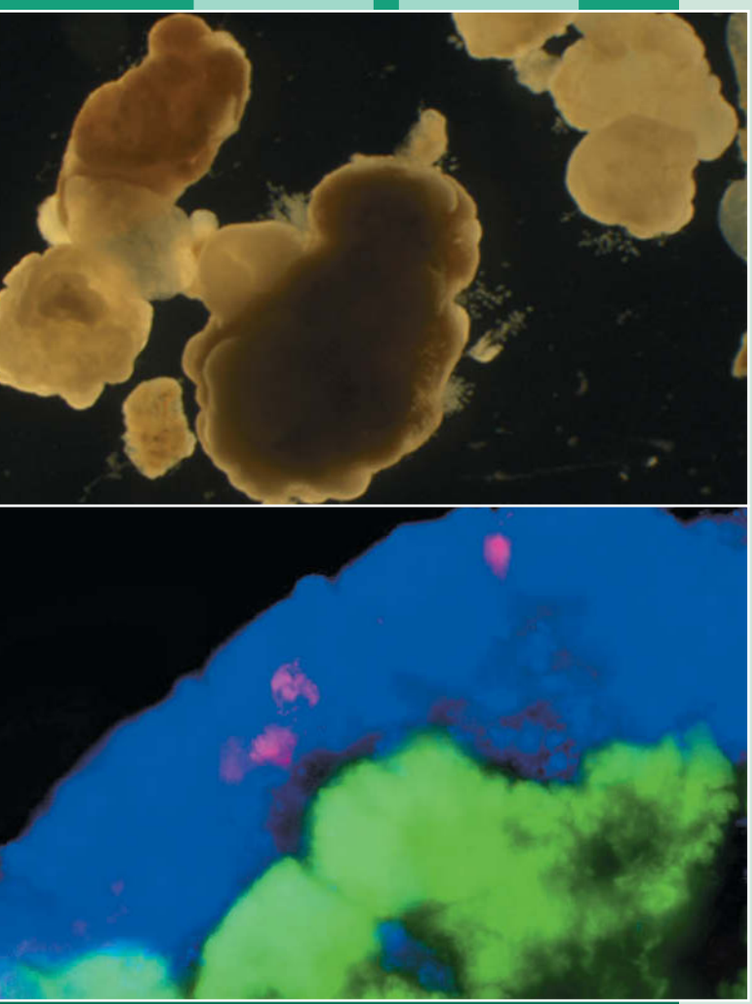
**Global warming** the predicted and ongoing warming of the atmosphere and oceans attributed to anthropogenic release of greenhouse gases, primarily $CO_2$, that trap infrared radiation emitted by Earth

**Humus** dead organic matter, some of which functions as electron shuttles for the microbial reduction of metal oxides

**Radiative forcing** the difference between sunlight energy absorbed by Earth and energy radiated back to space

**Syntrophy** the cooperation of two or more microorganisms to degrade anaerobically a substance neither can degrade alone

# 22 Microbiology of the Built Environment





## microbiologynow

### After the Toilet Flushes

Few give much thought to the fate of water leaving the toilet and the importance of sanitation technology in reducing the spread of infectious disease. However, sewage treatment technology is a critical part of our built environment without which large urban centers would simply not be possible.

Most wastewater treatment plants rely on a technology developed over a century ago called *activated sludge*, a process in which microbial degradation of organic material in sewage is promoted by vigorously mixing sewage in large, well-aerated reactor tanks. The resulting flocculated microbial biomass (sludge) is allowed to settle by gravity in large settling tanks, and the treated water is returned to the environment or directed to additional treatment processes. Both pathogenic microbes and most organic materials are removed in the activated sludge process.

Human population growth and demands for a more sustainable society have prompted the development of wastewater treatment systems that are simpler, more energy efficient, and require less space. A new wastewater treatment process called *aerobic granular sludge technology* (AGS) relies on microbes selected to grow as compact 1- to 2-millimeter granules (upper photo), rather than as flocs. The dense granules settle ten times faster than flocs (eliminating the need for large settling tanks), function in smaller reactors (a reduction of up to 80% in the wastewater built environment is possible), and generate a smaller volume of sludge needing disposal.

The slow diffusion of oxygen into the granules results in the formation of layered structures (lower photo, thin section of a FISH-stained granule) in which aerobes localize to the outer layer and anaerobes to the interior, and this makes the single reactor system ideal for the complete removal of nitrogen from wastewater. For example, aerobic ammonia-oxidizers (blue) in the outer layers of each AGS granule supply nitrite to anaerobic denitrifying microbes found mostly in the interior (green and red). The combined activities of these two physiological groups convert $NH_3$—an excellent microbial N source—into $N_2$, and thus when the treated effluent is released into the environment, it will not trigger massive microbial growth. The lower operational cost and complexity of AGS technology also make it ideal for use in small communities and developing countries.

**Source:** van Loosdrecht, M.C.M., and Brdjanovic, D. 2014. Anticipating the next century of wastewater treatment. *Science 344:* 1452–1453.

In this chapter we address the microbiology of "built" systems. These include the infrastructure for drinking water and wastewater distribution and treatment, gas and oil transmission, building materials, private and public spaces, and environments modified for mineral extraction or for the cleanup of pollutants. By their very nature, built systems create new microbial habitats, and these promote both desired and undesired microbial activities. From the standpoint of microbial ecology, these activities are simply the natural result of microbes exploiting resources provided to them.

Examples of built systems designed for desirable microbial activities include the construction of biological reactors for the treatment of wastewater and the stimulation of microbial activity in aquifers to clean up environmental pollutants. A notable example of an unwanted activity is microbially influenced corrosion of the pipelines used for transmission of wastewater, drinking water, and oil. Essential infrastructure costing several billion dollars is lost every year to microbially influenced corrosion. For example, the American Association of Civil Engineers estimates that between now and the year 2050, 30% of the drinking water distribution system in the United States will need to be replaced at an annual cost of about $11 billion.

# I • Mineral Recovery and Acid Mine Drainage

The biogeochemical capacities of microorganisms seem almost limitless, and it is often said that microorganisms are "Earth's greatest chemists." The activities of these great little chemists have been exploited in many ways. Here we consider how microbial activities help extract valuable metals from low-grade ores.

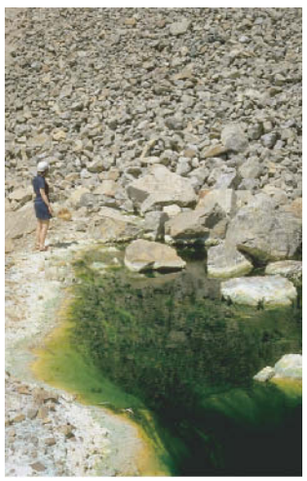
## 22.1 Mining with Microorganisms

One of the most common forms of iron in nature is **pyrite** ($FeS_2$), which is often present in coal and in metal ores. Sulfide ($HS^-$) also forms insoluble minerals with many metals, and many ores mined as sources of these metals are sulfide ores. If the concentration of metal in the ore is low, it may be economically feasible to mine the ore only if the desired metals are first concentrated by **microbial leaching** (Figure 22.1). The promotion of acid production and dissolution of $FeS_2$ by acidophilic bacteria such as *Acidithiobacillus ferrooxidans* is used to leach the metal ores in large-scale mining operations. Leaching is especially useful for copper ores because copper sulfate ($CuSO_4$), formed during the oxidation of copper sulfide ores, is very water-soluble. Indeed, approximately a quarter of all copper mined worldwide is obtained by microbial leaching.

### The Leaching Process

The susceptibility to oxidation varies among minerals, and those minerals that are most readily oxidized are most amenable to microbial leaching. Thus, iron and copper sulfide ores such as pyrrhotite (FeS) and covellite (CuS) are readily leached, whereas lead and molybdenum ores are much less so. In microbial leaching, low-grade ore is dumped in a large pile called the *leach dump* and a dilute sulfuric acid solution at pH 2 is percolated down through the pile (Figure 22.1). The liquid emerging from the


(a)




(b)


(c)


(d)

**Figure 22.1 The leaching of low-grade copper ores using iron-oxidizing bacteria.** *(a)* A typical leaching dump. The low-grade ore has been crushed and dumped in such a way that the surface area exposed is as high as possible. Pipes distribute the acidic leach water over the surface of the pile. The acidic water slowly percolates through the pile and exits at the bottom. *(b)* Effluent from a copper leaching dump. The acidic water is very rich in $Cu^{2+}$. *(c)* Recovery of copper as metallic copper ($Cu^0$) by passage of the $Cu^{2+}$-rich water over metallic iron in a long flume. *(d)* A small pile of metallic copper removed from the flume, ready for further purification.

bottom of the pile (Figure 22.1*b*) is rich in dissolved metals and is transported to a precipitation plant (Figure 22.1*c*) where the desired metal is precipitated and purified (Figure 22.1*d*). The liquid is then pumped back to the top of the pile and the cycle repeated. As needed, acid is added to maintain an acidic pH.

We illustrate microbial leaching of copper with the common copper ore CuS, in which copper exists as $Cu^{2+}$. *A. ferrooxidans* oxidizes the sulfide in CuS to $SO_4^{2-}$, releasing $Cu^{2+}$ as shown in Figure 22.2. However, this reaction can also occur spontaneously. Indeed, the key reaction in copper leaching is actually not the bacterial oxidation of sulfide in CuS but the spontaneous oxidation of sulfide by ferric iron ($Fe^{3+}$) generated from the bacterial oxidation of ferrous iron ($Fe^{2+}$) (Figure 22.2). In any copper ore, $FeS_2$ is also present, and its oxidation by bacteria leads to the formation of $Fe^{3+}$. The spontaneous reaction of CuS with $Fe^{3+}$ proceeds in the absence of $O_2$ and forms $Cu^{2+}$ plus $Fe^{2+}$; importantly for efficiency of the leaching process, this reaction can take place deep in the leach dump where conditions are anoxic (Figure 22.2).

## Metal Recovery

The precipitation plant is where the $Cu^{2+}$ from the leaching solution is recovered (Figure 22.1*c*, *d*). Shredded scrap iron (a source of elemental iron, $Fe^0$) is added to the precipitation pond to recover copper from the leach liquid by the chemical reaction shown in the lower part of Figure 22.2. This results in a $Fe^{2+}$-rich liquid that is pumped to a shallow oxidation pond where iron-oxidizing chemolithotrophs oxidize the $Fe^{2+}$ to $Fe^{3+}$. This now ferric-iron-rich acidic liquid is pumped to the top of the pile and the $Fe^{3+}$ is used to oxidize more CuS (Figure 22.1). The entire CuS leaching operation is thus driven by the oxidation of $Fe^{2+}$ to $Fe^{3+}$ by iron-oxidizing bacteria.

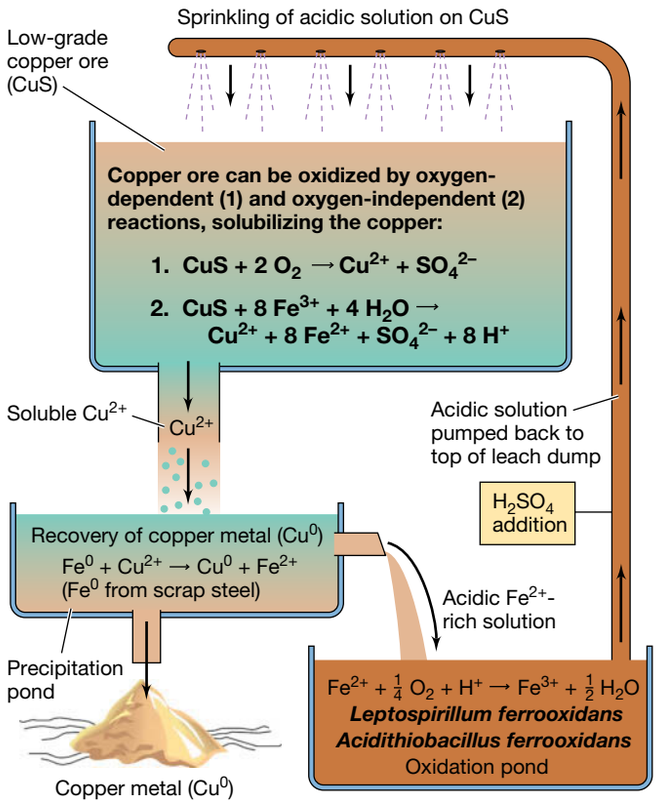



**Figure 22.2 Arrangement of a leaching pile and reactions in the microbial leaching of copper sulfide minerals to yield metallic copper.** Reaction 1 occurs both biologically and chemically. Reaction 2 is strictly chemical and is the most important reaction in copper-leaching processes. For reaction 2 to proceed, it is essential that the $Fe^{2+}$ produced from the oxidation of sulfide in CuS to sulfate be oxidized back to $Fe^{3+}$ by iron chemolithotrophs (see chemistry in the oxidation pond).

Temperatures rise in a leaching dump and this leads to shifts in the iron-oxidizing microbial community. *A. ferrooxidans* is a mesophile, and when heat generated by microbial activities raises temperatures above about 30°C inside a leach dump, this bacterium is outcompeted by mildly thermophilic iron-oxidizing chemolithotrophic *Bacteria* such as *Leptospirillum ferrooxidans* and *Sulfobacillus*. At even higher temperatures (60–80°C), hyperthermophilic *Archaea* such as *Sulfolobus* (Section 17.9) predominate in the leach dump.

## Other Microbial Leaching Processes: Uranium and Gold

Bacteria are also used in the leaching of uranium (U) and gold (Au) ores. In uranium leaching, *A. ferrooxidans* oxidizes $U^{4+}$ to $U^{6+}$ with $O_2$ as an electron acceptor. However, U leaching depends more on the abiotic oxidation of $U^{4+}$ by $Fe^{3+}$ with *A. ferrooxidans* contributing to the process mainly through the reoxidation of $Fe^{2+}$ to $Fe^{3+}$, as in copper leaching (Figure 22.2). The reaction observed is as follows:

$$UO_2 + Fe_2(SO_4)_3 \rightarrow UO_2SO_4 + 2\,FeSO_4$$
$$(U^{4+})\ (Fe^{3+}) \qquad (U^{6+}) \qquad (Fe^{2+})$$

Unlike $UO_2$, the uranyl sulfate ($UO_2SO_4$) formed is highly soluble and is concentrated by other processes.

Gold is typically present in nature in deposits associated with minerals containing arsenic (As) and $FeS_2$. *A. ferrooxidans* and related bacteria can leach the arsenopyrite minerals, releasing the trapped Au:

$$2\,FeAsS[Au] + 7\,O_2 + 2\,H_2O + H_2SO_4 \rightarrow Fe_2(SO_4)_3 + 2\,H_3AsO_4 + [Au]$$

The Au is then complexed with cyanide ($CN^-$) by traditional gold-mining methods. Unlike copper leaching, which is done in a huge dump (Figure 22.1*a*), gold leaching is done in small bioreactor tanks (Figure 22.3), where more than 95% of the trapped Au can be released. Moreover, the potentially toxic As and $CN^-$ residues from the mining process are removed in the gold-leaching bioreactor. Arsenic is removed as a ferric precipitate, and $CN^-$ is removed by its bacterial oxidation to $CO_2$ plus urea in later stages of the Au recovery process. Small-scale microbial-bioreactor leaching has thus become popular as an alternative to the environmentally devastating gold-mining techniques that leave a toxic trail of As and $CN^-$ at the extraction site. Pilot processes are also being developed for bioreactor leaching of zinc, lead, and nickel ores.

### MINIQUIZ

- What is required to oxidize CuS under anaerobic conditions?
- What key role does *Acidithiobacillus ferrooxidans* play in the copper leaching process?

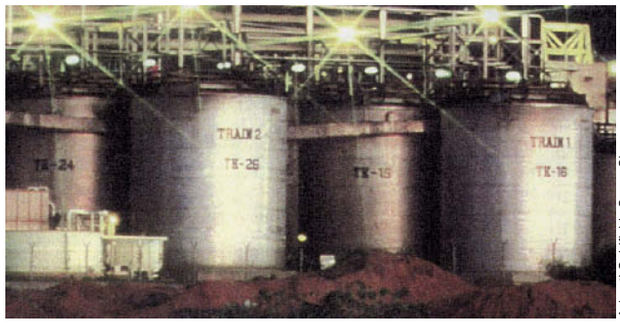



**Figure 22.3 Gold bioleaching.** Gold leaching tanks in Ghana (Africa). Within the tanks, a mixture of *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans*, and *Leptospirillum ferrooxidans* solubilizes the pyrite/arsenic mineral containing trapped gold, which releases the gold.

## 22.2 Acid Mine Drainage

Although microbial leaching has tremendous value in mining operations, the same process has contributed to extensive environmental destruction where mining operations improperly handle or dispose of pyrite-containing coal and mineral deposits. Bacterial and spontaneous oxidation of sulfide minerals is the major cause of **acid mine drainage**, an environmental problem worldwide caused by surface mining operations. As described for the oxidation of copper sulfides promoted in microbial mining (Section 22.1), the oxidation of $FeS_2$ is a combination of chemically and bacterially catalyzed reactions, and two electron acceptors participate in the process: $O_2$ and $Fe^{3+}$. When $FeS_2$ is first exposed in a mining operation (**Figure 22.4*b***), a slow chemical reaction with $O_2$ begins (Figure 22.4*c*). This reaction, called the *initiator reaction*, leads to the oxidation of $HS^-$ to $SO_4^{2-}$ and the development of acidic conditions as $Fe^{2+}$ is released. *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* then oxidize $Fe^{2+}$ to $Fe^{3+}$, and the $Fe^{3+}$ formed under these acidic conditions, being soluble, reacts spontaneously with more $FeS_2$ and oxidizes the $HS^-$ to sulfuric acid ($H_2SO_4$), which immediately dissociates into $SO_4^{2-}$ and $H^+$:

$$FeS_2 + 14\,Fe^{3+} + 8\,H_2O \rightarrow 15\,Fe^{2+} + 2\,SO_4^{2-} + 16\,H^+$$

Again, the bacteria oxidize $Fe^{2+}$ to $Fe^{3+}$, and this $Fe^{3+}$ reacts with more $FeS_2$. Thus, there is a progressive, rapidly increasing rate at which $FeS_2$ is oxidized, called the *propagation cycle* (Figure 22.4*c*). Under natural conditions some of the $Fe^{2+}$ generated by the bacteria leaches away and is subsequently carried by anoxic groundwater into surrounding streams. However, bacterial or spontaneous oxidation of $Fe^{2+}$ then takes place in the aerated streams, and because $O_2$ is present, the insoluble $Fe(OH)_3$ is formed.

As we have seen (Figure 22.4*c*), the breakdown of $FeS_2$ ultimately leads to the formation of $H_2SO_4$ and $Fe^{2+}$; in waters in which these products have formed, pH values can be lower than 1. Mixing of acidic mine waters into rivers (**Figure 22.5**) and lakes seriously degrades water quality because both the acid and the dissolved metals (in addition to iron, there is aluminum, and heavy metals such as cadmium and lead) are toxic to aquatic organisms.

The $O_2$ requirement for the oxidation of $Fe^{2+}$ to $Fe^{3+}$ explains how acid mine drainage develops. As long as the pyritic material is not mined, $FeS_2$ cannot be oxidized because $O_2$, water, and the bacteria cannot reach it. However, when a mineral or coal seam is exposed

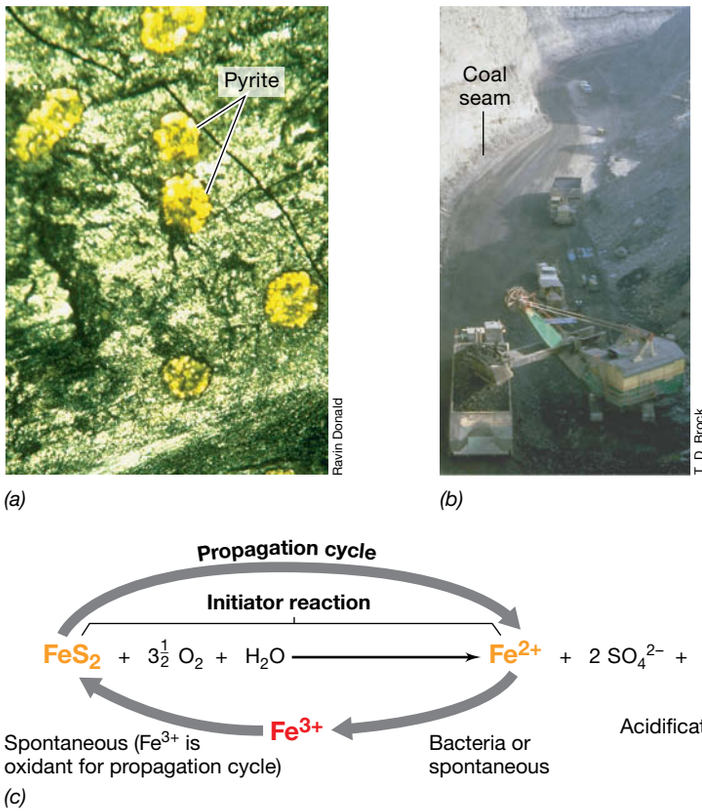



**Figure 22.4 Coal and pyrite.** *(a)* Coal from the Black Mesa formation in northern Arizona (USA); the gold-colored spherical discs (about 1 mm in diameter) are particles of pyrite ($FeS_2$). *(b)* A coal seam in a surface coal-mining operation. Exposing the coal to oxygen and moisture stimulates the activities of iron-oxidizing bacteria growing on the pyrite in the coal. *(c)* Reactions in pyrite degradation. The primarily abiotic initiator reaction sets the stage for the primarily bacterial oxidation of $Fe^{2+}$ to $Fe^{3+}$. The $Fe^{3+}$ attacks and oxidizes $FeS_2$ abiotically in the propagation cycle.

(Figure 22.4*b*), $O_2$ and water are introduced, making both spontaneous and bacterial oxidation of $FeS_2$ possible. The acid formed can then leach into surrounding aquatic systems (Figure 22.5).


**Figure 22.5 Acid mine drainage from a surface coal-mining operation.** The yellowish-red color is due to the precipitated iron oxides in the drainage (see Figure 22.4*c* for the reactions in acid mine drainage).

Where acid mine drainage is extensive and $Fe^{2+}$ levels high, a strongly acidophilic species of *Archaea, Ferroplasma*, is often present. This aerobic iron-oxidizing organism is capable of growth at pH 0 and at temperatures up to 50°C. Cells of *Ferroplasma* lack a cell wall and are phylogenetically related to *Thermoplasma*, also a cell-wall-lacking and strongly acidophilic (but chemoorganotrophic) member of the *Archaea* (Section 17.3).

**MINIQUIZ**

- In what oxidation state is iron in the mineral $Fe(OH)_3$? In FeS? How is $Fe(OH)_3$ formed?
- Natural pyritic deposits, such as underground coal seams, do not contribute to acid mine drainage; why not?

# II • Bioremediation

The term **bioremediation** refers to the microbial cleanup of oil, toxic chemicals, or other environmental pollutants, usually by stimulating the activities of indigenous microorganisms in some way. These pollutants include both natural materials, such as petroleum products, and **xenobiotic** chemicals, synthetic chemicals not produced by organisms in nature.

Although bioremediation of many toxic substances has been proposed, most successes have been in cleaning up spills of crude oil or the leakage of hydrocarbons from bulk storage tanks. More recently, the targeted destruction of chlorinated environmental pollutants, including commonly used solvents and pesticides, has become more amenable to bioremediation as a result of a better understanding of associated microbiology. There has also been increasing success in the bioremediation of uranium-contaminated environments, many of which are the legacy of poorly regulated past mining of uranium for nuclear fuel and weapons. We begin here with a consideration of this very toxic pollutant.

## 22.3 Bioremediation of Uranium-Contaminated Environments

Major classes of inorganic pollutants are metals and radionuclides that cannot be destroyed, but only altered in chemical form. Often the extent of environmental pollution is so great that physical removal of the contaminated material is impossible. Thus, *containment* is the only real option, and a common goal in the bioremediation of inorganic pollutants is to change their mobility, making them less likely to move with groundwater and so contaminate surrounding environments. Here we consider how the radioactive element uranium can be contained by the activities of bacteria.

### Bioremediation of Uranium

Uranium contamination of groundwater has occurred at sites in the United States and elsewhere where uranium ores have been processed or stored (Figure 22.6), and the movement of radioactive materials offsite via groundwater is a threat to environmental and human health. Because the contamination is often widespread, making mechanical methods of recovery very expensive, microbiologists have joined forces with engineers to develop biological treatments that exploit the ability of some bacteria to reduce $U^{6+}$ to $U^{4+}$. Uranium as $U^{6+}$ is soluble, whereas $U^{4+}$ forms an immobile uranium mineral called *uraninite*, thus limiting the movement of U into groundwater and potential contact with humans and other animals.


**Figure 22.6 Uranium bioremediation.** An experimental plot at a United States Department of Energy uranium-contaminated site. Organic carbon (acetate) is being infused into the site (see inset photo) and travels in groundwater in the direction of the arrow shown in the main photo. Acetate is an electron donor for reduction of $U^{6+}$ to $U^{4+}$, which immobilizes the uranium.

### Bacterial Transformations of Uranium

The major strategy for immobilizing uranium has been to use bacteria to change the oxidation state of U in major uranium contaminants to a form that will stabilize the element. In this regard, *Bacteria*, including metal-reducing *Shewanella* and *Geobacter* species (Section 15.14) and sulfate-reducing *Desulfovibrio* species (Section 15.9), couple the oxidation of organic matter and $H_2$ to the reduction of $U^{6+}$ to $U^{4+}$.

Field studies in which organic electron donors have been injected into uranium-contaminated aquifers to stimulate $U^{6+}$ reduction have shown that this approach can lower U levels to below the U.S. Environmental Protection Agency's drinking water standard of 0.126 µM. However, even though uraninite is stable under reducing conditions, if conditions become oxic, it reoxidizes. Thus, much ongoing uranium bioremediation research is focused on questions of whether microbially reduced uranium is stable if the composition of the


US Environmental Protection Agency

(a)


US Environmental Protection Agency

(b)


Bassam Lahoud, Lebanese American University

(c)

**Figure 22.7 Environmental consequences of large oil spills and the effect of bioremediation.** *(a)* A contaminated beach along the coast of Alaska containing oil from the *Exxon Valdez* spill of 1989. *(b)* The rectangular plot (arrow) was treated with inorganic nutrients to stimulate bioremediation of spilled oil by microorganisms, whereas areas above and to the left were untreated. *(c)* Oil spilled into the Mediterranean Sea from the Jiyeh (Lebanon) power plant that flowed to the port of Byblos during the 2006 war in Lebanon.

microbial community changes or if oxidants, such as $O_2$, $NO_3^-$, and $Fe^{3+}$, are introduced via groundwater. This is obviously an important question because uraninite stability must be targeted for the long term in order to account for the long half-life of nuclear decay of uranium.

**MINIQUIZ**

- Which reaction, oxidation or reduction, is key to uranium bioremediation?
- Why is immobilization a good strategy for dealing with uranium pollution?

## 22.4 Bioremediation of Organic Pollutants: Hydrocarbons

Organic pollutants, unlike inorganic pollutants, can generally be completely degraded by microorganisms, eventually to $CO_2$. This is true of petroleum released in oil spills (**Figure 22.7**), which can be attacked by many different microorganisms. These organisms have been exposed to complex mixtures of hydrocarbons through natural oil seeps for millennia and thus have evolved the catabolic machinery necessary to degrade this naturally occurring pollutant. In contrast, xenobiotic pollutants tend to be more persistent and are degraded by more specialized groups of microorganisms. In this section we focus on hydrocarbons and in the next section on xenobiotics.

### Petroleum and Hydrocarbon Bioremediation

Petroleum is a rich source of organic matter, and because of this, microorganisms readily attack hydrocarbons when petroleum is pumped to Earth's surface and comes into contact with air and moisture. Under some circumstances, such as in bulk petroleum storage tanks, microbial growth is undesirable. However, in oil spills, biodegradation is desirable and can be promoted by the addition of inorganic nutrients to balance the huge influx of organic carbon from the oil (Figure 22.7).

The biochemistry of hydrocarbon catabolism was covered in Sections 14.24 and 14.25. Both oxic and anoxic biodegradation is possible. We emphasized that under oxic conditions, oxygenase enzymes play an important role in introducing oxygen atoms into the hydrocarbon. Our discussion here will focus on *aerobic* processes, because it is only when $O_2$ is present that oxygenase enzymes can function and hydrocarbon bioremediation can be effective in a relatively short time.

Diverse bacteria, fungi, and a few green algae can oxidize petroleum products aerobically. Small-scale oil pollution of aquatic and terrestrial ecosystems from human as well as natural activities is common. Oil-oxidizing microorganisms develop rapidly on oil films and slicks, and hydrocarbon oxidation is most extensive if the temperature is warm enough and supplies of inorganic nutrients (primarily N and P) are sufficient. Moreover, because oil is insoluble in water and is less dense, it floats to the surface and forms slicks. There, hydrocarbon-degrading bacteria attach to the oil droplets (**Figure 22.8**) and eventually decompose the oil and disperse the slick. Certain oil-degrading bacteria are specialist species; for example, the bacterium *Alcanivorax borkumensis* grows only on hydrocarbons, fatty acids, or pyruvate. This organism produces surfactant chemicals that help break up the oil and solubilize it. Once solubilized, the oil can be incorporated more readily and catabolized as an electron donor and carbon source.

In large surface oil spills such as those shown in Figure 22.7, volatile hydrocarbons, both aliphatic and aromatic, evaporate quickly without bioremediation, leaving nonvolatile components

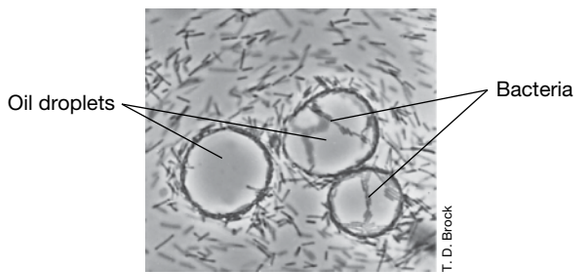



**Figure 22.8 Hydrocarbon-oxidizing bacteria in association with oil droplets.** The bacteria are concentrated in large numbers at the oil–water interface, but are actually not within the droplet itself.

for cleanup crews and microorganisms to tackle. Microorganisms consume oil by oxidizing it to $CO_2$. When bioremediation activities are promoted by inorganic nutrient application, oil-oxidizing bacteria typically develop quickly after an oil spill (Figure 22.7*b*), and under ideal conditions, 80% or more of the nonvolatile oil components can be oxidized within one year. However, certain oil fractions, such as those containing branched-chain and polycyclic hydrocarbons, are not preferred microbial substrates and remain in the environment much longer. Spilled oil that finds its way into sediments is even more slowly degraded and can have a significant long-term impact on fisheries that depend on unpolluted waters for productive yields.

A notable exception to the more common surface spill of oil was the 2010 sinking of the Deepwater Horizon offshore drilling platform in the Gulf of Mexico, resulting in the rupture of the wellhead at a depth of 1.5 km and release of over 4 million barrels (635 million liters) of oil into the deep ocean (Section 20.9 and Figure 20.21). About 35% of the resulting hydrocarbon plume was comprised of low-molecular-weight components and natural gas (methane, ethane, propane). The availability of these more easily degraded oil components is thought to have accelerated the natural degradation process by stimulating the development of a large bloom of bacteria having the capacity to oxidize both the easily degraded and more recalcitrant hydrocarbon components. It remains uncertain whether the industry decision to promote dispersal of the oil (which was intended to increase the oil's surface area and bioavailability) by injecting thousands of gallons of chemical dispersants directly into the plume actually accelerated microbial degradation. Regardless, although some legacy of this major oil spill remains, much of the oil did disappear from a combination of volatilization and microbial activities.

### Degradation of Stored Hydrocarbons

Interfaces where oil and water meet often form on a large scale. Besides water that separates from crude petroleum during storage and transport, moisture can condense inside bulk fuel storage tanks (**Figure 22.9**) where there are leaks. This water eventually accumulates in a layer beneath the petroleum. Gasoline and crude oil storage tanks are thus potential habitats for hydrocarbon-oxidizing microorganisms. If sufficient sulfate ($SO_4^{2-}$) is present in the oil, as it often is in crude oils, sulfate-reducing bacteria can grow in the tanks, consuming hydrocarbons under anoxic conditions (Sections 14.25 and 15.9). The sulfide ($H_2S$) produced is highly corrosive and causes pitting and subsequent leakage of the tanks along with souring of the fuel. Aerobic degradation of stored fuel components is less of a problem because the storage tanks are sealed and the fuel itself contains little dissolved $O_2$.

**Figure 22.9 Bulk petroleum storage tanks.** Fuel tanks often support microbial growth at oil–water interfaces.

**MINIQUIZ**

- Why do petroleum-degrading bacteria need to attach to the *surface* of oil droplets?
- What is unique about the physiology of the bacterium *Alcanivorax*?

## 22.5 Bioremediation of Organic Pollutants: Pesticides and Plastics

Unlike hydrocarbons, many chemicals that humans put into the environment have never been there before. These are the xenobiotics, and we consider their microbial degradation here.

### Pesticide Catabolism

Xenobiotics include pesticides, polychlorinated biphenyls (PCBs), munitions, dyes, and chlorinated solvents, among many other chemicals. Some xenobiotics differ chemically in such major ways from anything organisms have experienced in nature that they

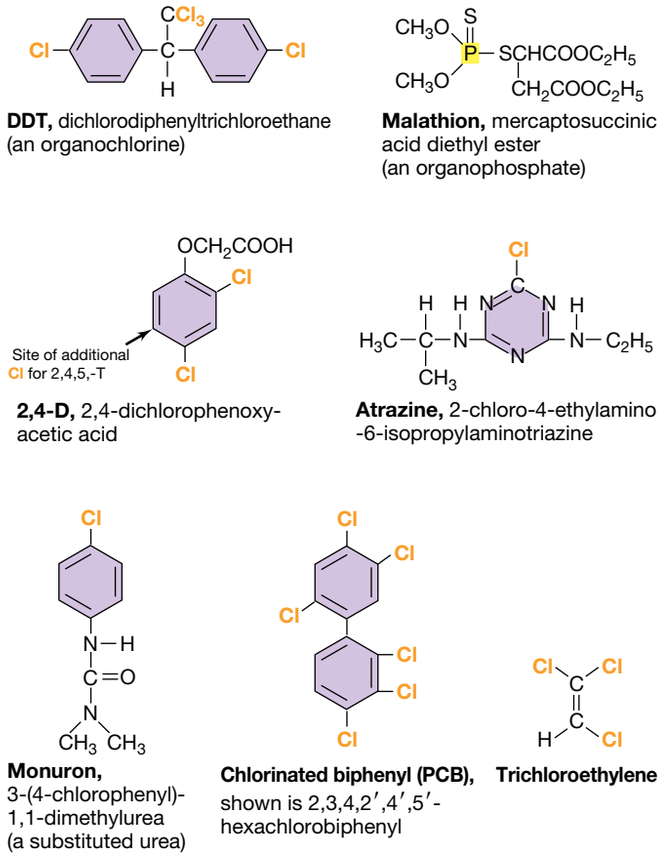



**Figure 22.10 Examples of xenobiotic compounds.** Although none of these compounds exist naturally, microorganisms exist that can break them down.

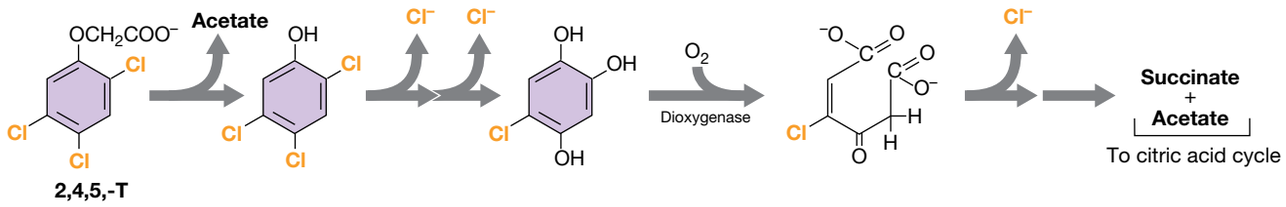



**Figure 22.11 Biodegradation of the herbicide 2,4,5-T.** Pathway of aerobic 2,4,5-T biodegradation; note the importance of a dioxygenase enzyme (Section 14.24) in the degradation process.

biodegrade extremely slowly, if at all. Other xenobiotics are structurally related to one or more natural compounds and can sometimes be degraded slowly by enzymes that normally degrade the structurally related natural compounds. We focus here on pesticide bioremediation.

Over 1000 pesticides have been marketed worldwide for pest control purposes. Pesticides include *herbicides, insecticides*, and *fungicides*. Pesticides display a wide variety of chemistries, and include chlorinated, aromatic, and nitrogen- and phosphorus-containing compounds (**Figure 22.10**). Some of these substances can be used as carbon and energy sources by microorganisms, whereas others are utilized only poorly or not at all. Highly chlorinated compounds are typically the pesticides most resistant to microbial attack. However, related compounds may differ remarkably in their degradability. For example, chlorinated compounds such as DDT persist relatively unaltered for years in soils, whereas chlorinated compounds such as 2,4-D are significantly degraded in just a few weeks.

Environmental factors, such as temperature, pH, aeration, and organic content of the soil, influence the rate of pesticide decomposition, and some pesticides can disappear from soils nonbiologically by volatilization, leaching, or spontaneous chemical breakdown. In addition, some pesticides are degraded only when other organic material is present that can be used as the primary energy source, a phenomenon called *cometabolism*. In most cases, pesticides that are cometabolized are only partially degraded, generating new xenobiotic compounds that may be even more toxic or difficult to degrade than the original compound. Thus, from an environmental standpoint, cometabolism of a pesticide is not always a good thing.

## Dechlorination

Many xenobiotics are chlorinated compounds and their degradation proceeds through *dechlorination*. For example, the bacterium *Burkholderia* dechlorinates the pesticide 2,4,5-T aerobically, releasing chloride ion ($Cl^-$) in the process (**Figure 22.11**); this reaction is catalyzed by oxygenase enzymes (Section 14.24). Following dechlorination, a dioxygenase enzyme breaks the aromatic ring to yield compounds that can enter the citric acid cycle and yield energy.

Although the aerobic breakdown of chlorinated xenobiotics is undoubtedly ecologically important, **reductive dechlorination** may be even more so because of the rapidity with which anoxic conditions develop in polluted microbial habitats. We previously described reductive dechlorination as a form of anaerobic respiration in which chlorinated organic compounds such as chlorobenzoate ($C_7H_4O_2Cl^-$) are terminal electron acceptors and when reduced, release chloride ($Cl^-$), a nontoxic substance (Section 14.15).

Many compounds can be reductively dechlorinated including dichloro-, trichloro-, and tetrachloro- (perchloro-) ethylene, chloroform, dichloromethane, and polychlorinated biphenyls (Figure 22.10). In addition, several brominated and fluorinated organic compounds can be dehalogenated in analogous fashion. Many of these chlorinated or halogenated compounds are highly toxic and some have been linked to cancer (particularly trichloroethylene). Some of these compounds, such as PCBs, have been widely used as insulators in electrical transformers and enter anoxic environments from slow leakage of the transformer or from leaking storage containers. Eventually these compounds end up in groundwater or sediment, where they are among the most common contaminants detected in the United States. There is therefore great interest in reductive dechlorination as a bioremediation strategy for their removal from anoxic environments.

## Plastics

Plastics are classic examples of xenobiotics, and the plastics industry worldwide produces about 300 million tons of plastic per year, almost half of which are discarded rather than recycled. Plastics are polymers of various chemistries (**Figure 22.12*a***).

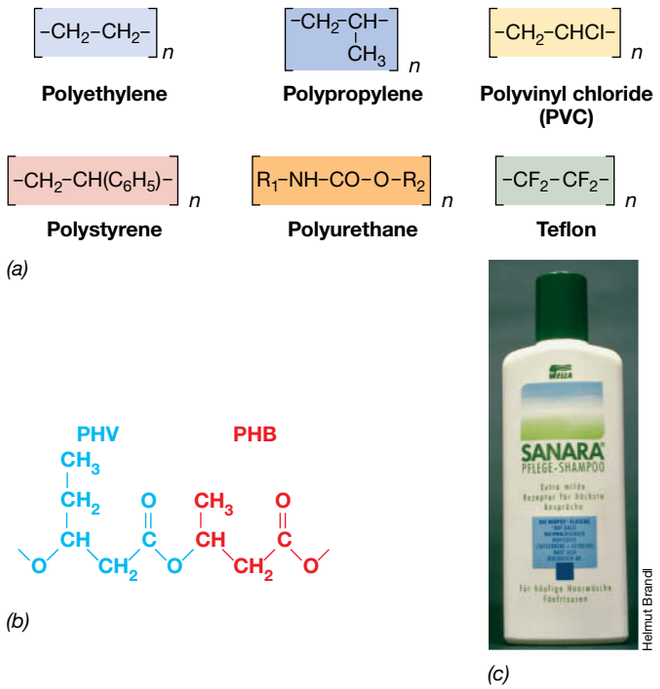



**Figure 22.12 Synthetic and microbial plastics.** *(a)* The monomeric structure of several synthetic plastics. *(b)* Structure of the copolymer of poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV). *(c)* A brand of shampoo previously marketed in Germany and packaged in a bottle made of the PHB/PHV copolymer.

UNIT 5

Many plastics remain essentially unaltered for long periods in landfills, refuse dumps, and as litter in the environment. As much as 9 million metric tons of plastic per year enters the marine environment, and this is of particular environmental concern. Weathering of plastic debris in the ocean causes fragmentation into small particles that small marine invertebrates can ingest, possibly disrupting important marine food webs. This problem has fueled the search for biodegradable alternatives called **microbial plastics** as replacements for some synthetic plastics.

Polyhydroxyalkanoates (PHAs) are a common bacterial storage polymer (Section 2.8), and these readily biodegradable polymers have many of the desirable properties of xenobiotic plastics. PHAs can be biosynthesized in various chemical forms, each with its own unique physical properties (stiffness, shear and impact strength, and the like). A PHA *copolymer* containing equal amounts of poly-β-hydroxybutyrate and poly-β-hydroxyvalerate (Figure 22.12*b*) has been marketed in Europe as a container for personal care products and has had the greatest success as a plastic substitute thus far (Figure 22.12*c*). However, because synthetic plastics are currently less expensive than microbial plastics, synthetic petroleum-based plastics make up virtually the entire plastics market today.

The bacterium *Ralstonia eutropha* has been used as a model organism for the commercial production of PHAs. This genetically manipulable and metabolically diverse bacterium produces PHAs in high yield, and specific copolymers can be obtained by simple nutritional modifications. Nevertheless, the microbial plastics industry is burdened by the reality that the best substrates for PHA biosyntheses are glucose and related organic compounds, substances obtained from corn or other crops. And even when the price of oil is high, plant products cannot compete with oil as feedstocks for the plastics industry.

**MINIQUIZ**

- Why might the addition of inorganic nutrients stimulate oil degradation whereas the addition of glucose would not?
- What is reductive dechlorination and how does it differ from the reactions shown in Figure 22.11?
- What main advantage do microbial plastics have over synthetic plastics?

# III • Wastewater and Drinking Water Treatment

Water is the most important potential common source of infectious diseases and a potential source for chemically induced intoxications (Chapter 32). This is because a single water source often serves large numbers of people, as, for example, in large cities. Everyone in these cities must use the available water, and contaminated water has the potential to spread disease to all exposed individuals. Similarly, appropriate treatment of wastewater is essential for maintaining environmental quality and for reducing the spread of disease. Thus, the microbiology of water, water transport systems, and water treatment are of the utmost importance to public health.

The outbreak of cholera in Haiti following the 2010 earthquake is a reminder of the importance of well-maintained waste and drinking water treatment systems in ensuring public health (Sections 29.8 and 32.3). Here we examine systems built for the chemical and biological treatment of water and the transmission systems used for delivering treated water to consumers. We also examine the human health significance of the microbial communities that develop within the pipes of municipal water distribution systems and premise plumbing.

## 22.6 Primary and Secondary Wastewater Treatment

**Wastewater** is domestic sewage or liquid industrial waste that cannot be discarded in untreated form into lakes or streams because of public health, economic, environmental, and aesthetic considerations. Wastewater treatment employs physical and chemical methods as well as industrial-scale use of microorganisms. Wastewater enters a treatment plant and, following treatment, the **effluent water**—treated wastewater discharged from the wastewater treatment facility—is suitable for release into surface waters such as lakes and streams or to drinking water purification facilities (**Figure 22.13**).

### Wastewater and Sewage

Wastewater from domestic sewage or industrial sources cannot be discarded in untreated form into lakes or streams. **Sewage** is liquid effluent contaminated with human or animal fecal materials. Wastewater may also contain potentially harmful inorganic and organic compounds as well as pathogenic microorganisms. Wastewater treatment can use physical, chemical, and biological (microbiological) processes to remove or neutralize contaminants.

On average, each person in the United States uses 100–200 gallons (380–760 liters) of water every day for washing, cooking, drinking, and sanitation. Wastewater collected from these activities must be treated to remove contaminants before it can be released into surface waters. About 16,000 publicly owned treatment works (POTW) operate in the United States. Most POTWs are fairly small, treating 1 million gallons (3.8 million liters) or less of wastewater per day. Collectively, however, these plants treat about 32 billion gallons (121 billion liters) of wastewater daily. Wastewater plants are usually constructed to handle both domestic and industrial wastes. Domestic wastewater is made up of sewage, "gray water" (the water resulting from washing, bathing, and cooking), and wastewater from small-scale food processing in homes and restaurants.

Industrial wastewater includes liquid discharged from the petrochemical, pesticide, food and dairy, plastics, pulp and paper, pharmaceutical, and metallurgical industries. Industrial wastewater may contain toxic substances; some manufacturing and processing plants are required by the U.S. Environmental Protection Agency (EPA) to pretreat toxic or heavily contaminated discharges before they enter POTWs. Pretreatment may

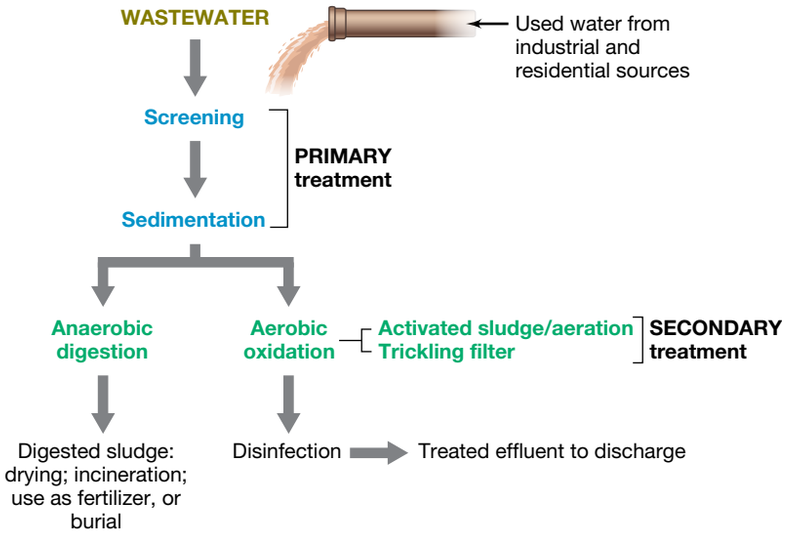



**Figure 22.13 Wastewater treatment processes.** Effective water treatment plants use the primary and secondary treatment methods shown here. Tertiary treatment may also be used to reduce nutrient levels in waters released to the environment, reducing biochemical oxygen demand (BOD), nitrogen, and phosphorus to very low to undetectable levels.

involve mechanical processes in which large debris is removed. Some wastewaters are pretreated biologically or chemically to remove highly toxic substances such as cyanide; heavy metals such as arsenic, lead, and mercury; or organic materials such as acrylamide, atrazine (a herbicide), and benzene. These substances are converted to less toxic forms by treatment with chemicals or microorganisms capable of neutralizing, oxidizing, precipitating, or volatilizing these wastes. The pretreated wastewater can then be released to the POTW.

## Wastewater Treatment and Biochemical Oxygen Demand

The goal of a wastewater treatment facility is to reduce organic and inorganic materials in wastewater to a level that no longer supports microbial growth and to eliminate other potentially toxic materials. The efficiency of treatment is expressed in terms of a reduction in the **biochemical oxygen demand (BOD)**, the relative amount of dissolved oxygen consumed by microorganisms to completely oxidize all organic and inorganic matter in a water sample (Section 20.8). High levels of organic and inorganic materials in the wastewater result in a high BOD.

Typical values for domestic wastewater, including sewage, are approximately 200 BOD units. For industrial wastewater from sources such as dairy plants, the values can be as high as 1500 BOD units. An efficient wastewater treatment facility reduces BOD levels to less than 5 BOD units in the final treated water. Wastewater facilities are designed to treat both low-BOD sewage and high-BOD industrial wastes.

Treatment is a multistep operation employing a number of independent physical and biological processes (Figure 22.13). *Primary*, *secondary*, and sometimes additional treatments are employed to reduce biological and chemical contamination in the wastewater, and each higher level of treatment employs more complex technologies.

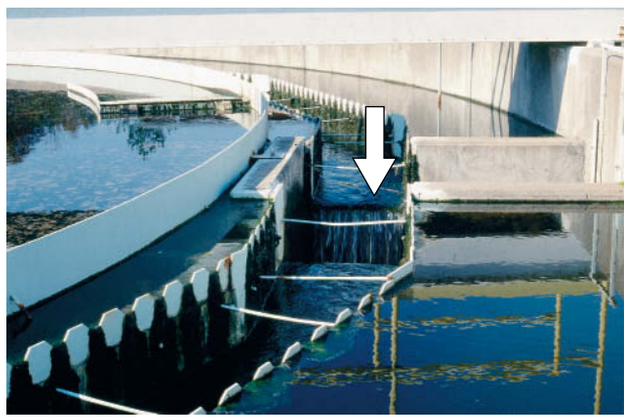
## Primary Wastewater Treatment

**Primary wastewater treatment** uses only physical separation methods to separate solid and particulate organic and inorganic materials from wastewater. Wastewater entering the treatment plant is passed through a series of grates and screens that remove large objects. The effluent is allowed to settle for a few hours. Solids settle to the bottom of the separation reservoir and the effluent is drawn off to be discharged or for further treatment (Figure 22.14).

Municipalities that provide only primary treatment, as is true for the city of Victoria (British Columbia, Canada), discharge extremely polluted water with high BOD into adjacent waterways; high levels of soluble and suspended organic matter and other nutrients remain in water following primary treatment. These nutrients trigger undesirable microbial growth, further reducing water quality. Most treatment plants employ secondary and even *tertiary* (Section 22.7) treatments to reduce the organic content of the wastewater before release to natural waterways. Secondary treatment processes use both aerobic and anaerobic microbial digestion to further reduce organic nutrients in wastewater.

## Secondary Aerobic Wastewater Treatment

**Secondary aerobic wastewater treatment** uses oxidative degradation reactions carried out by microorganisms under *oxic* conditions to treat wastewater containing low levels of organic materials (Figure 22.15*a*, *b*). In general, wastewaters that originate from residential sources can be treated efficiently using only aerobic treatment. Several aerobic degradative processes can be used for wastewater treatment; *activated sludge* methods are the most common (Figure 22.15*a*, *b*). Here, wastewater is continuously mixed and aerated in large tanks. Slime-forming aerobic bacteria, including *Zoogloea ramigera* and others, grow and form aggregated



John M. Martinko and Deborah O. Jung

**Figure 22.14 Primary treatment of wastewater.** Wastewater is pumped into the reservoir (left) where solids settle. As the water level rises, the water spills through the grates to successively lower levels. Water at the lowest level, now virtually free of solids, enters the spillway (arrow) and is pumped to a secondary treatment facility.

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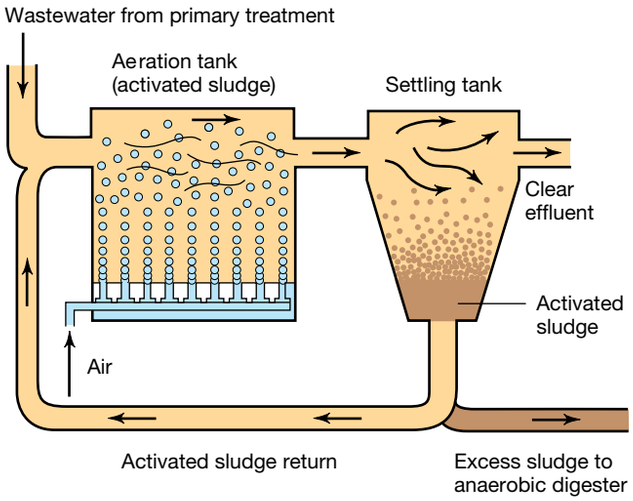
(a)




(b)

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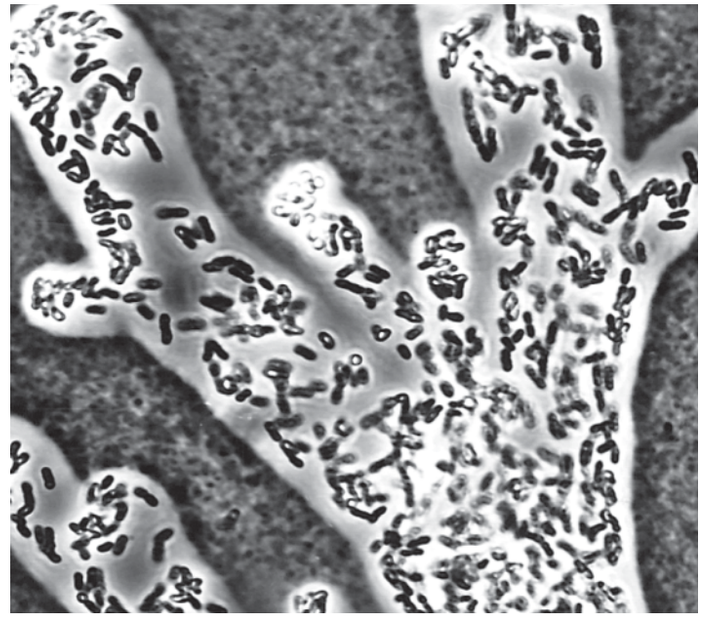
(c)

**Figure 22.15 Secondary aerobic wastewater treatment processes.** Parts *a* and *b* show the activated sludge method. *(a)* Aeration tank of an activated sludge installation in a metropolitan wastewater treatment plant. The tank is 30 m long, 10 m wide, and 5 m deep. *(b)* Wastewater flow through an activated sludge installation. Recirculation of activated sludge to the aeration tank introduces microorganisms responsible for oxidative degradation of the organic components of the wastewater. *(c)* Trickling filter method. The booms rotate, distributing wastewater slowly and evenly on the rock bed. The rocks are 10–15 cm in diameter and the bed is 2 m deep.



Richard Unz

**Figure 22.16 A wastewater floc formed by the bacterium *Zoogloea ramigera*.** Floc formed in the activated sludge process consists of a large number of small, rod-shaped cells of *Z. ramigera* surrounded by a polysaccharide slime layer, arranged in characteristic fingerlike projections in this negative stain with India ink.

masses called flocs (**Figure 22.16**). The biology of *Zoogloea* is discussed in Section 16.2. Protists, small animals, filamentous bacteria, and fungi attach to the flocs. Oxidation of organic matter occurs on the floc as it is agitated and exposed to air. The aerated effluent containing the flocs is pumped into a holding tank or clarifier where the flocs settle. Some of the floc material (called activated sludge) is then returned to the aerator as inoculum for new wastewater, and the rest is pumped to an anaerobic sludge digester (see Figure 22.17), or removed, dried, and burned, or is used for fertilizer.

Wastewater normally stays in an activated sludge tank for 5–10 hours, a time too short for complete oxidation of all organic matter. However, during this time much of the soluble organic matter is adsorbed to the floc and incorporated by the microbial cells. The BOD of the liquid effluent is considerably reduced (up to 95%) when compared to the incoming wastewater; most of the material with high BOD is now in the settled flocs. The flocs can then be transferred to the anoxic sludge digester for conversion to $CO_2$ and $CH_4$.

Activated sludge treatment performance is sometimes diminished by the overgrowth of certain filamentous microbes (commonly members of the *Actinobacteria*, Section 16.12) that cause slow settling of the flocs, a problem called *sludge bulking*. This persistent problem, as well as the large reactor volumes required for activated sludge treatment, has prompted the development of new types of reactors that promote the growth of dense microbial aggregates, called *granular sludge*. The individual dense microbial granules (of several mm size) in granular sludge have both high metabolic activity and good gravity settling properties, greatly reducing the size and energy costs of a treatment facility. Although much of this work is still laboratory based, there should be a relatively rapid transfer of this technology to practice (see page 708).

The *trickling filter* is an alternative method of aerobic secondary treatment (Figure 22.15*c*). A trickling filter is a bed of crushed rocks, about 2 m thick. Wastewater is sprayed on top of the rocks and slowly passes through the bed. The organic material in the wastewater adsorbs to the rocks, and microorganisms grow and form biofilms on the large, exposed rock surfaces. The complete mineralization of organic matter to $CO_2$, ammonia, nitrate, sulfate, and phosphate takes place in the extensive microbial biofilm that develops on the rocks.

Most treatment plants chlorinate the effluent after secondary treatment to further reduce the possibility of biological contamination. The treated effluent can then be discharged into streams or lakes. In the eastern United States, many wastewater treatment facilities use ultraviolet (UV) radiation to disinfect effluent water. Ozone ($O_3$), a strong oxidizing agent that is an effective bactericide and viricide (Sections 5.15–5.17), is also used for wastewater disinfection in some treatment plants in the United States.

### Secondary or Tertiary Anaerobic Treatment

**Anaerobic treatment** involves a series of catabolic reactions carried out by various *Bacteria* and *Archaea* under *anoxic* conditions. Anaerobic treatment is typically used to treat wastewater containing large quantities of insoluble organic matter (and therefore having a very high BOD) such as fiber and cellulose waste from food and dairy plants. It is also used for additional treatment of the sludge originating from secondary aerobic wastewater treatment (Figure 22.15*b*). In that case, the additional step is called **tertiary treatment**, defined as any treatment process in which unit operations are added for the further processing of the secondary treatment effluent or solids.

The anaerobic degradation process is carried out in large, enclosed tanks called *sludge digesters* or *bioreactors* (**Figure 22.17**). The process requires the collective activities of many different microbes and the major reactions are summarized in Figure 22.17*c*. First, anaerobes use polysaccharidases, proteases, and lipases to digest suspended solids and large macromolecules into soluble components. These soluble components are then fermented to yield a mixture of fatty acids, $H_2$, and $CO_2$; the fatty acids are further fermented by the cooperative actions of syntrophic bacteria (Sections 14.23 and 21.2) to produce acetate, $CO_2$, and $H_2$. These products are then used as substrates by methanogenic *Archaea* (Sections 17.2 and 21.2), fermenting acetate to produce methane ($CH_4$) and $CO_2$, the major products of anoxic sewage treatment (Figure 22.17*c*). The $CH_4$ is burned off or used as fuel to heat and power the wastewater treatment plant.

**MINIQUIZ**

- What is biochemical oxygen demand (BOD), and why is its reduction important in wastewater treatment?
- How do primary and secondary wastewater treatment methods differ?
- Other than treated water, what are the final products of wastewater treatment? How might these end products be used?

## 22.7 Advanced Wastewater Treatment

Advanced wastewater treatment is any process designed to produce an effluent of higher quality than normally achieved by secondary treatment. This includes tertiary treatment, physical–chemical treatment, or combined biological–physical treatment. Typical goals of advanced treatment include additional removal of organic matter and suspended solids, removal of key inorganic nutrients required for microbial growth (including ammonia, nitrate, nitrite, phosphorus, and dissolved organic carbon), and degradation of any potentially toxic materials. Advanced water treatment is the most complete method of treating sewage but has not been widely adopted because of the costs associated with such complete nutrient removal. Here we examine biological removal of phosphorus, nitrogen, and trace contaminants, three areas of advanced treatment of increasing importance to wastewater treatment.

### Biological Phosphorus Removal

Conventional secondary biological treatment removes only about 20% of phosphorus from wastewater, necessitating additional

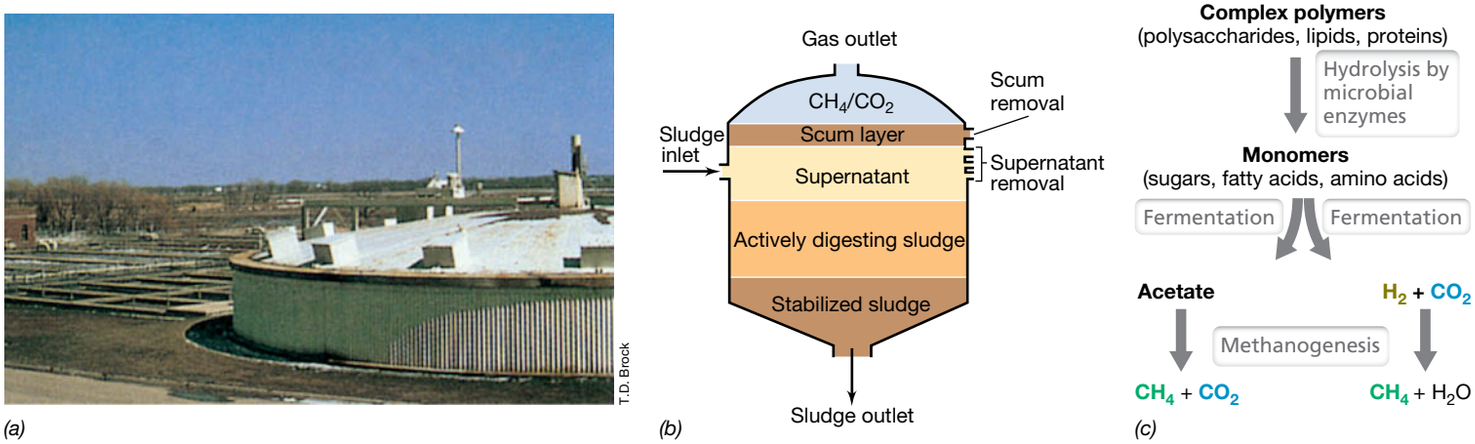



**Figure 22.17 Anaerobic treatment.** *(a)* Anaerobic sludge digester. Only the top of the tank is shown; the remainder is underground. *(b)* Inner workings of a sludge digester. *(c)* Major microbial processes in anaerobic sludge digestion. Methane ($CH_4$) and carbon dioxide ($CO_2$) are the major products of anaerobic biodegradation.

UNIT 5

chemical or biological treatment. Chemical precipitation is the most commonly used process, removing up to 90% of the influent phosphorus. Removal is accomplished by the addition of either Fe or Al as chloride or sulfate salts, with $Fe^{2+}$ or $Fe^{3+}$ salts more commonly used. At near-neutral pH, the $Fe^{3+}$ forms insoluble ferric phosphate ($FePO_4$) or ferric hydroxide-phosphate complexes. These then precipitate and are removed as sludge.

The chemical precipitation process results in up to 95% more sludge, contributing to additional disposal problems. As an alternative, tertiary treatment that encourages the growth of phosphorus-accumulating bacteria can also remove up to 90% of phosphorus, a process called *enhanced biological phosphorus removal* (*EBPR*). Here the waste stream is processed by sequential passage through anaerobic and aerobic bioreactors (**Figure 22.18**). In the anaerobic reactor, *phosphorus-accumulating organisms* (*PAOs*) use energy available from stored polyphosphate to assimilate short-chain fatty acids, and produce intracellular polyhydroxyalkanoates (PHAs) (Figure 22.18*a*; Section 2.8); as this occurs, soluble orthophosphate ($PO_4^{3-}$) is released. During the following aerobic phase of treatment, the stored PHA is metabolized, providing energy and carbon for new cell growth. The energy is used to form intracellular polyphosphate, removing orthophosphate from solution (Figure 22.18*a*). The new biomass (sludge) with high polyphosphate content is then collected for phosphorus removal (Figure 22.18*b*).

The EBPR process sometimes fails as a result of the overgrowth of competing microbial populations, commonly microorganisms that accumulate glycogen as opposed to phosphorus, thus rendering the process less efficient. Hence, better control of the process will require improved understanding of the ecology and physiology of the PAOs. Recent progress in this area has been made with identification of one of the principal PAOs, the appropriately named bacterium *Accumulibacter phosphatis*. *A. phosphatis* is part of a clade of related phosphorus-accumulating *Betaproteobacteria* (Section 16.2) that have been identified in different EBPR systems. Although no pure cultures are yet available, laboratory reactor systems enriched in these organisms are now providing insight into operating conditions necessary for stable operation of the EBPR.

## Biological Nitrogen Removal: The Conventional Process

The strict regulatory limitations on release of nitrogen as ammonia or nitrate/nitrite, also called *reactive nitrogen* (*Nr*), from wastewater treatment facilities reflects their adverse health effects on human and aquatic life and contribution to eutrophication of receiving water bodies (Section 20.8). Thus, there is increasing use of tertiary treatment to remove remaining Nr from wastewater following secondary treatment or anaerobic sludge digestion (Figure 22.15).

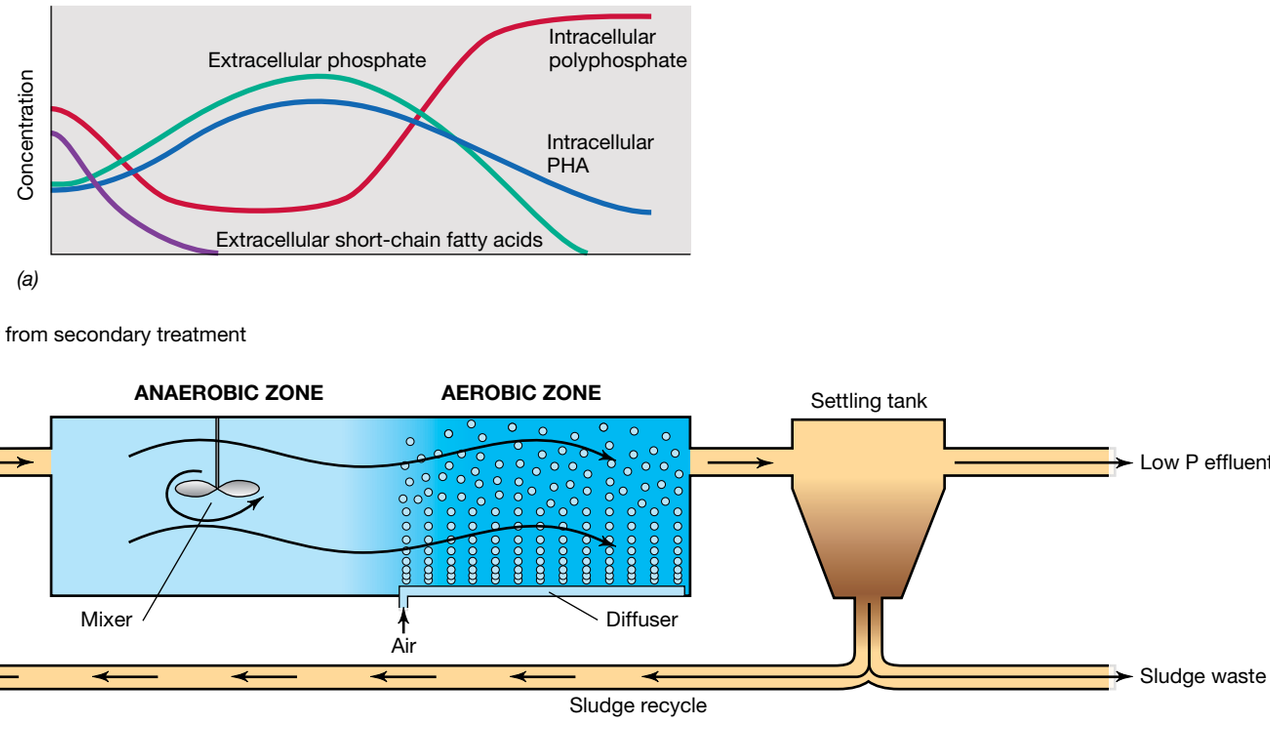



**Figure 22.18 Enhanced biological phosphorus removal process.** *(a)* Carbon and phosphate transitions during the treatment process. *(b)* Wastewater processing. During the passage of wastewater through the reactor system, the microbial community transitions from anaerobic to aerobic growth. In the anaerobic zone, short-chain fatty acids are taken up and internal stores of polyphosphate (polyP) are released as extracellular orthophosphate. In the aerobic zone, the extracellular phosphate is reassimilated as polyP and the intracellular stores of polyhydroxyalkanoates (PHAs) are metabolized. High-phosphorus sludge is harvested for disposal.

Conventional ("classical") treatment converts the Nr to its inert atmospheric form ($N_2$) using a combination of nitrification and denitrification (**Figure 22.19*a***). Nitrification (↔ Section 14.11) is first used to convert ammonia to nitrate followed by anaerobic conversion of nitrate to $N_2$ (and some $N_2O$) by denitrification (↔ Section 14.13). In the following reactions, organic carbon is represented by chemical oxygen demand (COD, ↔ Section 20.8), the mass of oxygen that is reduced by a specific amount of organic matter; for example, a COD of 4 g reduces 4 g of $O_2$ (0.125 moles) to water:

$$\text{Ammonia oxidation: } NH_4^+ + 1.5\,O_2 \rightarrow NO_2^- + H_2O + 2\,H^+$$

$$\text{Nitrite oxidation: } NO_2^- + 0.5\,O_2 \rightarrow NO_3^-$$

$$\text{Denitrification: } NO_3^- + 40\,g\,COD + H^+ \rightarrow 0.5\,N_2 + 15\,g\,biomass$$

$$\text{Combined: } NH_4^+ + 2\,O_2 + 40\,g\,COD \rightarrow 0.5\,N_2 + H_2O + H^+ + 15\,g\,biomass$$

Although the primarily autotrophic ammonia- and nitrite-oxidizing microbes require little or no reduced carbon for growth, a carbon source is required for the subsequent microbial reduction of nitrate to $N_2$ by denitrification. This may require supplementation with additional carbon for wastewaters having a low ratio of organic carbon to reactive nitrogen (C/N). Supplementation is commonly accomplished by adding a relatively inexpensive carbon source, such as methanol. However, because a typical treatment plant treats well over a million gallons (3.8 million liters) of wastewater a day, organic carbon addition can add significant costs, and this has fueled the development of less costly advanced treatment processes.

## Biological Nitrogen Removal: Partial Nitrification–Denitrification and Anammox

The cost of Nr removal can be reduced through advanced treatment processes that limit the activities of nitrite-oxidizing bacteria. This leaves more of the oxidized $NH_4^+$ in the form of $NO_2^-$ than $NO_3^-$ and thus less organic matter is needed to denitrify:

$$\text{Ammonia oxidation: } NH_4^+ + 1.5\,O_2 \rightarrow NO_2^- + H_2O + 2\,H^+$$

$$\text{Denitrification: } NO_2^- + 24\,g\,COD + H^+ \rightarrow 0.5\,N_2 + 9\,g\,biomass$$

$$\text{Combined: } NH_4^+ + 1.5\,O_2 + 24\,g\,COD \rightarrow 0.5\,N_2 + H_2O + H^+ + 9\,g\,biomass$$

By limiting the oxidation of ammonia to nitrite in the wastewater, the requirement for carbon is reduced by ~40%, oxygen by ~25%, and biomass by ~40%. This makes nitrogen removal from low C/N wastewater less costly. The only complication to this treatment strategy is the need to suppress nitrite-oxidizing bacteria (NOB), since they generally have higher substrate utilization rates than the ammonia-oxidizing bacteria (AOB). Achieving cost-effective control of partial ammonia oxidation is therefore key to advanced nitrogen removal treatment systems.

Suppression of nitrite oxidation has been achieved by adjusting growth conditions that differentially affect AOB and NOB, primarily those of pH and temperature. AOB grow faster than NOB at temperatures above room temperature, having a specific growth rate (↔ Section 5.4) approximately two times that of NOB at 35°C. For example, one widely used process relies on washing out NOB from a reactor operated at 30–40°C

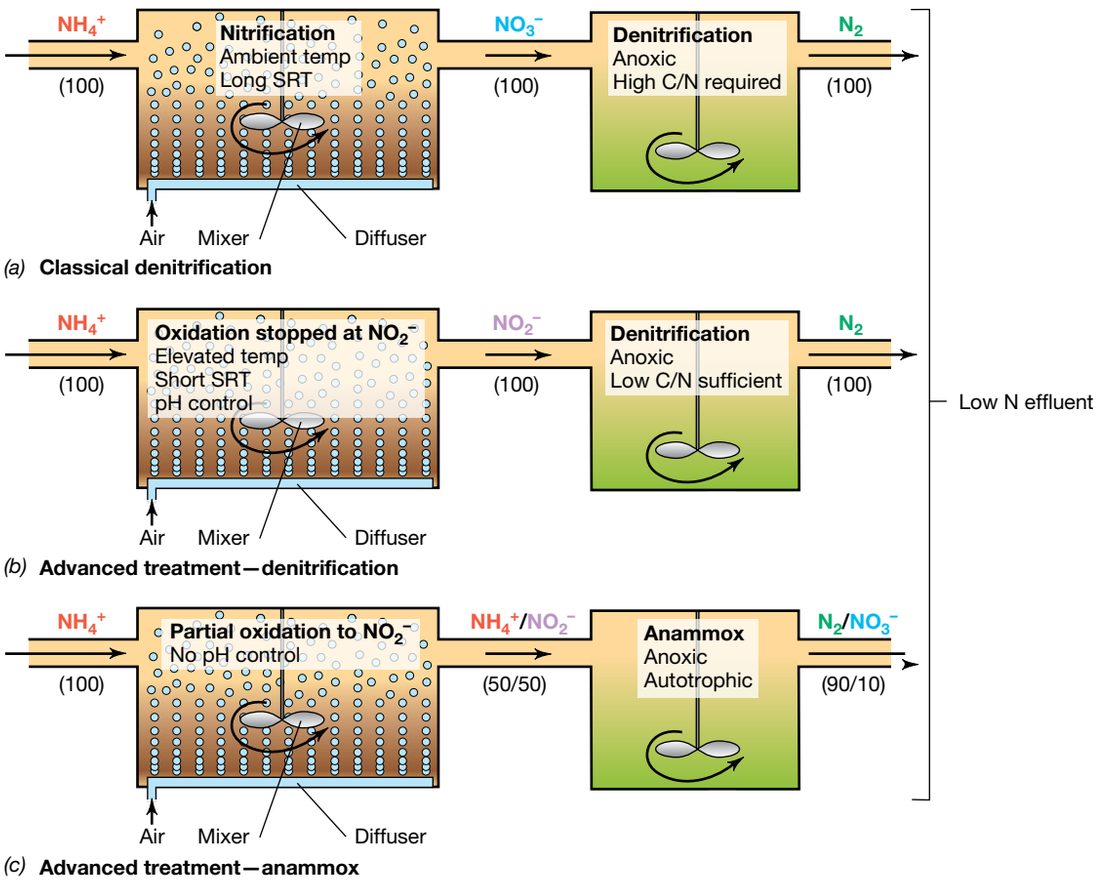



**Figure 22.19 Alternative treatment processes for nitrogen removal from wastewater.** Boxes show the conceptual—not full-scale operational—design of biological reactors and operating conditions that promote the indicated processes. *(a)* Classic denitrification process. *(b, c)* Advanced treatment processes for more efficient and economical removal of nitrogen. Numbers in parentheses correspond to percentages of each form of nitrogen entering and leaving a reactor. Since $NO_2^-$ is also used as a source of electrons for autotrophic carbon fixation by anammox bacteria, the product of its oxidation ($NO_3^-$) is also generated in the anammox reactor. SRT, Sludge retention time.

and a short sludge retention time of 1–1.5 days (Figure 22.19*b*). The retention time can be controlled by the rate the reactor is fed wastewater or by allowing sludge to settle during a period without mixing before withdrawing the treated effluent. Control of reactor pH provides another way to achieve partial nitrification, as an alkaline pH (7.5–8.5) favors the growth of AOB over NOB (Figure 22.19*b*).

Anammox bacteria (Section 14.12) are now increasingly used to remove Nr from concentrated ammonia streams of low C/N, such as is found in anaerobic sludge digester liquor and animal production wastewater. The following reaction includes the production of cellular biomass during growth of anammox bacteria:

$$\text{Anammox: } NH_4^+ + 1.32\,NO_2^- + 0.066\,HCO_3^- + 0.13\,H^+ \rightarrow$$
$$1.02\,N_2 + 0.26\,NO_3^- + 2.03\,H_2O + 0.066\,C_{biomass}$$

Since anammox bacteria are chemolithotrophic autotrophs and also anaerobes, they do not depend on organic carbon as an electron donor and $O_2$ is not needed in the reaction. Anammox also reduces the biomass generated and the emission of greenhouse gases (primarily $CO_2$ and $N_2O$, Section 21.8). When pH is not controlled in the ammonia-oxidation process, the primary reactor will generate a nearly equal molar ratio of ammonia and nitrite. This is because after about half the ammonia is oxidized, the resulting drop in pH prevents further ammonia oxidation (Figure 22.19*c*). The N-rich effluent is then treated in a separate reactor that separates partial nitrification in the first reactor from anammox in the second (Figure 22.19*c*).

### Contaminants of Emerging Concern

As one can imagine, a wide variety of chemicals end up in wastewaters. Until recently, studies of the environmental fate of chemicals in wastewaters have focused primarily on priority pollutants, including heavily used agricultural products and chemicals that demonstrate acute toxicity or carcinogenicity. However, it is now clear that new bioactive pollutants are entering the environment that will pose new challenges for microbial bioremediation, and most of these enter the environment in wastewaters. These pollutants include pharmaceuticals, active ingredients in personal care products, fragrances, household products, sunscreens, prescription and other medications, and many other unusual or xenobiotic molecules.

Unlike pesticides, these "new" pollutants are more or less continuously discharged into the environment primarily through release of treated or untreated sewage and, because of this, they do not need to persist to have environmental effects. For example, it is known that low levels of synthetic estrogen compounds, excreted in the urine of women taking birth control pills and eventually discharged in active form from wastewater treatment plants, can activate estrogen response genes in aquatic animals such as fish and contribute to the feminization of males.

Wastewater treatment plants were originally designed to handle natural materials, primarily human and industrial wastes, but now there is a growing interest in carefully researching the design of a new generation of treatment facilities to stimulate bioremediation of these emerging contaminants. Because these contaminants are often present in very low concentrations and are often new classes of xenobiotic chemicals, they may not actually support microbial growth but rather may be degraded only by cometabolism (Section 22.5) or by highly specialized species. We can therefore expect that the bioremediation of emerging contaminants will be an active area of microbiological research and environmental public policy in coming years.

**MINIQUIZ**

- What are the advantages of enhanced biological phosphorus removal (EBPR) relative to traditional chemical removal of phosphorus? Are there any disadvantages?
- Why is the incomplete oxidation of ammonia useful in tertiary treatment of wastewater? What advantages does the anammox process have over classical wastewater treatments for N removal?
- Give an example of an "emerging" contaminant.

## 22.8 Drinking Water Purification and Stabilization

Wastewater treated by secondary methods can usually be discharged into rivers and streams. However, such water is not **potable** (safe for human consumption). The production of potable water requires further treatment to remove potential pathogens, eliminate taste and odor, reduce nuisance chemicals such as iron and manganese, and decrease **turbidity**, which is a measure of suspended solids. **Suspended solids** are small particles of solid pollutants that resist separation by ordinary physical means.

Intestinal infections due to waterborne pathogens are still common, even in developed countries (Section 32.1), and some estimates indicate that waterborne diseases impact the health of several million people each year in the United States alone. Water treatment practices, however, have significantly improved access to safe water, starting with public works projects coupled with the application and development of water microbiology in the early twentieth century.

A century ago, water purification in the United States was limited to *filtration* to reduce turbidity, and this resulted in high rates of waterborne disease. Although filtration significantly decreased the microbial load of water, many microorganisms still passed through the filters. However, around 1913, **chlorination** using $Cl_2$ came into use as a disinfectant for large water supplies. Chlorine gas was an effective and inexpensive general disinfectant for drinking water, and its use quickly reduced the incidence of waterborne disease (Section 32.2). Major improvements in public health in the United States were largely due to the adoption of water filtration and disinfection treatment procedures. Public works engineering and microbiology working hand in hand were thus the major contributors to the dramatic advances in public health in the United States and other developed countries in the twentieth century.

### Physical and Chemical Purification

A typical city installation for drinking water treatment is shown in Figure 22.20*a*. Figure 22.20*b* shows the process that purifies **raw water** (also called **untreated water**) that flows through the treatment plant. Raw water is first pumped from the source, in this

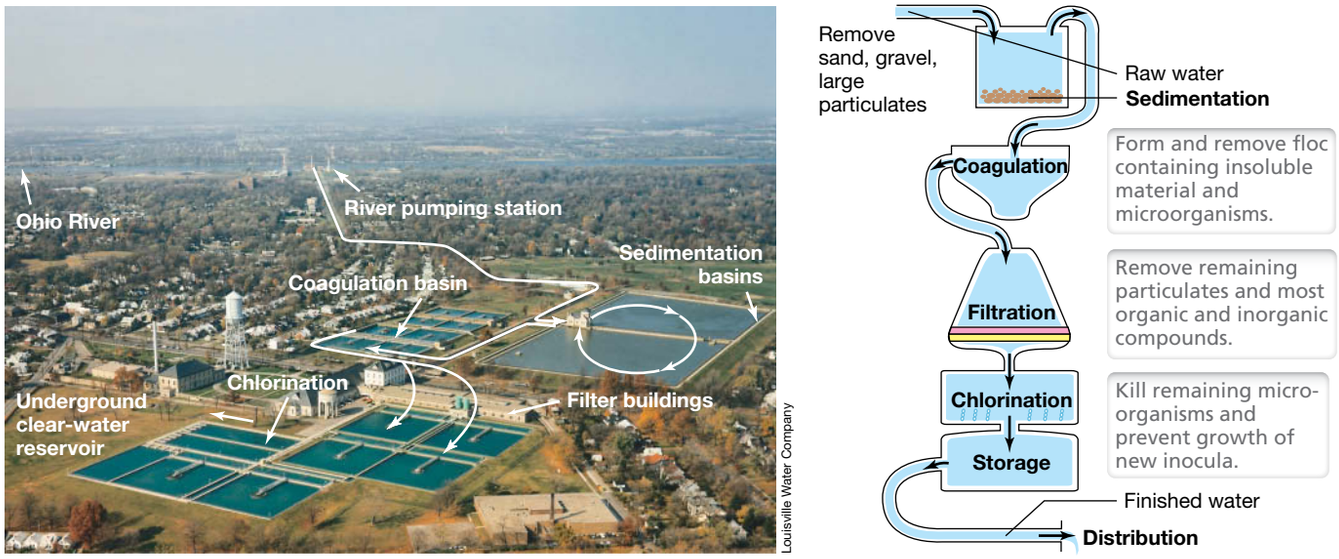



**Figure 22.20 Water purification plant.** *(a)* Aerial view of a water treatment plant in Louisville, Kentucky, USA. The arrows indicate direction of flow of water through the plant. *(b)* Schematic overview of a typical community water purification system.

case a river, to a sedimentation basin where anionic polymers, alum (aluminum sulfate), and chlorine are added. **Sediment**, including soil, sand, mineral particles, and other large particles, settles out. The sediment-free water is then pumped to a **clarifier** or coagulation basin, which is a large holding tank where **coagulation** takes place. The alum and anionic polymers form large particles from the much smaller suspended solids. After mixing, the particles continue to interact, forming large, aggregated masses, a process called **flocculation**. The large, aggregated particles (floc) settle out by gravity, trapping microorganisms and adsorbing suspended organic matter and sediment.

After coagulation, flocculation, and sedimentation, the clarified water undergoes **filtration** through a series of filters designed to remove organic and inorganic solutes, as well as remaining suspended particles and microorganisms. The filters typically consist of thick layers of sand, activated charcoal, and ion exchangers. After this step and the previous purification steps, the filtered water is free of particulate matter, most organic and inorganic chemicals, and nearly all microorganisms.

## Disinfection

Clarified, filtered water must be disinfected before it is released to the supply system as pure, potable **finished water**. **Primary disinfection** is the introduction of sufficient disinfectant into clarified, filtered water to kill existing microorganisms and inhibit further microbial growth. Chlorination is the most common method of primary disinfection. In sufficient doses, chlorine kills most microorganisms within 30 minutes. A few pathogenic protists such as *Cryptosporidium*, however, are not easily killed by chlorine treatment (Section 33.4). In addition to killing microorganisms, chlorine oxidizes and effectively neutralizes many organic compounds. Since most taste- and odor-producing chemicals are organic compounds, chlorination thus improves water taste and smell as well as disinfecting.

Chlorine is added to water either from a concentrated solution of sodium hypochlorite or calcium hypochlorite, or as chlorine gas from pressurized tanks. Chlorine gas is commonly used in large water treatment plants because it is most amenable to automatic control. When dissolved in water, chlorine gas is extremely volatile and dissipates within hours from treated water. To maintain adequate levels of chlorine for primary disinfection, many municipal water treatment plants introduce ammonia gas with the chlorine to form the more stable, nonvolatile chlorine-containing compound **chloramine**, $HOCl + NH_3 \rightarrow NH_2Cl + H_2O$.

Chlorine is consumed when it reacts with organic materials. Therefore, sufficient quantities of chlorine must be added to finished water containing organic materials so that a small amount, called the *chlorine residual*, remains. The chlorine residual reacts to kill any remaining microorganisms. The water plant operator performs chlorine analyses on the treated water to determine the level of chlorine to be added for **secondary disinfection**, the maintenance of sufficient chlorine residual or other disinfectant residual in the water distribution system to inhibit microbial growth. A chlorine residual level of 0.2–0.6 mg/liter is suitable for most water supplies. After chlorine treatment, the now potable water is pumped to storage tanks from which it flows by gravity or pumps through a **distribution system** of storage tanks and supply lines to the consumer. Residual chlorine levels inhibit growth of bacteria in the finished water prior to the water reaching the consumer. It does not protect against catastrophic system failures such as a broken pipe in the distribution system.

Ultraviolet (UV) radiation is also used as an effective means of disinfection. As we discussed in Section 5.16, UV radiation is used to treat secondarily treated effluent from water treatment plants.

In Europe, UV irradiation is commonly used for drinking water applications, and it is increasingly used in the United States. For disinfection, UV light is generated from mercury vapor lamps. Their major energy output is at 253.7 nm, a wavelength that is bactericidal and may also kill cysts and oocysts of protists such as *Giardia* and *Cryptosporidium*, important eukaryotic pathogens in water (Section 33.4). Viruses, however, are more resistant.

UV radiation has several advantages over chemical disinfection procedures like chlorination. First, UV irradiation is a physical process that introduces no chemicals into the water. Second, UV radiation–generating equipment can be used in existing flow systems. Third, no disinfection by-products are formed with UV disinfection. Especially in smaller systems where finished water is not pumped long distances or held for long periods (reducing the need for residual chlorine), UV disinfection may be preferable to reduce dependence on chlorination.

**MINIQUIZ**

- What specific purposes do sedimentation, coagulation, filtration, and disinfection accomplish in the drinking water treatment process?
- What general procedures are used to reduce microbial numbers (microbial load) in water supplies?
- What are the advantages of UV disinfection versus, or as a complement to, chemical disinfection with chlorine?

## 22.9 Water Distribution Systems

Once drinking water leaves the treatment facility, the water often travels through many miles of municipal and premise distribution pipes from the facility to the consumer (Figure 22.21). In addition to taste and odor problems often associated with source water, the long transit and residence times may also contribute to undesirable taste and odors from biological and chemical processes. Although undesirable, taste and odor alone usually do not signal a health threat. However, water distribution systems may also promote the growth of obligate or opportunistic pathogens (Section 25.4), sequester and protect pathogens, or select for more pathogenic and resistant forms of microorganisms. Even though drinking water–associated disease often goes unreported, in the United States alone in 2009 and 2010, 33 disease outbreaks associated with drinking water affected over 1000 persons and were linked to nine deaths.

### The Microbiology of Municipal Water Distribution Systems

Microbial growth in drinking water distribution systems can be eliminated only through complete nutrient removal (elimination of growth substrates originating from the source water and from distribution system structural materials) or by maintaining appropriate residual chlorine levels throughout the distribution system. In reality, neither of these is attainable. Growth is unavoidable as a consequence of reduction in chlorine concentration with increasing distance from the point of production together with the tendency for microorganisms to form biofilms on the pipe

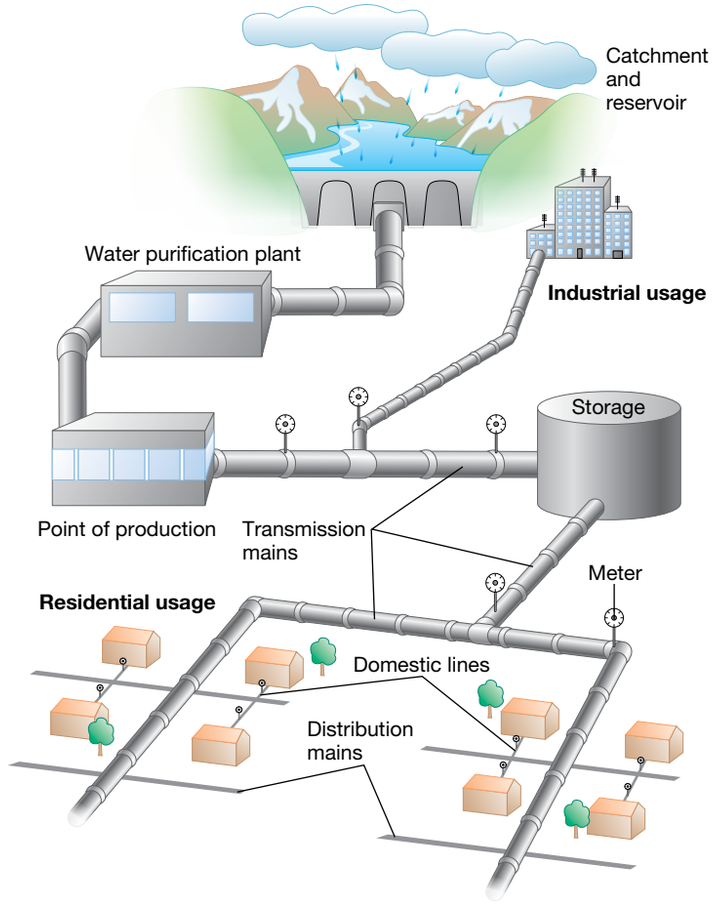



**Figure 22.21 Drinking water distribution system.** A municipal distribution system includes a surface reservoir, water purification plant, distribution mains, and domestic lines that encompass many miles of pipes in a typical community.

walls. Microorganisms in biofilms are more resistant to disinfection (Sections 7.9 and 20.4) and significant microbial accumulation is found in all distribution systems, over 90% of which is in the form of biofilms that coat the pipe walls.

Only recently have culture-independent molecular techniques, including 16S rRNA sequence analysis (Section 19.6), begun to fully resolve the species that commonly colonize water distribution pipes. Although these studies are showing that pathogenic species are rare, some opportunistic pathogens (Section 25.4) are present and can infect susceptible humans, including infants and the elderly or individuals with compromised immune systems. Opportunistic pathogens that have been found in water distribution systems include (1) nontuberculous mycobacteria (including *Mycobacterium avium, M. intracellulare, M. kansasii*, and *M. fortuitum*) associated with many thousands of clinical cases each year in the United States; (2) *Legionella pneumophila* (the causative agent of Legionnaires' disease, Section 32.4); (3) *Pseudomonas aeruginosa* (which can infect the eyes, ears, skin, and lungs); and (4) opportunistic protozoan pathogens such as *Naegleria* and *Acanthamoeba* (Section 33.3) that can cause keratitis and encephalitis.

Because infection by these and other opportunistic pathogens is often of unclear origin and much waterborne disease goes

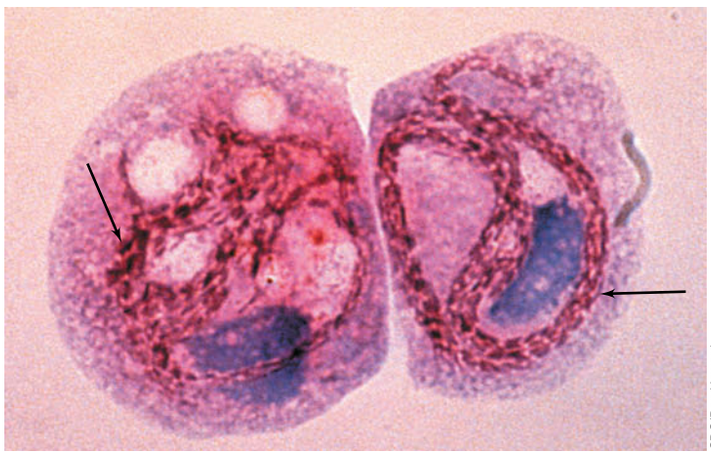
unreported, the significance of water distribution systems as a source (or reservoir) for pathogenic microorganisms is unclear. However, because of the potential large-scale health risk, the issue of pathogens in drinking water has been receiving much greater attention in recent years, including the use of molecular microbial ecology (Chapter 19) to investigate the problem.

Water distribution systems also support numerous grazing protists that subsist by consuming bacteria. For example, as many as 300 amoebae/cm$^2$ have been observed in some water distribution systems. Bacteria that survive and replicate following ingestion by these protists are potentially also less susceptible to clearance by the mammalian immune system. The best example of this is *Legionella*, an opportunistic pathogen that has emerged as a relatively new public health risk because of its ability to establish residence and replicate in protists inhabiting water-handling systems (**Figure 22.22**), including premise plumbing, shower heads, and air-conditioning systems. The basic cellular mechanisms *Legionella* uses to gain entry and replicate in a broad variety of protists (including *Acanthamoeba, Hartmannella, Naegleria*, and *Tetrahymena*) also allow it to more easily infect human cells. It has even been suggested that protists have been the driving force in the evolution of pathogenic *Legionella*. Opportunistic pathogens now recognized to have the ability to survive or grow within protists include *Legionella, Pseudomonas*, and *Mycobacterium* species.

### The Microbiology of Premise Water Distribution Systems

One of the best-recognized microbiological concerns with premise water is *L. pneumophila* (Section 32.4). This pathogen multiplies in premise water systems at temperatures between 20 and 46°C. It survives for months in drinking water and its survival is augmented by the presence of other bacteria and protozoa—in which intracellular growth is possible (Figure 22.22)—and also through sequestration in biofilms. Temperatures greater than 50°C lead to a decrease in numbers, and temperatures greater than 60°C result in rapid elimination (cell death). Thus, to prevent growth of *L. pneumophila*, premise water must be kept below 20°C or above 50°C from storage units to the tap.

Nontuberculous mycobacteria (including the species *Mycobacterium avium, M. intracellulare, M. kansasii*, and *M. fortuitum*) are also more resistant to chlorine disinfection and protozoal grazing and are enriched in showerheads receiving municipal water that still maintains a chlorine residual. As yet the significance of showers as a reservoir of opportunistic pathogens is unknown. However, the increasing frequency of showering as opposed to bathing, and possible aerosolization of opportunistic pathogens through showering, has prompted additional research in this area. The general picture that is emerging is that changes in treatment processes and the architecture of water distribution systems, coupled with the aging condition of some systems, can compromise human health (see Figure 22.23*a*).



CDC/Don Howard

**Figure 22.22 Protists as reservoirs of *Legionella*.** Two cells of the protist *Tetrahymena* contain chains of the rod-shaped pathogen *Legionella pneumophila* (arrows). In premise water systems, protists can persist and be reservoirs of bacterial pathogens. *L. pneumophila* and legionellosis are discussed in Section 32.4.

**MINIQUIZ**

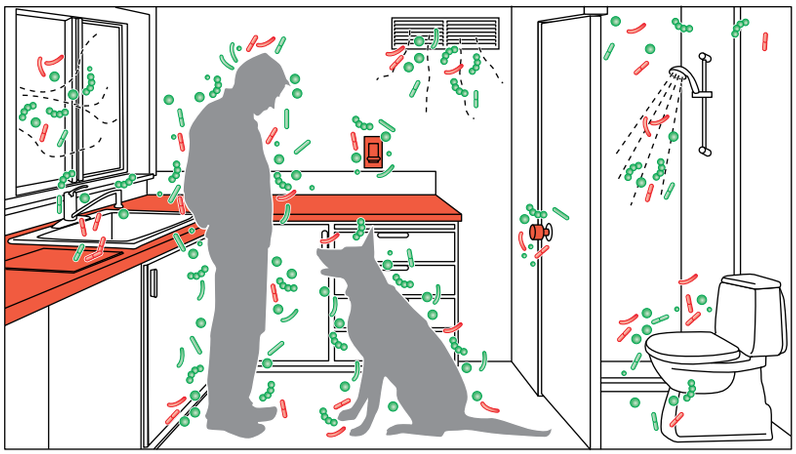
- Trace the treatment of water through a drinking water treatment plant, from the inlet to the final distribution point (faucet).
- What features of a premise water distribution system might encourage the growth of *Legionella*? Suppress growth?

# IV • Indoor Microbiology and Microbially Influenced Corrosion

Although one might think that being indoors protects a person from the microbial world, nothing could be farther from the truth. Indoor air, both in private dwellings and public places, as well as surfaces of the structures themselves, can be teeming with microbes. In addition, billions of dollars' worth of metal, stone, and concrete infrastructure in dwellings and buried pipes is lost every year from corrosion catalyzed by microbial activities. Microbial metabolism accelerates corrosion through alteration of pH or redox, production of corrosive metabolites, and creation of corrosive microenvironments in biofilms. In the final part of this chapter we examine the microbiology of indoor microbial ecosystems and a few cases in which the microbial contribution to corrosion is relatively well understood.

## 22.10 The Microbiology of Homes and Public Spaces

Humans in urban environments spend the majority of their life indoors. They share this indoor environment with a microbiota that inhabit the air, dust, surfaces, and ventilation and water systems (**Figure 22.23**). The health effects of indoor microbial exposure may be positive or negative (Figure 22.23*a*). Pathogens, such as antibiotic-resistant *Staphylococcus aureus*, may be elevated in the indoor environment as a consequence of shedding from human skin. In contrast, the increased incidence of allergies and autoimmune disorders in children in developed countries has



(a)

(b)

**Figure 22.23 Sources of airborne and surface-associated microorganisms in the built environment.** *(a)* Sources of microorganisms in a typical household, including surfaces, humans, pets, plumbing systems, and outdoor air. The colors correspond to microorganisms or spores that may be beneficial or not harmful (green) or potentially detrimental (red) to human health. *(b)* Air particle collector (arrow) deployed in the New York City subway system for surveying the diversity of airborne bacteria by 16S rRNA gene sequencing (Section 19.6).

been in part attributed to the "too-clean" indoor environments that reduce exposure to microbes important for "training" the immune system early in life. Thus, a microbially depleted indoor environment may actually be detrimental to human health.

In addition to living with microbes in our home environment, what microbial exposures does a person experience in major public spaces, such as subway systems, the supermarket, or even the classroom? These questions can now be addressed using powerful culture-independent molecular methods to quantify microbial diversity and abundance patterns using 16S (or 18S) rRNA gene sequencing of DNA isolated from samples collected from different parts of the indoor environment (Section 19.6).

## Microbiology of the Indoor Air in Private Dwellings

Although dedicated studies of premise microbiology are as yet limited, some general trends are emerging. A study of dust collected from upper trims of inside and outside doors of over a thousand homes throughout the USA revealed distinct indoor and outdoor microbial communities composed of fungi and bacteria. The outdoor fungi closely resemble the outdoor fungal populations found in different geographical regions, whereas the indoor bacterial communities more strongly reflect the type and number of occupants, including pets. The overall indoor diversity of bacteria and fungi is greater than that found outdoors, reflecting a mixing of indoor and outdoor sources. A small number of fungal species are more abundant inside the house, including common molds such as *Aspergillus, Penicillium, Alternaria*, and *Fusarium*. The incidence of these fungal populations increases with the age of the dwelling and whether or not a basement is present (basements are often damp and this increases mold abundance).

Bacteria that are characteristic of the human skin (gram-positive bacteria such as *Staphylococcus, Streptococcus, Corynebacterium*, and *Propionibacterium*), feces (*Bacteroides, Faecalibacterium, Ruminococcus*), or the vagina (*Lactobacillus, Bifidobacterium, Lactococcus*) are much more commonly found inside a house than outdoors. The increased incidence of species of the bacteria *Prevotella, Porphyromonas, Moraxella*, and *Bacteroides* is typically associated with the presence of domestic animals—in particular dogs or cats—in a home. In fact, it is possible to predict with near certainty whether a home has these pets based only on a molecular analysis of household microbiota. Certain skin-associated taxa such as *Corynebacterium* and *Dermabacter* are elevated in homes with more men than women, possibly reflecting the known tendency for men to shed more skin than do women.

In addition to the surveys of dust described above, molecular surveys of home surfaces show that the microbial communities of human hands, noses, and bare feet (Sections 9.11 and 24.5, and Figure 9.31) closely resemble the organisms found on household surfaces, including floors, light switches, countertops, and door knobs (Figure 22.23). Moreover, the surface-associated microbiota of a home is highly predictive of a specific family, and the composition of the microbial community has been found to shift within days of a change in home occupants.

## Microbiology of Public Places

Public buildings, office spaces, and transit systems are another important component of the built environment. The heavily trafficked New York City municipal subway system alone moves over 1.5 billion passengers a year. Similar to homes, subways and offices contain a mixture of airborne microorganisms sourced from humans and the outdoor air. Because air exchange in a subway system must be extensive, most airborne microorganisms in subway systems are typical of those found outdoors. In addition, however, about 5% of the microbial population in subway air is composed of microbes that normally reside on the feet, hands, arms, and heads of humans (Section 24.5); these are most likely shed from the more exposed areas of the subway ridership.

Indoor plumbing of public and private buildings is another well-recognized point of microbial exposure. Each flush of a toilet generates over 100,000 small (<5-μm) aerosol particles. Since aerosolized bacteria and viruses can remain viable for hours after they deposit on bathroom surfaces, flushing is a potential mechanism of enteric pathogen transmission as well as a means of transmitting harmless saprophytes from person to person. Transmission of human diseases caused by direct contact (such as sexually transmitted diseases) is not a major issue with bathroom fixtures because pathogens such as *Neisseria gonorrhoeae* (gonorrhea) and *Treponema pallidum* (syphilis) are very sensitive to drying.

However, because of the enormous numbers of bacteria shed in feces, transmission of enteric bacteria by bathroom aerosols is a distinct possibility.

As ongoing studies of indoor microbiology continue to reveal the types and origins of microorganisms we spend much of our day in contact with, we can also anticipate important future changes in construction that will enhance beneficial exposures and limit detrimental exposures to microorganisms in the built environment. At this point in our understanding, indoor air, per se, seems relatively innocuous. However, and depending greatly on the degree of cleanliness of the structure, certain areas in the home and public buildings can be more dangerous than others.

**MINIQUIZ**

- How can a microbial inventory reveal information about the presence or absence of household pets?
- Which room(s) in a private dwelling are potentially the most dangerous from a microbiology perspective, and why?

## 22.11 Microbially Influenced Corrosion of Metals

Iron is the most commonly used metal in the built environment. On a global basis, millions of miles of water, gas, and oil distribution pipelines are made of metal, and their corrosion contributes to the greatest loss of infrastructure in the built environment. Corrosion of iron by oxygen in air is thought to be solely an electrochemical process. However, much critical iron-containing infrastructure is buried or submerged, restricting exposure to oxygen. At near-neutral pH, in the absence of oxygen, corrosion of iron and steel is significantly accelerated by **microbially influenced corrosion (MIC)**. Microbial groups implicated in MIC include sulfate-reducing bacteria (Sections 14.14 and 15.9), ferric-iron-reducing bacteria (Sections 15.14 and 21.5), ferrous-iron-oxidizing bacteria (Sections 14.10, 15.15, and 21.5), and methanogens (Sections 14.17, 17.2, and 21.2).

### Metal Corrosion by Sulfate-Reducing Bacteria

Metal structures submerged in the marine environment and pipelines used for transmission of low-grade oil are particularly subject to MIC through the activities of sulfate-reducing bacteria. Corrosion by sulfate-reducing bacteria is partly attributable to the chemically corrosive nature of hydrogen sulfide ($H_2S$), the product of their metabolism. Crude oils containing more than about 0.5% sulfur by weight are called "sour" and may be naturally corrosive because of the $H_2S$ that is present. In oil fields near the ocean, such as in the Middle East and Alaska, seawater is injected to maintain reservoir pressure and force oil into the producing well. Since seawater contains nearly 30 mM sulfate, an undesirable consequence of injection is further souring by stimulating the growth of sulfate-reducing bacteria.

A strategy now used by the petroleum industry to control souring is inclusion of nitrate ($NO_3^-$) in the injection water, stimulating the growth of nitrate-reducing bacteria. Since nitrate respiration is energetically more favorable than sulfate respiration (Sections 14.13 and 20.2), the nitrate reducers outcompete sulfate reducers for usable organic electron donors in the oil. Nitrate also stimulates the growth of sulfide-oxidizing, nitrate-reducing chemolithotrophs (Sections 14.9 and 15.11), thereby reversing souring by removing the sulfide.

### Mechanisms of Metal Corrosion

At least two mechanisms have been described by which sulfate reducers could corrode iron. In the first mechanism, $H_2$ consumption by the sulfate reducer accelerates electrochemical pitting of the iron surface (**Figure 22.24a**). This model is based on the capacity of many sulfate reducers to use hydrogen ($H_2$) as an electron donor, thereby accelerating the energetically favorable but kinetically slow $H_2$ production originating from the chemical oxidation of iron ($Fe^0 + 2\ H^+ \rightarrow Fe^{2+} + H_2$). The overall stoichiometry of this reaction

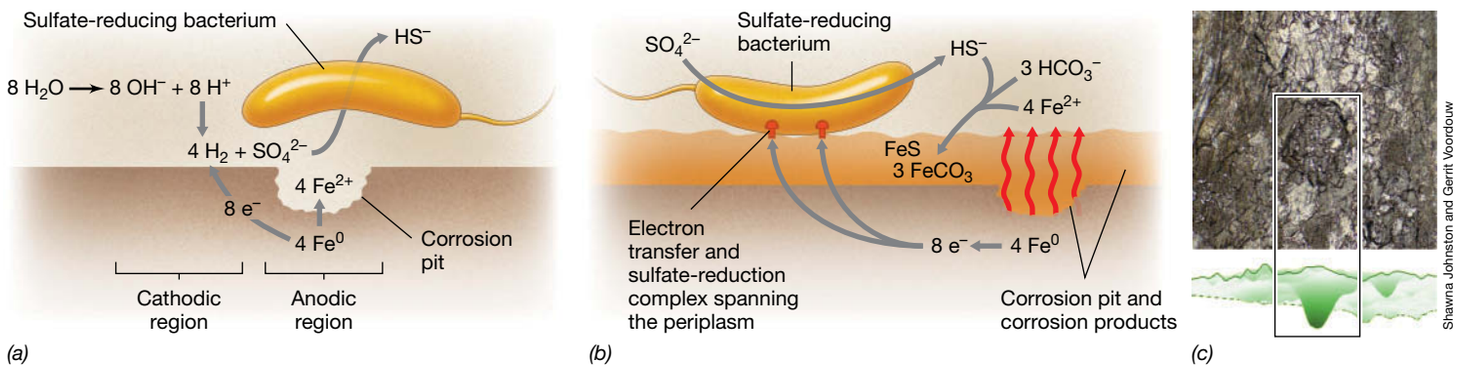



**Figure 22.24 Corrosion of iron by sulfate-reducing bacteria.** Two models for the activities of sulfate-reducing bacteria in metal corrosion. *(a)* Accelerating oxidation of metallic iron by consuming $H_2$ produced abiotically by proton reduction at the metal surface. *(b)* Direct electron transfer from the metal using electron-conductive outer cell wall structures connecting to an electron transfer system spanning the periplasm. *(c)* Top: photo of a model iron surface undergoing sulfidic corrosion. Bottom: scan of a side view of the metal surface in the photo revealing the areas where corrosion and pitting of the metal surface has occurred.

shows that $Fe^{2+}$ formed from pitting reacts with sulfide from sulfate reduction and that the reaction is energetically favorable:

$$4\,Fe^0 + SO_4^{2-} + 3\,HCO_3^- + 5\,H^+ \rightarrow FeS + 3\,FeCO_3 + 4\,H_2O$$
$$(\Delta G^{0\prime} = -925\,kJ)$$

This mechanism, although feasible, has been questioned because $H_2$ formation from the iron surface at neutral pH is an intrinsic bottleneck, controlled by the limited availability of protons required for the reactions generating $H_2$.

Detailed electrochemical studies have shown that some sulfate-reducing bacteria, such as *Desulfopila corrodens*, have the capacity to take up electrons directly from the metal ($Fe^0$, Figure 22.24*b*). In this mechanism, the sulfate reducers attached to the metal surface engage in direct (cathodic) electron uptake from the metal through an electroconductive sulfidic corrosion layer (Figure 22.24*b*). A similar ability to take up electrons directly from $Fe^0$ has been observed for a *Methanobacterium* species that produces methane ($CH_4$) rather than sulfide from growth on $Fe^0$. The direct electron uptake model also suggests that associated with the cell surface are redox-active proteins, or other conductive structures, that conduct electrons from the corrosion layer to the cell. This represents yet another of a growing number of examples of the microbial use of conductive cellular structures for the oxidation or reduction of insoluble electron acceptors or electron donors, respectively (Sections 15.14 and 21.5, and see Explore the Microbial World, "Microbially Wired," in Chapter 21).

**MINIQUIZ**

- How does a nitrate addition prevent sulfide souring of crude oil?
- Why is accelerated microbial corrosion of iron metal thought to require a direct interaction between the sulfate reducers and the metal surface?

## 22.12 Biodeterioration of Stone and Concrete

In the same way that microorganisms contribute to soil formation through the dissolution of mineral and rock surfaces by their physical and metabolic activities (Section 20.6), buildings or other structures composed of natural stone or concrete are also subject to microbial colonization that may contribute to a slow loss of structural integrity through the microbes' metabolic activities. This degradative process is called *biodeterioration*.

### Biodeterioration of Stone Building Materials

Microbial colonization of natural and structural stone building material is ubiquitous. Microbes can colonize the surface and penetrate several millimeters into rocky material depending on its physical characteristics (e.g., surface roughness, porosity, light penetration). Microbes can also grow on and within the facades of buildings constructed of limestone, sandstone, granite, basalt, and soapstone. These "within stone," or *endolithic*, communities are phylogenetically diverse, comprised of chemoorganotrophic and chemolithotrophic *Bacteria* and *Archaea*, microbial eukaryotes including fungi and algae, and cyanobacteria. The cyanobacteria and algae primarily nourish the community, living in close or symbiotic association with other microbial members. For example, endolithic fungi have been observed to enclose the phototrophs in lichen-like associations (Figure 23.2).

Although not generally included in discussions of "extreme environments," life on and within stone building materials requires adaptation to multiple extreme conditions, including intense solar radiation, desiccation, temperature and moisture fluctuations, and lack of nutrients. Protection from solar radiation is conferred by production of UV-absorbing pigments (for example, melanin, mycosporines, and carotenoids) by fungi and other community members. The fungi also play a central role in this process of slow biodeterioration through the production of oxalic acid, which dissolves and mobilizes mineral constituents of the stone. Mineral dissolution and mobilization provides the communities with nutrients and increases habitability by enlarging pore spaces within the stone and thereby accelerating deterioration.

### Crown Corrosion of Wastewater Distribution Systems

A very rapid form of microbial biodeterioration is observed in the **crown corrosion** of concrete sewer tiles, a process leading ultimately to the collapse of the pipe. Crown corrosion is a consequence of interactions between sulfate-reducing bacteria (Sections 14.14 and 15.9) and chemolithotrophic sulfur-oxidizing bacteria (Sections 14.9 and 15.11) in these underground wastewater transmission systems (**Figure 22.25**).

The first step in crown corrosion is the reduction of sulfate in the sewage to $H_2S$ by sulfate-reducing bacteria, using primarily organic electron donors available in the waste stream water for

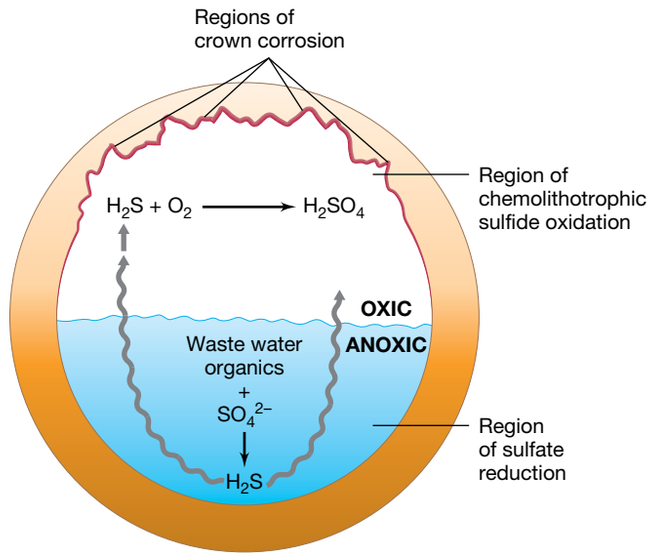



**Figure 22.25 Crown corrosion of concrete sewer pipes.** Corrosion is the result of a microbial sulfur cycle that develops within the transmission pipe. Sulfate-reducing bacteria consume organic material in the anoxic wastewater, producing $H_2S$. The latter is oxidized by sulfur-oxidizing chemolithotrophic bacteria that attach to the oxic upper (crown) pipe surface, accelerating corrosion from the production of $H_2SO_4$ (sulfuric acid).

sulfate reduction. The $H_2S$ is then released into the headspace of the pipe where conditions are oxic. The sulfide, or partially oxidized intermediates such as thiosulfate or sulfur, is then oxidized by neutrophilic thiobacilli such as *Thiobacillus thioparus* (Section 15.11). As the pH drops to 4–5 with continued microbial production of sulfuric acid, acidophilic sulfur-oxidizing species such as *Acidithiobacillus thiooxidans* displace the neutrophilic species. Destruction and ultimate structural failure of the concrete results from the reaction of sulfuric acid with the free lime in the concrete, producing $CaSO_4 \cdot 2H_2O$ (gypsum) that penetrates into the concrete. The gypsum then reacts with calcium aluminate in the concrete, leading to the production of the calcium aluminum sulfate mineral ettringite $[(CaO)_3 \cdot (Al_2O_3) \cdot (CaSO_4)_3 \cdot (32H_2O)]$, which by increasing internal pressure contributes to cracking and further acceleration of the corrosion process.

A series of steps and microbial ecology similar to that of crown corrosion contributes to the corrosion of concrete holding tanks and cooling towers, particularly those in or near the marine environment where sulfate levels are typically high. In the United States alone such corrosion costs billions of dollars a year for replacement structures and control of the progressing corrosion.

**MINIQUIZ**

- How does the production of oxalic acid by fungi contribute to the deterioration of stone building materials?
- Prior to better regulatory control of metal release into domestic wastewater systems, crown corrosion of sewer tiles was less of a problem. Why?



# Chapter Review

## I • Mineral Recovery and Acid Mine Drainage

**22.1** The capacity of bacteria to oxidize $Fe^{2+}$ aerobically at acidic pH is used to mine metals, principally copper-, uranium-, and gold-containing low-grade ores, through a process called microbial leaching. Bacterial oxidation of $Fe^{2+}$ to $Fe^{3+}$ is the key reaction in most microbial leaching processes because $Fe^{3+}$ can oxidize sulfide ores, liberating extractable metals in the ores under either oxic or anoxic conditions.

**Q Which crucial step in the oxidation of copper ores is carried out by *Acidithiobacillus ferrooxidans*? How is copper recovered from copper solutions produced by leaching?**

**22.2** Spontaneous microbial oxidation of ferrous iron in pyritic ore or coal that has been exposed to air and water, such as occurs during some coal-mining operations, causes a type of pollution called acid mine drainage.

**Q Which *Bacteria* and *Archaea* play a major role in acid mine drainage? Why do they carry out the reactions that they do? Why is air necessary for this process?**

## II • Bioremediation

**22.3** Although an inorganic pollutant such as uranium cannot be destroyed, containment is possible by reducing its mobility. For example, metal-reducing microorganisms in a region of uranium contamination can be stimulated to reduce $U^{6+}$ to $U^{4+}$, forming the immobile uranium mineral *uraninite* that does not move into the groundwater.

**Q What could thwart microbial bioremediation of a site that contains buried nuclear weapons that are leaking uranium?**

**22.4** Hydrocarbons are excellent carbon sources and electron donors for bacteria and are readily oxidized when $O_2$ is available. Hydrocarbon-oxidizing bacteria bioremediate spilled oil, and their activities can be assisted by addition of inorganic nutrients.

**Q What physical and chemical conditions are necessary for the rapid microbial degradation of oil in aquatic environments? Design an experiment that would allow you to test which conditions optimized the oil oxidation process.**

**22.5** Some xenobiotics (chemicals new to nature) persist, whereas others are readily degraded, depending on their chemistries. Dechlorination is a major means of detoxifying xenobiotics that reach anoxic environments. With the exception of readily degradable microbial plastics, recalcitrant synthetic plastics are major environmental concerns.

**Q Why are some bacterial transformations of xenobiotics possible only through cometabolism?**

## III • Wastewater and Drinking Water Treatment

**22.6** Sewage and industrial wastewater treatment reduces the BOD of wastewater. Primary, secondary, and tertiary wastewater treatment employs physical, biological, and physicochemical processes. After secondary or tertiary treatment, effluent water has significantly reduced BOD and is suitable for release into the environment.

**Q Trace the treatment of wastewater in a typical plant from incoming water to release. What is the overall reduction in the BOD for typical household wastewater? What is the overall reduction in the BOD for typical industrial wastewater?**

**22.7** Advanced wastewater treatment, such as enhanced biological phosphorus removal, is used to improve the quality of the treated wastewater. Of increasing concern are pharmaceuticals and ingredients in personal care products that are not degraded by conventional treatment systems and that can have adverse environmental effects, even at very low concentrations.

**Q Given its merits, why has advanced wastewater treatment not been widely adopted?**

**22.8** Drinking water purification plants employ industrial-scale physical and chemical systems that remove or neutralize biological, inorganic, and organic contaminants from natural, community, and industrial sources. Water purification plants employ clarification, filtration, and chlorination processes to produce potable water.

**Q Identify (stepwise) the process of purifying drinking water. What important contaminants are targeted by each step in the process?**

**22.9** The many miles of pipes for municipal drinking water distribution systems and premise plumbing have created new microbial habitats. Most microbes here are associated with the pipe walls as biofilms, resulting in a community that is more resistant to chlorine and that can sustain or sequester opportunistic pathogenic bacteria, such as *Mycobacterium, Legionella*, and *Pseudomonas*. The ability of some of these to grow within protist cells may increase their pathogenicity.

**Q What features of municipal and premise water distribution systems might contribute to a microbial health hazard? Why might showering increase your exposure to opportunistic pathogens?**

## IV • Indoor Microbiology and Microbially Influenced Corrosion

**22.10** The indoor air and surfaces of dwellings and other buildings contain a diversity of mostly harmless saprophytic microbes that are for the most part a reflection of the humans and animals that reside there. However, the microbiota of certain parts of the indoor built environment, such as bathrooms and toilets, may contain enteric pathogens from aerosols generated there.

**Q From the perspective of a child's health, can a home be "too clean"? Explain.**

**22.11** Corrosion of metal structures exposed to the environment can be accelerated by microbial activity during microbially influenced corrosion. Structures in or near seawater are particularly prone to corrosion as a consequence of the direct and indirect activities of sulfate-reducing bacteria.

**Q How does microbial metabolism accelerate the corrosion of various metals?**

**22.12** Microbial contribution to the structural degradation of stone and concrete is called biodeterioration. Complex microbial communities colonize the stone and produce substances that dissolve and mobilize its mineral constituents. Crown corrosion of concrete sewer lines results from the concerted activities of sulfate-reducing and sulfur-oxidizing bacteria growing within the wastewater and the headspace of sewer pipes, respectively. The resulting sulfuric acid is primarily responsible for the destruction of the concrete.

**Q How have fungi adapted in order to survive harsh conditions on stone building materials?**



# Application Questions

1. Acid mine drainage is in part a chemical process and in part a biological process. Discuss the chemistry and microbiology that lead up to acid mine drainage and point out the key reactions that are biological. What ways can you think of to prevent acid mine drainage? How might you prevent further generation of acid drainage?
2. Why is reduction of BOD in wastewater a primary goal of wastewater treatment? What are the consequences of releasing wastewater with a high BOD into local water sources such as lakes or streams?
3. Discuss the microbial ecology contributing to crown corrosion of concrete sewer lines. In consideration of this ecology, what intervention strategies might be useful in reducing or eliminating corrosion?

# Chapter Glossary

**Acid mine drainage** acidic water containing $H_2SO_4$ derived from the microbial and spontaneous oxidation of iron sulfide minerals released by coal mining

**Anaerobic treatment** degradative and fermentative reactions carried out by microorganisms under anoxic conditions to treat sludge solids or wastewater containing high levels of insoluble organic materials

**Biochemical oxygen demand (BOD)** the relative amount of dissolved oxygen consumed by microorganisms for complete oxidation of bioavailable organic and inorganic material in a water sample

**Bioremediation** the cleanup of oil, toxic chemicals, and other pollutants by organisms, usually microorganisms

**Chloramine** a disinfectant chemical manufactured on-site by combining chlorine and ammonia at precise ratios

**Chlorination** disinfecting water with $Cl_2$ at a sufficiently high concentration that a residual level is maintained throughout the distribution system

**Clarifier** a reservoir in which suspended solids in raw water are coagulated and removed through precipitation

**Coagulation** the formation of large, insoluble particles from much smaller, colloidal particles by the addition of aluminum sulfate and anionic polymers

**Crown corrosion** the destruction of the upper half, or crown, of concrete wastewater pipes by sulfuric acid produced through the concerted activities of sulfate-reducing and sulfur-oxidizing bacteria

**Distribution system** water pipes, storage reservoirs, tanks, and other equipment used to deliver drinking water to consumers or store it before delivery

**Effluent water** treated wastewater discharged from a wastewater treatment facility

**Filtration** the removal of suspended particles from water by passing it through one or more permeable membranes or media (e.g., sand, anthracite, or diatomaceous earth) and ion exchangers

**Finished water** water delivered to the distribution system after treatment

**Flocculation** the water treatment process after coagulation that uses gentle stirring to cause suspended particles to form larger, aggregated masses (flocs)

**Microbial leaching** the extraction of valuable metals such as copper from sulfide ores by microbial activities

**Microbial plastics** polymers consisting of microbially produced (and thus biodegradable) substances, such as polyhydroxyalkanoates

**Microbially influenced corrosion (MIC)** the contribution of microbial metabolic activities to accelerating the corrosion of metal and concrete structures

**Potable** drinkable; safe for human consumption

**Primary disinfection** the introduction of sufficient chlorine or other disinfectant into clarified, filtered water to kill existing microorganisms and inhibit further microbial growth

**Primary wastewater treatment** physical separation of wastewater contaminants, usually by separation and settling

**Pyrite** a common iron-containing ore, $FeS_2$

**Raw water** surface water or groundwater that has not been treated in any way (also called untreated water)

**Reductive dechlorination** an anaerobic respiration in which a chlorinated organic compound is used as an electron acceptor, usually with the release of $Cl^-$

**Secondary aerobic wastewater treatment** oxidative reactions carried out by microorganisms under aerobic conditions to treat wastewater containing low levels of organic materials

**Secondary disinfection** the maintenance of sufficient chlorine or other disinfectant residual in the water distribution system to inhibit microbial growth

**Sediment** soil, sand, minerals, and other large particles found in raw water

**Sewage** liquid effluents contaminated with human or animal fecal material

**Suspended solid** a small particle of solid pollutant that resists separation by ordinary physical means

**Tertiary treatment** any treatment process in which unit operations are added for the further processing of the secondary treatment effluent or solids

**Turbidity** a measurement of suspended solids in water

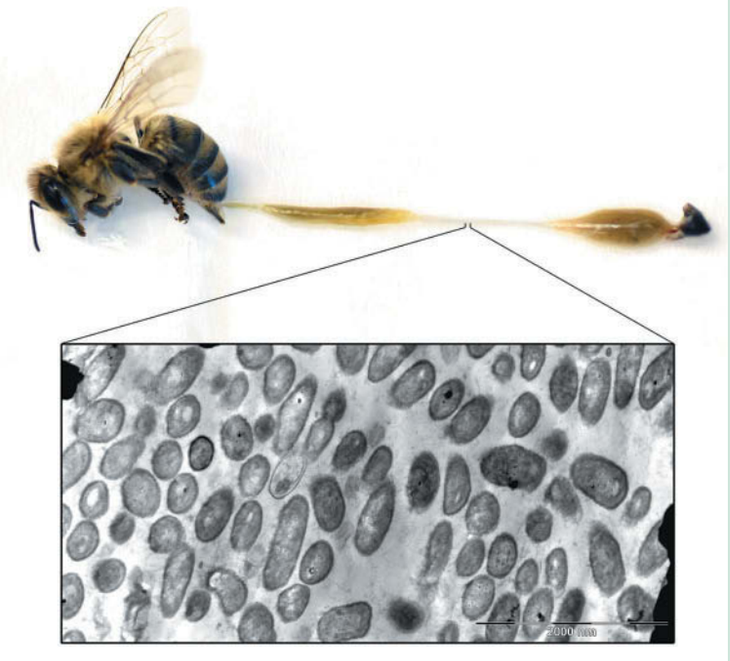
**Untreated water** surface water or groundwater that has not been treated in any way (also called raw water)

**Wastewater** liquid derived from domestic sewage or industrial sources, which cannot be discarded in untreated form into lakes or streams

**Xenobiotic** a synthetic compound not produced by organisms in nature

# 23

# Microbial Symbioses with Microbes, Plants, and Animals





## microbiologynow

### The Inner Life of Bees

Bees and other animal pollinators provide vital ecosystem services. Thus, the loss of nearly 60% of honeybee colonies in the USA since 1947 is a major concern. Several factors are thought to contribute to this decline including fragmentation of habitats, pesticides, pollution, land use changes, and climate change. The decline of bee populations has also drawn attention to the importance of the bee gut microbiome for combating pathogens and environmental stressors. For example, when the European bumblebee is experimentally deprived of its gut microbiota, the insect is much more susceptible to infection with a common protozoan parasite.

The gut communities of bumblebees and honeybees (photo) are surprisingly simple and consist of only five dominant and culturable bacterial species. These include two gram-negative *Proteobacteria* (*Snodgrassella alvi* and *Gilliamella apicola*), two *Lactobacillus* species, and a *Bifidobacterium* species. *S. alvi* and *G. apicola* pack the lumen of the honeybee gut (see transmission electron micrograph). Although different strains of each honeybee gut microbe are closely related in 16S rRNA gene sequence, their genomes have diverged significantly through coevolution with bees over millions of years. As a result, strains have become host-specific. For example, *S. alvi* isolated from honeybees will not colonize bumblebees, and vice versa.

Genome sequence analyses have also identified metabolic interdependencies among microbes and the bee. For instance, *S. alvi* oxidizes the fermentation products of *G. apicola*, and *G. apicola* has genes for the degradation of pectin, a polysaccharide in the cell wall of pollen grains. Because bees do not produce pectinase and pollen is a key component of their diet, bee gut microbiota are essential for utilization of this important food source. Some bee gut microbes also carry genes for the utilization of uncommon sugars such as arabinose and raffinose that are indigestible and potentially toxic to the bee.

The relative simplicity of the bee gut microbial community has made this insect an excellent model for animal–microbe associations in general and the microbiology of bee health in particular. Bees are major pollinators of fruits and other plants important in the human diet. Thus, studies of the bee microbiome highlight both the environmental and economic importance of developing a better understanding of the "microbial life" of bees.

**Source:** Kwong, W.K., P. Engel, H. Koch, and N.A. Moran. 2014. Genomics and host specialization of honey bee and bumble bee gut symbionts. *Proc. Natl. Acad. Sci. USA 111:* 11509–11514.

In this chapter we consider relationships of microorganisms with other microorganisms or with macroorganisms—prolonged and intimate relationships of a type called **symbiosis**, a word that means "living together." In Chapter 24 we examine microbial symbioses with humans.

Microorganisms that live within or on plants and animals are grouped according to how they affect their hosts. *Parasites* are microorganisms that benefit at some expense to the host, *pathogens* actually cause a disease in the host, *commensals* have no discernible effect on the host, and *mutualists* are beneficial to the host. In one way or another, all microbial symbioses benefit the microorganism. In this chapter we focus on **mutualisms**—relationships in which both partners benefit. We view the microorganisms as intimate evolutionary partners that influence both the evolution and physiology of their hosts. Most mutualistic symbioses of microbes with plants or animals had their origins many millions of years ago and have evolved to benefit the physiology of both partners, a process called **coevolution**. Over time, the changes in both partners may be so extensive that the symbiosis becomes obligate—neither the microbe nor the host can survive in the absence of the other.


(a)


(b)

**Figure 23.1 Lichens.** *(a)* A lichen growing on a branch of a dead tree. *(b)* Lichens coating the surface of a large rock.

# I • Symbioses between Microorganisms

Many microbial species have intimate and beneficial associations with other microbial species. Direct microscopic observations of natural samples show that many microbes are not solitary entities but are associated with other microbes on surfaces or as suspended aggregates of cells. In most cases, the advantages conferred by an association are unknown. Because microbial ecologists have recognized that *communities* of interacting microbial populations—not individual organisms—control critical environmental processes, research to discover the nature of strictly microbial symbioses has increased. We present here two microbial mutualisms where the advantages to both partners are clear.

## 23.1 Lichens

**Lichens** are readily visible, leafy or encrusting microbial symbioses often found growing on bare rocks, tree trunks, house roofs, and bare soils—surfaces where other organisms typically do not grow (**Figure 23.1**). A lichen is a mutualistic association between two dominant microorganisms, a fungus, usually an ascomycete (Section 18.12), and either an alga or a cyanobacterium. The alga or cyanobacterium is the phototrophic partner and produces organic matter that feeds the fungus. The fungus, unable to carry out photosynthesis, provides a firm anchor within which the phototrophic partner can grow, protected from erosion by rain or wind. Cells of the phototroph are embedded in defined layers or clumps among cells of the fungus (**Figure 23.2**). The characteristic morphology of any given lichen is primarily determined by the fungus, and many fungi (more than 18,000 named species) are able to form lichen associations. Diversity among the phototrophs is much lower, and thus many different kinds of lichens have the same phototrophic partner. Many cyanobacteria that partner in lichens are nitrogen-fixing species, organisms such as *Anabaena* or *Nostoc* (Sections 14.6 and 15.3).

The fungus clearly benefits from associating with the phototroph in the lichen symbiosis, but how does the phototroph benefit besides just having a sturdy substratum? *Lichen acids*, complex organic compounds secreted by the fungus, promote the dissolution and chelation of inorganic nutrients from the rock or other surface that are needed by the phototroph. Another role of the fungus is to protect the phototroph from drying; most of the habitats

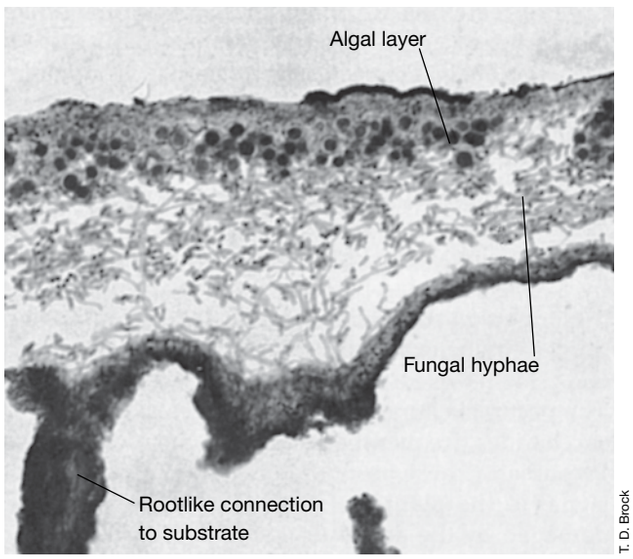



**Figure 23.2 Lichen structure.** Photomicrograph of a cross section through a lichen. The algal layer is positioned within the lichen structure near the top so as to receive the most sunlight. The fungal partners form the structural layers (cortex) positioned above and below the algal layer.

in which lichens live are dry, and fungi are, in general, better able to tolerate dry conditions than are the phototrophs. The fungus actually facilitates the uptake of water and sequesters some for the phototroph. Lichens typically grow quite slowly. For example, a lichen 2 cm in diameter growing on the surface of a rock may be several years old. Lichen growth varies from 1 mm or less per year to over 3 cm per year, depending on the organisms composing the symbiosis and the amount of rainfall and sunlight received.

The general view that lichens are simple two-partner assemblages has been challenged by recent culture-independent studies (Chapter 19) showing that lichens also host a bacterial and archaeal microbiota that may benefit the association by supplying additional nutrients such as vitamin $B_{12}$, protection from toxic compounds, and metabolites that have antimicrobial activity. Another remarkable recent discovery based on culture-independent analyses overturns the accepted understanding that only a single fungal species is a partner in this symbiosis. The cortex of many lichens, formed by fungal structural tissue bounding the phototroph layer (Figure 23.2), is now known to be composed of a basidiomycete yeast in addition to the known ascomycete (Chapter 18). This previously unrecognized yeast partner may account for the inability to reconstitute natural lichen thalli in axenic conditions from only the ascomycete and phototroph. These examples demonstrate how recent advances in culture-independent methods, despite being in an early stage, are bringing new understanding even to well-studied symbioses.

**MINIQUIZ**

- What two microbes form a partnership in the lichen symbiosis? What are the benefits to both partners?
- Besides organic compounds, of what benefit to the fungus is a mutualism with *Anabaena*?

## 23.2 *"Chlorochromatium aggregatum"*

Microbial mutualisms called **consortia** form in freshwater environments. A commonly observed consortium develops between nonmotile green sulfur bacteria (phototrophs that are colored either green or brown) and certain motile, nonphototrophic bacteria. These consortia are found worldwide in stratified sulfidic freshwater lakes and can account for up to 90% of the green sulfur bacteria present and nearly 70% of the bacterial biomass in these lakes. The basis of the mutualism of these consortia is in the phototrophic production of organic matter by the green sulfur bacterium and the motility and organic matter consumption of the chemotrophic partner organism. Each consortium has been given a genus and species name, but since these names do not denote true species (because they are not a single organism), the names are enclosed in quotation marks. We examined the general biology of these consortia in Section 15.6.

### Nature of the Consortium

The morphology of a green sulfur bacterial consortium depends upon its species composition. The consortium generally consists of 13–69 green sulfur bacteria, called *epibionts*, surrounding and attached to a central, colorless, flagellated, rod-shaped bacterium (Figure 23.3). Several distinct motile phototrophic consortia have been recognized based on the color, morphology, and presence or absence of gas vesicles (↔ Section 2.9) of the epibionts. For example, in "*Chlorochromatium aggregatum*" the central bacterium is surrounded by rod-shaped green bacteria, whereas in "*Pelochromatium roseum*" the epibiont is brown. The consortium "*Chlorochromatium glebulum*" is bent and includes green epibionts that contain gas vesicles (Figure 23.3).

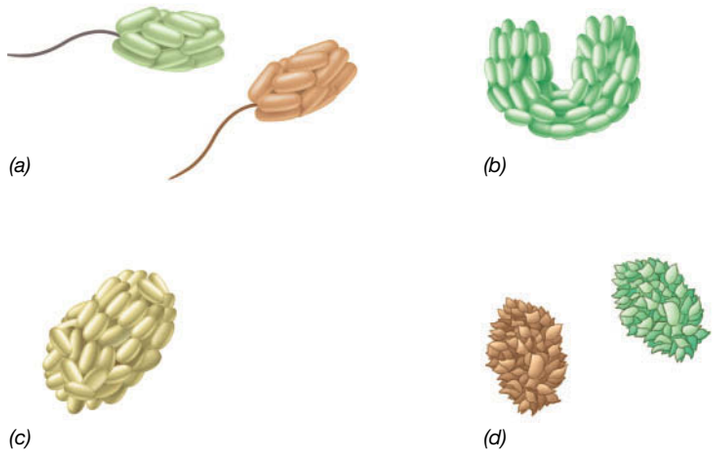
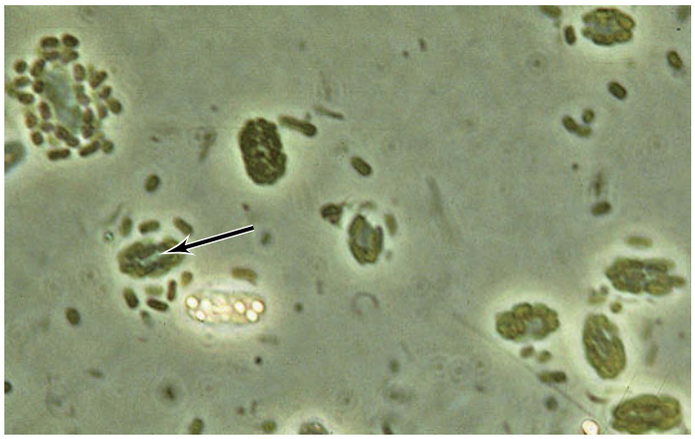



**Figure 23.3 Drawings of some motile phototrophic consortia found in freshwater lakes.** Green epibionts: *(a) "Chlorochromatium aggregatum," (b) "C. glebulum," (c) "C. magnum," (d) "C. lunatum."* Brown epibionts: *(a) "Pelochromatium roseum," (d) "P. selenoides."* The epibionts are 0.5–0.6 μm in diameter. Adapted from Overmann, J., and H. van Gemerden. 2000. *FEMS Microbiol. Rev. 24:* 591–599.

Green sulfur bacteria are obligately anaerobic phototrophs that form a distinct phylum (*Chlorobi*, ↔ Section 15.6). The green and brown species differ in the types of bacteriochlorophyll and carotenoids they contain. Both green and brown species are found in stratified lakes where light penetrates to depths at which the water contains hydrogen sulfide ($H_2S$), the primary electron donor for photosynthetic $CO_2$ fixation by the phototroph. In stratified lakes, the motile consortia reposition rapidly to remain in regions where conditions are most favorable for photosynthesis in the constantly changing gradients of light, oxygen, and sulfide that occur throughout the course of a day. Water samples collected from depths where these conditions are most favorable are enriched in this morphologically conspicuous consortium (Figure 23.4). The consortia show dark aversion (scotophobotaxis, ↔ Section 2.13) and positive chemotaxis toward sulfide.

Some free-living green sulfur bacteria, such as *Pelodictyon* (*Chlorobium*) *phaeoclathratiforme*, produce gas vesicles that regulate buoyancy and vertical position in the water column. However, the time they require for repositioning in the water column is from one to several days, which is not fast enough for tracking the more rapidly changing gradients. By contrast, motile consortia move up and down in the water column fast enough to follow the gradients of light and sulfide as they change on a diel basis.

Although green bacterial consortia were discovered almost a century ago, only with the advent of molecular techniques and newer culture methods has it become possible to study certain aspects of



J. Overmann and H. van Gemerden

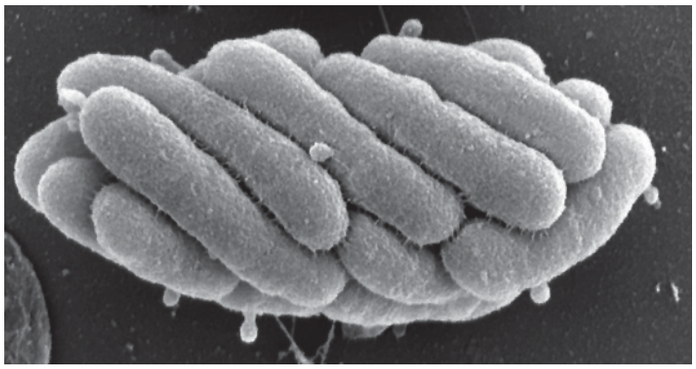
**Figure 23.4 Phase-contrast micrograph of "*Pelochromatium roseum*" from Lake Dagow (Brandenburg, Germany).** The preparation was compressed between a coverslip and microscope slide to reveal the central rod-shaped bacterium (arrow). A single consortium is about 3.5 μm in diameter. Used with permission from J. Overmann and H. van Gemerden. 2000. *FEMS Microbiol. Rev. 24*: 591–599.

these remarkable associations. Sequencing of 16S ribosomal RNA (rRNA) genes revealed a significant biogeography of epibionts in lakes of Europe and the United States. *Biogeography* is the study of the geographic distribution of organisms, in this case, the genetically distinct phototrophic consortia in different lakes. Epibionts in neighboring lakes have identical 16S rRNA gene sequences, whereas the sequences of morphologically similar epibionts in widely separated lakes differ. Phylogenetic analysis has shown that the mechanisms of cell–cell recognition responsible for stable morphology have evolved between particular epibionts and their central bacterium.

## Phylogeny and Metabolism of a Consortium

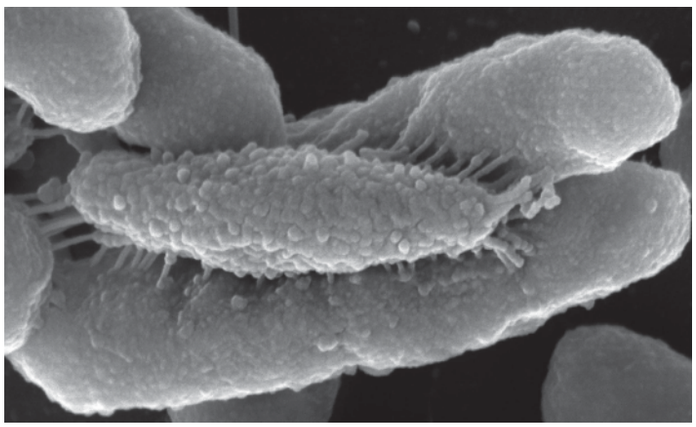
The epibiont of "*Chlorochromatium aggregatum*" has been isolated and grown in pure culture. Although this green sulfur bacterium, named *Chlorobium chlorochromatii*, can be grown in pure culture, no naturally free-living variant has been observed, supporting the view that in nature, a symbiotic lifestyle is obligate for epibionts. The central bacterium of "*Chlorochromatium aggregatum*" belongs to the *Betaproteobacteria* (Section 16.2). Interestingly, this bacterium requires α-ketoglutarate, an intermediate of the citric acid cycle (Section 3.9), and this is presumably supplied to it by the epibiont. However, the central cell only assimilates fixed carbon in the presence of light and sulfide—conditions in which the epibionts are active and can transfer nutrients to the central bacterium. Genomic analysis of the central bacterium of one consortium revealed massive gene loss, indicating that this organism is unable to grow independently of the green sulfur bacterium.

Recent studies comparing the transcriptome and proteome (Sections 9.9 and 9.10) of *C. chlorochromatii* growing alone or in association with the central rod bacterium have identified some features specifically related to the symbiosis. Approximately 50 proteins are unique to the symbiotic state. Most of approximately 350 differentially regulated genes are repressed when the organism is symbiotically associated, whereas only 19 genes are more highly expressed. Many of the more highly expressed genes encode proteins of amino acid metabolism and nitrogen regulation.



J. Overmann, with permission from J. Bacteriol

(a)



J. Overmann, with permission from J. Bacteriol

(b)

**Figure 23.5 Scanning electron micrographs of "*Chlorochromatium aggregatum*."** *(a) Chlorobium chlorochromatii* epibionts tightly clustered around a flagellated central bacterium. *(b)* The central bacterium exhibits numerous protrusions of its outer membrane that make intimate contact with the epibionts, possibly fusing the periplasms of the two gram-negative organisms. Cells of the epibiont are about 0.6 μm in diameter. Used with permission from G. Wanner, *et al.* 2008. *J. Bacteriol. 190:* 3721–3730.

These include the enzyme glutamate synthase (Section 3.14) and an ABC transporter of branched amino acids, suggesting that the metabolic coupling between the epibiont and central rod bacterium involves the exchange of amino acids.

Although it is not yet known whether the central bacterium transfers any organic compounds to the epibiont, this hypothesis can be tested now that the genome sequence of the central bacterium is known. Scanning electron microscopy of the consortium (Figure 23.5) has revealed that tubular extensions of the central bacterium's periplasm (Section 2.5) cover much of its surface and appear to fuse with the periplasm of the epibiont. If the two bacterial partners actually share a common periplasmic space, this would facilitate the transfer of nutrients from phototroph to chemotroph. The fact that the central bacterium is unable to grow without its phototrophic partner (while the phototroph can be grown in pure culture), and that organic compounds are only assimilated by the chemotroph in the light, is strong evidence that nutrients flow from the phototroph to feed the chemotroph and that the chemotroph is obligatorily dependent on its phototrophic partner.

**MINIQUIZ**

- What is the evidence that "*Chlorochromatium aggregatum*" is a stable product of evolution?
- What advantage does motility offer a phototrophic consortium?
- How might nutrients be shuttled between phototroph and chemotroph in the consortium?

# II • Plants as Microbial Habitats

Plants interact closely with microbes through their roots and leaf surfaces and even more intimately within their vascular tissue and cells. Most mutualisms between plants and microorganisms increase nutrient availability to the plants or defend them against pathogens. We consider three examples in the next three sections: (1) a mutualism where the nature of the symbiosis is understood in exquisite detail (root nodules), (2) a mutualism in which plants expand and interconnect their root system through association with a fungus (mycorrhizae), and (3) a symbiosis that is harmful to the plant (crown gall disease).

## 23.3 The Legume–Root Nodule Symbiosis

A plant–bacterial mutualism of great importance to humans is that of leguminous plants and nitrogen-fixing bacteria. *Legumes* are flowering plants that bear their seeds in pods and include such agriculturally important members as soybeans, clover, alfalfa, beans, and peas. These crops are key commodities for the food and agricultural industries, and the ability of legumes to grow without added nitrogen saves farmers millions of dollars in fertilizer costs yearly and reduces the polluting effects of fertilizer runoff.

The partners in a symbiosis are called *symbionts*, and most nitrogen-fixing bacterial symbionts of plants are collectively called *rhizobia*, derived from the name of a major genus, *Rhizobium*. Species of rhizobia are *Alpha-* or *Betaproteobacteria* (↔ Sections 16.1 and 16.2) (**Figure 23.6**) that can grow freely in soil or infect leguminous plants and establish a symbiotic relationship. The same genus (or even species) of legume can contain both rhizobial and nonrhizobial strains. Infection of legume roots by rhizobia leads to the formation of **root nodules** (**Figure 23.7**) in which the bacteria fix gaseous nitrogen ($N_2$) (↔ Section 14.6). Nitrogen fixation in root nodules accounts for a fourth of the $N_2$ fixed annually on Earth and is of enormous agricultural importance, as it increases the fixed nitrogen content of soil. Nodulated legumes can grow well on unfertilized bare soils that are nitrogen deficient, while other plants grow only poorly on them (**Figure 23.8**).


Joe Burton

**Figure 23.7 Soybean root nodules.** The nodules developed from infection by *Bradyrhizobium japonicum*. The main stem of this soybean plant is about 0.5 cm in diameter.

### Leghemoglobin and Cross-Inoculation Groups

In the absence of its bacterial symbiont, a legume cannot fix $N_2$. Rhizobia, on the other hand, can fix $N_2$ when grown in pure culture under microaerophilic conditions (a low-oxygen environment is

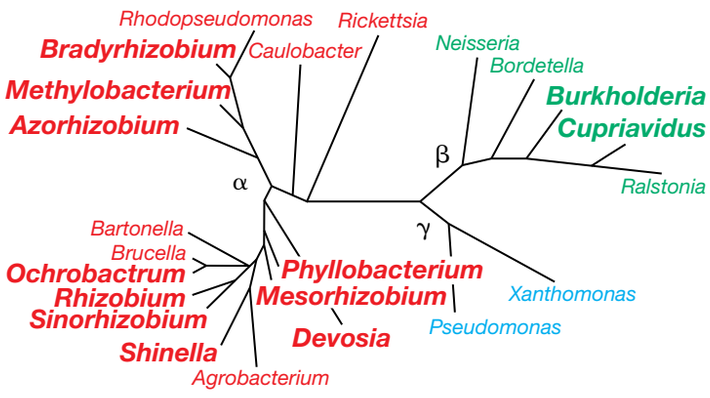



**Figure 23.6 Phylogeny of rhizobial (names in boldface) and related genera inferred from analysis of 16S rRNA gene sequences.** More than 70 species of rhizobia are found in 12 genera of *Alpha-* and *Betaproteobacteria*.


Ben B. Bohlool

**Figure 23.8 Effect of nodulation on plant growth.** A field of unnodulated (left) and nodulated (right) soybean plants growing in nitrogen-poor soil. The yellow color is typical of chlorosis, the result of nitrogen starvation.

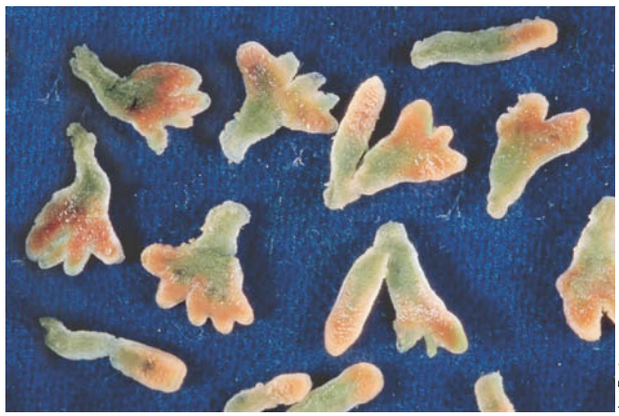



**Figure 23.9 Root nodule structure.** Sections of root nodules from the legume *Coronilla varia*, showing the reddish pigment leghemoglobin.

necessary because the key nitrogen-fixing enzyme, called *nitrogenase*, is inactivated by high levels of $O_2$, ↩ Section 14.6). In the nodule, $O_2$ levels are precisely controlled by the $O_2$-binding protein **leghemoglobin**. Production of this iron-containing protein in healthy $N_2$-fixing nodules (**Figure 23.9**) is induced through the interaction of the plant and bacterial partners. Leghemoglobin functions as an "oxygen buffer," cycling between the oxidized ($Fe^{3+}$) and reduced ($Fe^{2+}$) forms of iron to supply sufficient $O_2$ for bacterial respiration while keeping unbound $O_2$ within the nodule low. The ratio of leghemoglobin-bound $O_2$ to free $O_2$ in the root nodule is thus maintained on the order of 10,000:1.

There is a marked specificity between the species of legume and rhizobium that can establish a symbiosis. A particular rhizobial species is able to infect certain species of legumes but not others. A group of related legumes that can be infected by a particular rhizobial species is called a *cross-inoculation group*. Each group consists of all the legume species that will develop nodules when inoculated with rhizobia obtained from any other legume of the group (**Table 23.1**). If legumes are inoculated with the correct rhizobial strain, leghemoglobin-rich, $N_2$-fixing nodules develop on their roots (Figures 23.7–23.9).

**TABLE 23.1 Major cross-inoculation groups of leguminous plants**

| *Host plant* | *Nodulated by* |
|---|---|
| Pea | *Rhizobium leguminosarum* biovar *viciae*[a] |
| Bean | *Rhizobium leguminosarum* biovar *phaseoli*[a] |
| Bean | *Rhizobium tropici* |
| Lotus | *Mesorhizobium loti* |
| Clover | *Rhizobium leguminosarum* biovar *trifolii*[a] |
| Alfalfa | *Sinorhizobium meliloti* |
| Soybean | *Bradyrhizobium japonicum* |
| Soybean | *Bradyrhizobium elkanii* |
| Soybean | *Sinorhizobium fredii* |
| *Sesbania rostrata* (a tropical legume) | *Azorhizobium caulinodans* |

[a]Several varieties (biovars) of *Rhizobium leguminosarum* exist, each capable of nodulating a different legume.

## Steps in Root Nodule Formation

How root nodules form is well understood for most rhizobia (**Figure 23.10**). The steps are as follows:

1. Recognition of the correct partner by both plant and bacterium and attachment of the bacterium to the root hairs
2. Secretion of oligosaccharide signaling molecules (Nod factors) by the bacterium
3. Bacterial invasion of the root hair
4. Movement of bacteria to the main root by way of the infection thread
5. Formation of modified bacterial cells (bacteroids) within the plant cells, development of the $N_2$-fixing state, and continued plant and bacterial cell division forming the mature root nodule

Another mechanism of nodule formation that does not require Nod factors is used by some species of phototrophic rhizobia. This mechanism has yet to be fully elucidated, but appears to require

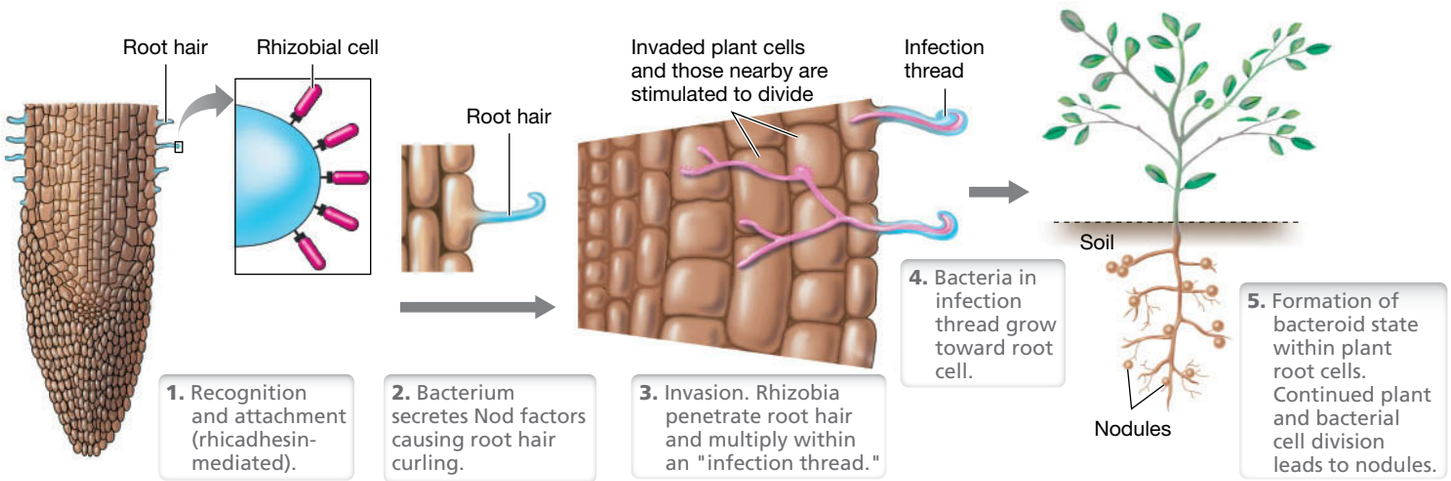



**Figure 23.10 Steps in the formation of a root nodule in a legume infected by *Rhizobium*.** Formation of the bacteroid state is a prerequisite for nitrogen fixation. The time course of nodulation events from infection to effective nodule is about 1 month for soybeans. See Figure 23.15 for physiological activities in the nodule.

the bacterial production of *cytokinins*. Cytokinins are plant hormones derived from adenine or phenylurea necessary for cell growth and differentiation.

## Attachment and Infection

The roots of leguminous plants secrete organic compounds that stimulate the growth of a diverse rhizosphere microbial community. If rhizobia of the correct cross-inoculation group are in the soil, they will form large populations and eventually attach to the root hairs that extend from the roots of the plant (Figure 23.10). An adhesion protein called *rhicadhesin* is present on the cell surfaces of rhizobia. Other substances, such as carbohydrate-containing proteins called *lectins* and specific receptors in the plant cytoplasmic membrane, also play roles in plant–bacterium attachment.

After attaching, a rhizobial cell penetrates into the root hair, which curls in response to substances (Nod factors) secreted by the bacterium. The bacterium then induces formation by the plant of a cellulosic tube, called the **infection thread** (Figure 23.11*a*), which spreads down the root hair. Root cells adjacent to the root hairs subsequently become infected by rhizobia, and plant cells divide. Continued plant cell division forms the tumorlike nodule (Figure 23.11*a*) consisting of plant cells filled with bacteroids (discussed below and Figure 23.11*b*). A different mechanism of infection is used by some rhizobia adapted to aquatic or semiaquatic tropical legumes (see Figure 23.16). These rhizobia enter the plant at the loose cellular junctions of roots emerging perpendicular from an established root (*lateral roots*). Following entry into the plant, some of the rhizobia develop infection threads, whereas others do not.

## Bacteroids

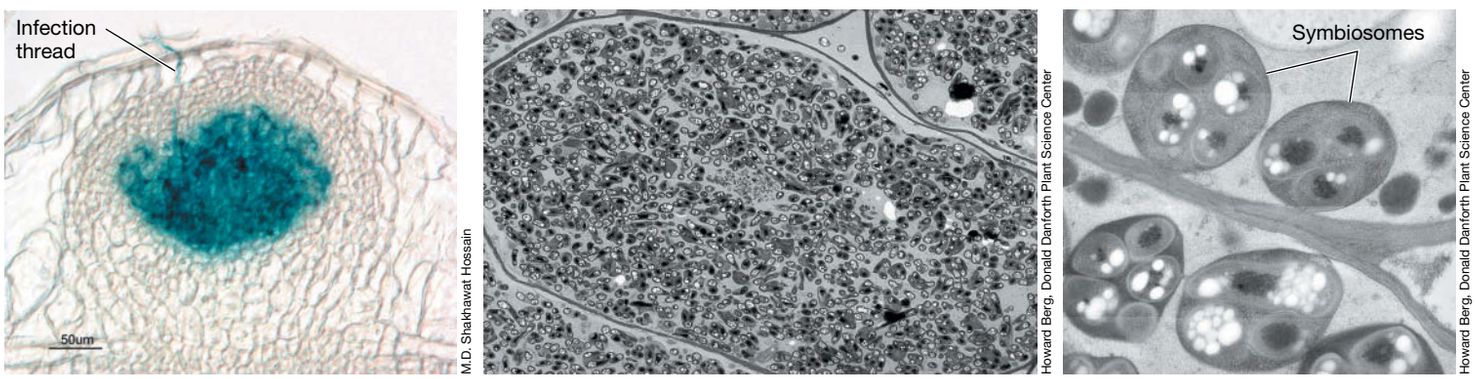
The rhizobia multiply rapidly within the plant cells and become transformed into swollen, misshapen, and branched cells called **bacteroids**. A microcolony of bacteroids becomes surrounded by portions of the plant cytoplasmic membrane to form a structure called the *symbiosome* (Figure 23.11*c*), and only after the symbiosome forms does $N_2$ fixation begin. Nitrogen-fixing nodules can be detected experimentally by the reduction of acetylene to ethylene (Section 14.6). When the plant dies, the nodule deteriorates, releasing bacteroids into the soil. Although bacteroids are incapable of division, a small number of dormant rhizobial cells are always present in the nodule. These now proliferate, using some of the products of the deteriorating nodule as nutrients. The bacteria can then initiate infection the next growing season or maintain a free-living existence in the soil.

## Nodule Formation: *nod* Genes, Nod Proteins, and Nod Factors

Rhizobial genes that direct the steps in nodulation of a legume are called *nod genes*. It is thought that the ability to form nodules has independently emerged multiple times through the horizontal transfer of such genes as *nod* and *nif* that are located on plasmids or transferable regions of chromosomal DNA. In *Rhizobium leguminosarum* biovar *viciae*, which nodulates peas, ten *nod* genes have been identified. The *nodABC* genes encode proteins that produce oligosaccharides called **Nod factors**; these induce root hair curling and trigger cell division in the pea plant, eventually leading to formation of the nodule (see Figure 23.15 for a description of root nodule biochemistry).

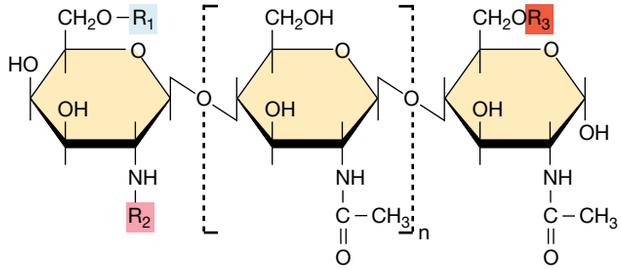
Nod factors are lipochitin oligosaccharides to which various substituents are bonded (Figure 23.12) that function as primary rhizobial signaling molecules triggering legumes to develop new plant organs: root nodules that host the bacteria as nitrogen-fixing bacteroids (Figure 23.11). Resolving the details of the signaling pathway triggered by Nod binding to cell surface receptors (NFR1 and NFR2) and leading to the induction of organogenesis (nodule formation) is an active area of research (Figure 23.13). Interestingly, many elements of the signaling pathway leading to nodulation are also used by the mycorrhizal fungi for infection of plant roots (Figure 23.13 and Section 23.4).

Which plants a given rhizobial species can infect is in part determined by the structure of the Nod factor it produces. Besides the *nodABC* genes, which are universal and whose




(a) (b) (c)

**Figure 23.11 The infection thread and formation of root nodules.** *(a)* Light micrograph of an early-stage nodule from a legume (*Lotus japonicus*) infected with a rhizobium strain containing a chromosomal copy of the *lacZ* gene. The nodule was sectioned and bacterial distribution (blue) determined using an activity stain (X-gal) that turns blue when cleaved by the enzyme β-galactosidase (Section 12.2 and Figure 12.8). An infection thread, consisting of a cellulosic tube through which bacteria move to root cells, is clearly visible extending from the surface to the interior. *(b)* Transmission electron micrograph through a soybean (*Glycine max*) nodule infected with *Bradyrhizobium japonicum*, showing bacteroid-filled plant cells. The plant cell is approximately 50 μm long. *(c)* Higher-magnification micrograph showing individual symbiosomes, each filled with several bacteroids. The clear areas in each bacteroid are the storage polymer poly-β-hydroxybutyrate (Section 2.8). Bacteroids are about 2 μm long.

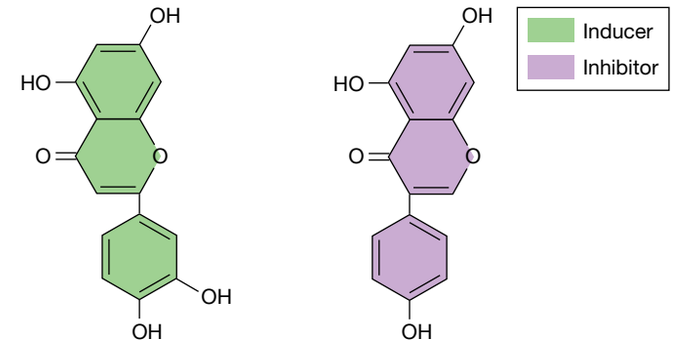



(a)

| Rhizobial or AM fungus species | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| *Sinorhizobium meliloti* (alfalfa) | Ac | C16:2 or C16:3 | $SO_3H$ |
| *Rhizobium leguminosarum* biovar *viciae* (pea) | Ac | C18:1 or C18:4 | H or Ac |
| *Glomus intraradices* (many agricultural crops) | H | C16 or C16:1 or C16:2 or C18 or C18:1Δ9Z | H or $SO_3H$ |

(b)

**Figure 23.12 Nod and Myc factors.** *(a)* General structure of Nod factors produced by rhizobia species (*Sinorhizobium meliloti* and *Rhizobium leguminosarum* biovar *viciae*) and the Myc factor produced by *Glomus intraradices*, an arbuscular mycorrhizal (AM) fungus (Section 23.4). The central hexose unit can repeat up to three times for different Nod factors, and repeat either two or three times for the different Myc factors. *(b)* Table of the structural differences ($R_1$, $R_2$, $R_3$) that define the precise signaling factors of each species. C16:1, C16:2, and C16:3, palmitic acid with either one, two, or three double bonds, respectively; C18:1, oleic acid with one double bond; C18:1Δ9Z, the *trans* isomer of oleic acid with one double bond at the 9th C–C bond; C18:4, oleic acid with four double bonds; Ac, acetyl.




5,7,3′,4′-Tetrahydroxyflavone (a)

5,7,4′-Trihydroxyisoflavone (b)

**Figure 23.14 Plant flavonoids and nodulation.** Structures of flavonoid molecules that are *(a)* an inducer of *nod* gene expression and *(b)* an inhibitor of *nod* gene expression in *Rhizobium leguminosarum* biovar *viciae*. Note the similarities in the structures of the two molecules. The common name of the structure shown in part *a* is *luteolin*, and it is a flavone derivative. The structure in part *b* is called *genistein*, an isoflavone derivative.

products synthesize the Nod backbone, each cross-inoculation group contains *nod* genes that encode proteins that chemically modify the Nod factor backbone to form its species-specific molecule (Figure 23.12). In *R. leguminosarum* biovar *viciae*, *nodD* encodes the regulatory protein NodD, which controls transcription of other *nod* genes. After interacting with inducer molecules, NodD *promotes* transcription and is thus a *positive* regulatory protein (Section 6.3). NodD inducers are plant flavonoids, organic molecules that are widely secreted by plants. Some flavonoids that are structurally very closely related to *nodD* inducers in *R. leguminosarum* biovar *viciae* inhibit *nod* gene expression in other rhizobial species (Figure 23.14). This indicates that part of the specificity observed between plant and bacterium in the rhizobia–legume symbioses is controlled by the chemistry of the flavonoids secreted by each species of legume.

## Biochemistry of Root Nodules

As discussed in Section 14.6, $N_2$ fixation requires the enzyme *nitrogenase*. Nitrogenase from bacteroids shows the same biochemical properties as the enzyme from free-living $N_2$-fixing bacteria, including $O_2$ sensitivity and the ability to reduce acetylene as well as $N_2$. Bacteroids are dependent on the plant for the electron donor for $N_2$ fixation. The major organic compounds transported across

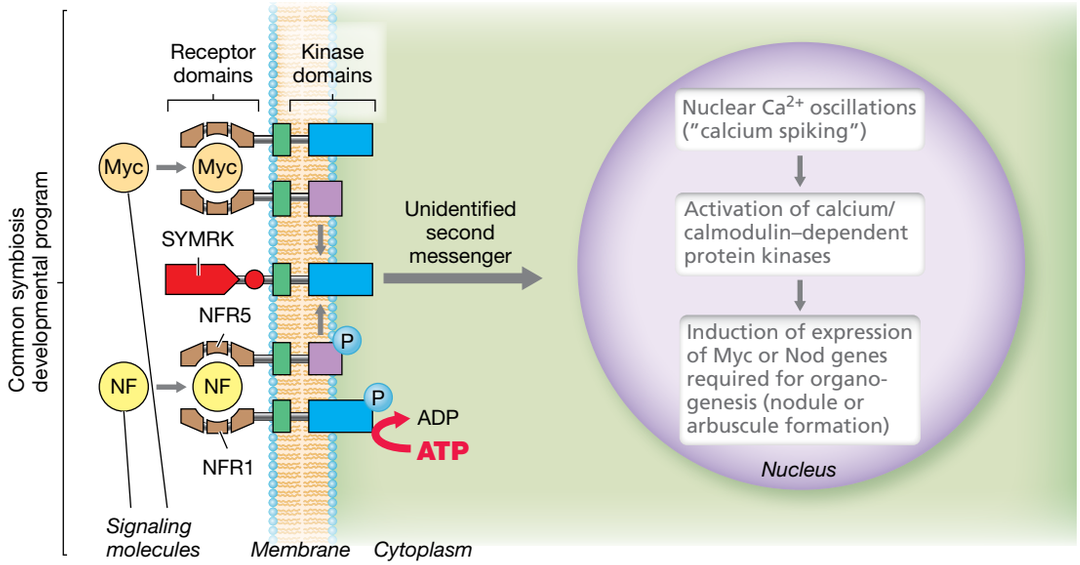



**Figure 23.13 Nod and Myc signaling pathways in root nodule and mycorrhizal arbuscule formation.** Nod factor (NF) signaling involves at least three membrane-associated receptors (NFR1, NFR5, and SYMRK) that together initiate nodulation via protein phosphorylation. NFR1 and SYMRK have active kinase domains (blue), whereas NFR5 kinase is inactive. The direct binding of NF to a complex of NFR1 and NFR5 at the plant cell cytoplasmic membrane initiates signal transduction by activation of the NFR1 kinase. The resulting phosphorylation of the NFR cytoplasmic domains triggers events leading to formation of the infection thread. Signal transduction to the SYMRK by the NFR1–NFR5–Nod factor complex (or by an unidentified receptor for the Myc factor) is part of a conserved symbiosis program in which induction of calcium signaling in the plant cell nucleoplasm triggers gene expression changes and production of plant growth hormones (cytokinins) required for nodule or arbuscule formation. See Section 23.4 for a discussion of mycorrhizae.

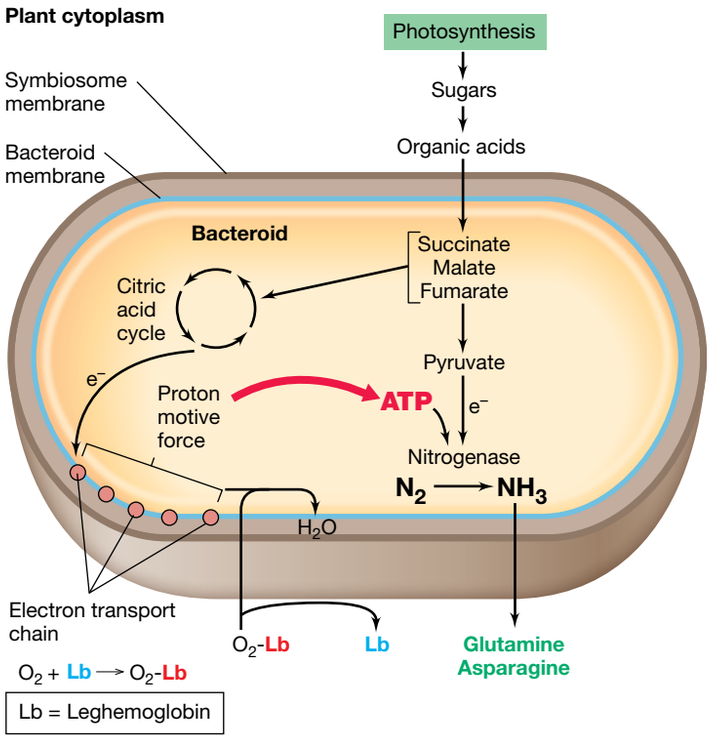



**Figure 23.15 The root nodule bacteroid.** Schematic diagram of major metabolic reactions and nutrient exchanges in the bacteroid. The symbiosome is a collection of bacteroids surrounded by a membrane originating from the plant (see Figure 23.11*c*).

the symbiosome membrane (Figure 23.11*c*) and into the bacteroid proper are citric acid cycle intermediates, in particular, the $C_4$ organic acids *succinate, malate*, and *fumarate* (Figure 3.16) (**Figure 23.15**). These are used as electron donors for ATP production and, following conversion to pyruvate, as the ultimate source of electrons for the reduction of $N_2$.

The product of $N_2$ fixation is ammonia ($NH_3$), and the plant assimilates most of this $NH_3$ by forming organic nitrogen compounds. The $NH_3$-assimilating enzyme glutamine synthetase is present in high levels in the plant cell cytoplasm and can convert glutamate and $NH_3$ into glutamine (Section 3.14). This and a few other organic nitrogen compounds transport bacterially fixed nitrogen throughout the plant.

## Stem-Nodulating Rhizobia

Although most leguminous plants form $N_2$-fixing nodules on their *roots*, a few legume species bear nodules on their *stems*. Stem-nodulated leguminous plants are widespread in tropical regions where soils are often nitrogen deficient because of leaching and intense biological activity. The best-studied system is the tropical aquatic legume *Sesbania*, which is nodulated by the bacterium *Azorhizobium caulinodans* (**Figure 23.16**). Stem nodules typically form in the submerged portion of the stems or just above the water level. The general sequence of events by which stem nodules form in *Sesbania* resembles that of root nodules: attachment, formation of an infection thread, and bacteroid formation.

Some stem-nodulating rhizobia produce bacteriochlorophyll *a* and thus have the potential to carry out anoxygenic photosynthesis (Section 14.3). Bacteriochlorophyll-containing rhizobia, called

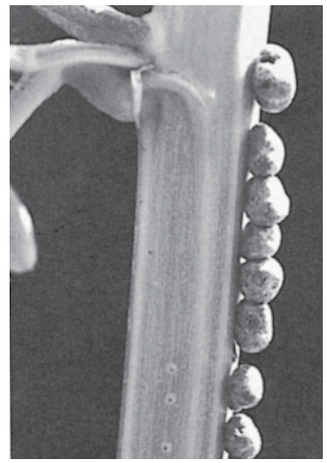



**Figure 23.16 Stem nodules formed by stem-nodulating *Azorhizobium*.** The right side of this stem of the tropical legume *Sesbania rostrata* was inoculated with *Azorhizobium caulinodans*, but the left side was not.

photosynthetic *Bradyrhizobium*, are widespread in nature, particularly in association with tropical legumes. In these species, light energy converted to chemical energy (ATP) in photosynthesis probably supplies part of the energy source needed by the bacterium to support $N_2$ fixation.

## Nonlegume $N_2$-Fixing Symbioses: *Azolla–Anabaena* and *Alnus–Frankia*

Various nonleguminous plants form $N_2$-fixing symbioses with bacteria other than rhizobia. For example, the water fern *Azolla* harbors within small pores of its fronds a species of heterocystous $N_2$-fixing cyanobacteria (Section 15.3) called *Anabaena azollae* (**Figure 23.17**). *Azolla* has been used for centuries to enrich Asian rice paddies with fixed nitrogen. Before planting rice, the farmer allows the surface of the rice paddy to become densely covered with *Azolla*. As the rice plants grow, they eventually crowd out the *Azolla*, causing its death and the release of its nitrogen, which is

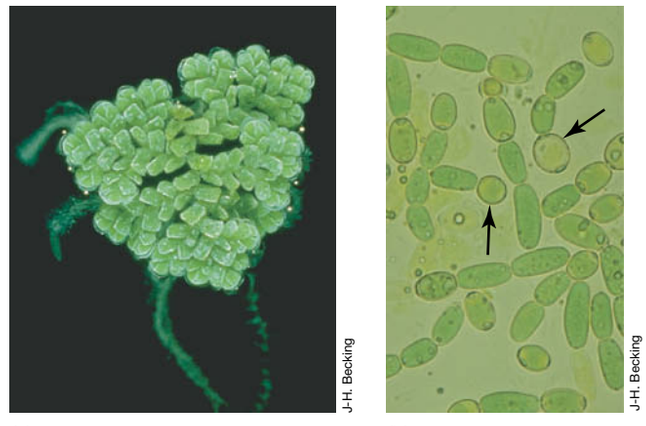
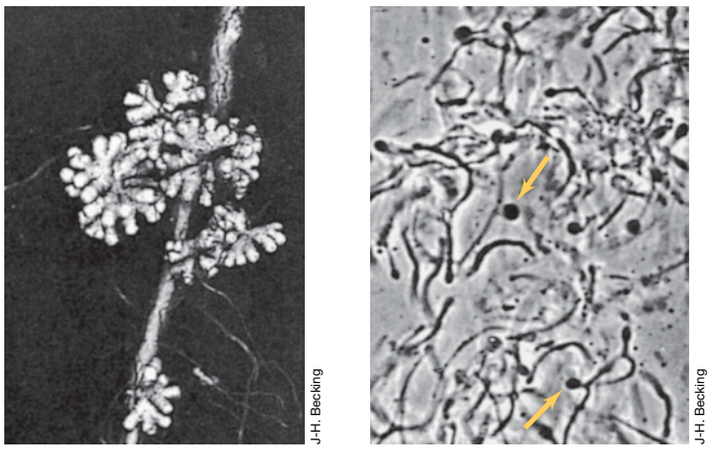



**Figure 23.17 *Azolla–Anabaena* symbiosis.** *(a)* Intact association showing a single plant of *Azolla pinnata*. The diameter of the plant is approximately 1 cm. *(b)* Cyanobacterial symbiont *Anabaena azollae* as observed in crushed leaves of *A. pinnata*. Single cells of *A. azollae* are about 5 μm wide. Vegetative cells are oblong; the spherical heterocysts (lighter color, arrows) are differentiated for nitrogen fixation.




(a) (b)

**Figure 23.18 *Frankia* nodules and *Frankia* cells.** *(a)* Root nodules of the common alder *Alnus glutinosa*. *(b) Frankia* culture purified from nodules of *Comptonia peregrina*. Note vesicles (arrows) on the tips of hyphal filaments.

assimilated by the rice plants. By repeating this process each growing season, rice farmers can obtain high yields of rice without applying nitrogenous fertilizers.

The alder tree (genus *Alnus*) has $N_2$-fixing root nodules (**Figure 23.18*a***) that harbor filamentous, $N_2$-fixing actinomycetes of the genus *Frankia*. When assayed in cell extracts the nitrogenase of *Frankia* is sensitive to $O_2$, but cells of *Frankia* fix $N_2$ at full oxygen tensions. This is because *Frankia* protects its nitrogenase from $O_2$ by localizing the enzyme in terminal swellings on the cells called *vesicles* (Figure 23.18*b*). The vesicles contain thick walls that retard $O_2$ diffusion, thus maintaining the $O_2$ tension within vesicles at levels compatible with nitrogenase activity. In this regard, *Frankia* vesicles resemble the heterocysts produced by some filamentous cyanobacteria as localized sites of $N_2$ fixation (Section 15.3).

Alder is a characteristic pioneer tree able to colonize nutrient-poor soils, probably because of its ability to enter into a symbiotic $N_2$-fixing relationship with *Frankia*. A number of other small or bushy, woody plants are nodulated by *Frankia*. As is the case for the rhizobial symbionts of leguminous plants, a single strain of *Frankia* can form nodules on several different species of plants.

**MINIQUIZ**

- How do rhizobial root nodules benefit a plant?
- What are Nod factors and what do they do?
- What is a bacteroid and what occurs within it? What is the function of leghemoglobin?
- What are the major similarities and differences between rhizobia and *Frankia*?

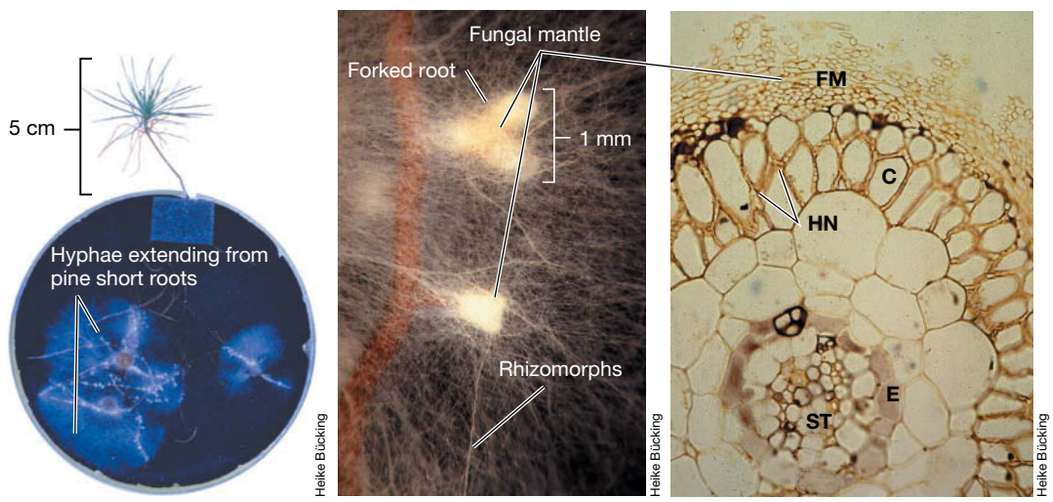
## 23.4 Mycorrhizae

**Mycorrhizae** are mutualisms between plant roots and fungi in which nutrients are transferred in both directions. The fungus transfers inorganic nutrients—in particular, phosphorus and nitrogen—from the soil to the plant, and the plant in turn transfers primarily carbohydrates to the fungus. These mutualisms are harnessed in agricultural applications. From fungal spores produced in culture or from root scrapings of infected plants, soil inoculants are produced that enhance plant growth.

### Classes of Mycorrhizae

There are two classes of mycorrhizae. In *ectomycorrhizae*, fungal cells form an extensive sheath (*fungal mantle*) around the outside of the root with only a slight penetration into the root cellular structure (**Figure 23.19**). In *endomycorrhizae*, a part of the fungus






(a) (b) (c)

**Figure 23.19 Ectomycorrhizal colonization of pine and beech tree roots.** *(a)* Colonization of pine (*Pinus sylvestris*) roots with the ectomycorrhizal fungus *Suillus bovinus*. *(b)* Ectomycorrhizal root tips of pine roots enclosed by a mantle of ectomycorrhizal fungi (white) and associated hyphae extending into the soil matrix. The ectomycorrhizal fungus *Suillus bovinus* also forms rhizomorphs, hyphal aggregations that are involved in the long-distance transport from the soil to the mycorrhizal root. *(c)* Cross section of a beech fine root showing the fungal mantle (FM) and Hartig net (HN) within the root cortex (C). The Hartig net is the location of nutrient exchange between the plant and fungus. Also shown are root vascular tissue (root stele, ST) and endodermis (E).

becomes deeply embedded within cells comprising the root tissue. Ectomycorrhizae are found mainly on the roots of forest trees, especially conifers, beeches, and oaks, and are most highly developed in boreal and temperate forests. In such forests, almost every root of every tree is mycorrhizal. The root system of a mycorrhizal tree such as a pine (genus *Pinus*) is composed of both long and short roots. The short roots, which are characteristically dichotomously branched in *Pinus* (Figure 23.19*b*), show typical fungal colonization, and long roots are also frequently colonized.

Ectomycorrhizal hyphae extending from the fungal mantle and penetrating between the epidermal and cortical cells form the *Hartig net* (Figure 23.19*c*). This network is where nutrient exchange between the fungus and the host plant occurs. Most mycorrhizal fungi do not catabolize cellulose and other leaf litter polymers. Instead, they catabolize simple carbohydrates and typically have one or more vitamin requirements. They obtain their carbon from root secretions and obtain inorganic minerals from the soil. Mycorrhizal fungi are rarely found in nature except in association with roots, and most are probably obligate symbionts.

Despite the close symbiotic association between fungus and root, a single species of tree can form multiple mycorrhizal associations. One pine species can associate with over 40 species of fungi. This relative lack of host specificity allows ectomycorrhizal mycelia to interconnect trees, providing linkages for transfer of carbon and other nutrients between trees of the same or different species. Nutrient transfer from well-illuminated overstory plants to shaded trees is thought to help equalize resource availability, subsidizing young trees and increasing biodiversity by promoting the coexistence of different species.

## Arbuscular Mycorrhizae

Although ectomycorrhizal fungi play a significant role in the ecology of forests, there is a greater diversity of endomycorrhizae. Most are *arbuscular mycorrhizae* (*AM*) that comprise a phylogenetically distinct fungal division, the *Glomeromycota* (Section 18.11), of which all or most species are obligate plant mutualists (the word *arbuscular* means "little tree"). AM colonize 70–90% of all terrestrial plants, including most grassland species and many crop species. The association between plants and the *Glomeromycota* is thought to be the ancestral type of mycorrhizae, established about 450 million years ago and an important evolutionary step in the successful invasion of dry land by terrestrial plants (see page 593).

It is now known that AM fungi produce lipochitin oligosaccharide signaling factors (**Myc factors**) very closely related to the Nod factors in the rhizobium–legume symbiosis (Section 23.3), and Myc factors initiate formation of the mycorrhizal state (Figures 23.12 and 23.13). Root colonization by an AM fungus begins with germination of a soilborne spore, producing a branched germination mycelium that recognizes the host plant through reciprocal chemical signaling. Spore germination and mycelial branching is induced by *strigolactones*, plant hormones released by the roots that also play a key role in plant development. When a plant is nutrient-limited, this hormone acts to repress aboveground plant growth (suppressing formation of secondary shoots) and to stimulate the growth of the root system, enhancing the production of lateral roots and root hairs. These developmental changes help the plant secure nutrients before using them later for aboveground growth.

The Myc factor produced by the AM fungal mycelium signals the plant to initiate the developmental process (Figure 23.13). The fungus then forms a contact structure called the *hyphopodium* with root epidermal cells (**Figure 23.20***c*). Penetrating hyphae extend into the plant from each hyphopodium, usually forging an intracellular path through epidermal and outer cortical cell layers of the root before forming dichotomously branched or coiled hyphal structures called **arbuscules** within plant inner cortex cells, near vascular tissues. However, the arbuscular hyphae remain separated from plant protoplasm by an extensive plant cytoplasmic membrane that forms a region called the *apoplast* (**Figure 23.21**), and this structure functions to increase the surface area of contact between plant and fungus. Inorganic nitrogen and phosphorus are then "mined" from the soil by the fungi, converted to arginine and polyphosphate, and translocated through the hyphae to the plant (Figure 23.21).

Myc factors are very similar to the rhizobial Nod factors and only relatively minor modifications of the chitin backbone structure confer specificity (Figure 23.12). It is now suspected that the basic signaling and developmental systems used in the legume–root nodule symbiosis (Section 23.3), which arose about 60 million years ago, first evolved in the

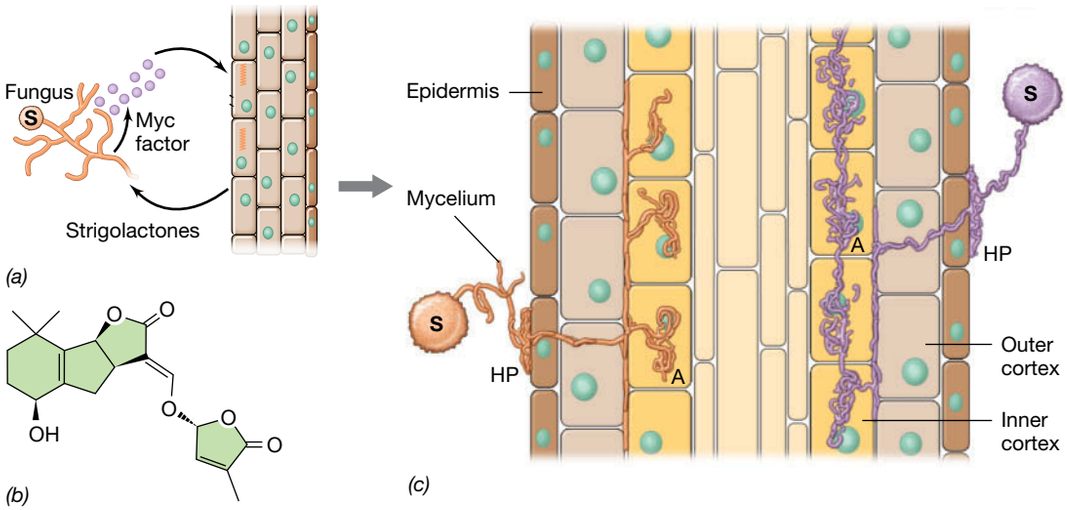



**Figure 23.20 Arbuscular mycorrhizal root colonization.** *(a)* A spore (S) recognizes the presence of a nearby root by sensing the presence of strigolactones released by the plant root. *(b)* The structure of strigol, one type of strigolactone. Strigolactones stimulate spore germination and mycelial branching. Myc factor (see Figure 23.12) produced by the growing fungal mycelium then initiates the infection process. *(c)* The mycelium forms an attachment structure called the hyphopodium (HP) and then enters the inner cortex region of the root by penetrating epidermal cells and cells of the outer cortex. Arbuscules (dichotomously branched invaginations, A) are formed by mycelia spreading either *inter*cellularly (left) or *intra*cellularly (right).

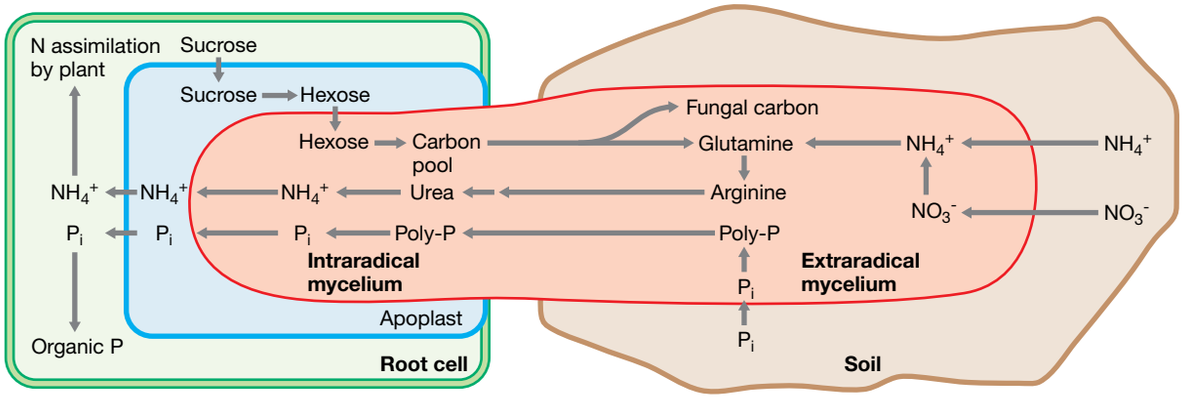



**Figure 23.21 Pathways of N, P, and C exchange between plant and arbuscular mycorrhizal fungi.** Inorganic nitrogen ($NH_4^+$ and $NO_3^-$) and phosphorus ($P_i$) mined from the soil by the extraradical (soil-associated) mycelia are translocated to the plant as arginine and polyphosphate (poly-P) through the mycelial network and delivered to the plant at the intraradical (plant cell–associated) mycelium. Ammonia and phosphate are regenerated in the intraradical mycelium for transfer to the plant cell. In exchange for the N and P, the plant provides organic carbon to the fungus.

much more ancient AM fungi–plant symbiosis. Apparently the AM fungal system was recruited and adapted for the legume–root nodule symbiosis (Figure 23.13).

Although the arbuscular mycorrhizae are a much more ancient and widely distributed microorganism–plant symbiosis, understanding of their signaling and developmental program has been much slower to develop because the AM fungi cannot be maintained in pure culture. AM fungi are obligately *biotropic* (meaning that they obtain their nutrients only from living cells of their symbiotic partner), and unlike rhizobia they have no supporting genetic system as has been exploited to help unravel the complex developmental steps leading to legume–root nodule formation.

## Benefits for the Plant

The beneficial effect of the mycorrhizal fungus on the plant is best observed in poor soils where plants that are mycorrhizal thrive, but nonmycorrhizal ones do not. For example, if trees planted in prairie soils, which ordinarily lack a suitable fungal inoculum, are artificially inoculated at the time of planting, they grow much more rapidly than uninoculated trees (**Figure 23.22**). The mycorrhizal plant can absorb nutrients from its environment more efficiently (Figure 23.21) and thus has a competitive advantage. This improved nutrient absorption is due to the greater surface area provided by the fungal mycelium. For example, in the pine seedling shown in Figure 23.19*a* and *b*, the ectomycorrhizal fungal mycelium makes up the overwhelming part of the absorptive capacity of the plant root system. The mycorrhizal plant is better able to function physiologically and compete successfully in a species-rich plant community, and the fungus benefits from a steady supply of organic nutrients.

In addition to helping plants absorb nutrients, mycorrhizae also play a significant role in supporting plant diversity. Field experiments have clearly shown a positive correlation between the abundance and diversity of mycorrhizae in a soil and the extent of the plant diversity that develops in it. However, although most mycorrhizae are true mutualistic symbioses, there are also parasitic mycorrhizae. In these less common mycorrhizal symbioses, either the plant parasitizes the fungus or, in some cases, the fungus parasitizes the plant. Obviously, there is much more to learn about fungal–plant symbioses, and such discoveries will be important not only in agriculture but also in facilitating the restoration of damaged ecosystems.

### MINIQUIZ

- How do endomycorrhizae differ from ectomycorrhizae?
- What features of mycorrhizal fungi might have assisted in colonization of dry land by plants?
- How do mycorrhizal fungi promote plant diversity?

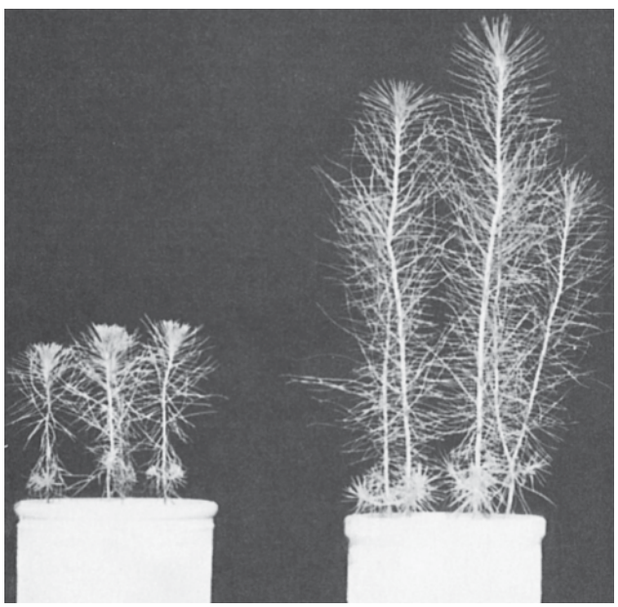



**Figure 23.22 Effect of mycorrhizal fungi on plant growth.** Six-month-old seedlings of Monterey pine (*Pinus radiata*) growing in pots containing prairie soil: left, nonmycorrhizal; right, mycorrhizal.

## 23.5 *Agrobacterium* and Crown Gall Disease

Some microorganisms develop parasitic symbioses with plants. The genus *Agrobacterium*, a relative of the root nodule bacterium *Rhizobium* (Section 23.3), is such an organism, causing the formation of tumorlike growths on diverse plants. The two species of *Agrobacterium* most widely studied are *Agrobacterium tumefaciens* (also called *Rhizobium radiobacter*), which causes *crown gall disease*, and *Agrobacterium rhizogenes* (also called *Rhizobium rhizogenes*), which causes *hairy root disease*.

### The Ti Plasmid

Although wounded plants often form a benign accumulation of tissue called a *callus*, the growth in crown gall disease (**Figure 23.23**) is different in that it is uncontrolled growth, resembling an animal tumor. *A. tumefaciens* cells induce tumor formation only if they contain a large plasmid called the **Ti plasmid** (Ti for *t*umor *i*nducing). In *A. rhizogenes*, a similar plasmid called the *Ri plasmid* is necessary for induction of hairy root disease. Following infection, a part of the Ti plasmid called the *transferred DNA* (T-DNA) is integrated into the plant's genome. T-DNA carries the genes for tumor formation and also for the synthesis of a number of modified amino acids called *opines*. *Octopine* [$N^2$-(1,3-dicarboxyethyl)-L-arginine] and *nopaline* [$N^2$-(1,3-dicarboxypropyl)-L-arginine] are two common opines. Opines are produced by plant cells transformed by T-DNA and are a source of carbon and nitrogen, and sometimes phosphate, for the parasitic *A. tumefaciens* cells. These nutrients are the benefits for the bacterial symbiont.


**Figure 23.23 Crown gall.** Photograph of a crown gall tumor (arrow) on a tobacco plant caused by *Agrobacterium tumefaciens*. The disease usually does not kill the plant but may weaken it and make it more susceptible to drought and diseases.

### Recognition and T-DNA Transfer

To initiate the tumorous state, *A. tumefaciens* cells attach to a wound site on the plant. Following attachment, the synthesis of cellulose microfibrils by the bacteria helps anchor them to the wound site, and bacterial aggregates form on the plant cell surface. This sets the stage for plasmid transfer from bacterium to plant.

The general structure of the Ti plasmid is shown in **Figure 23.24**. Only the T-DNA is actually transferred to the plant. The T-DNA contains genes that induce tumorigenesis. The *vir* genes on the Ti plasmid encode proteins that are essential for T-DNA transfer. Transcription of *vir* genes is induced by metabolites synthesized by wounded plant tissues. Examples of inducers include the phenolic compounds acetosyringone and ferulate. The transmissibility genes on the Ti plasmid (Figure 23.24) allow the plasmid to be transferred by conjugation from one bacterial cell to another.

The *vir* genes are the key to T-DNA transfer. The *virA* gene encodes a protein kinase (VirA) that interacts with inducer molecules and then phosphorylates the product of the *virG* gene (**Figure 23.25**). VirG is activated by phosphorylation and functions to activate other *vir* genes. The product of the *virD* gene (VirD) has endonuclease activity and nicks DNA in the Ti plasmid in a region adjacent to the T-DNA. The product of the *virE* gene is a DNA-binding protein that binds the single strand of T-DNA in the plant cell to protect it from destruction by nucleases. The *virB* operon encodes 11 different proteins that form a type IV secretion system (↔ Section 4.13) for single-strand T-DNA and protein transfer between bacterium and plant (Figure 23.25) and thus resembles bacterial conjugation (↔ Section 11.8). Laboratory studies of *A. tumefaciens* have shown that it can transfer T-DNA into many types of eukaryotic cells, including fungi, algae, protists, and even human cell lines.

Once inside the plant cell, T-DNA becomes inserted into the genome of the plant. Tumorigenesis (*onc*) genes on the Ti plasmid (Figure 23.24) encode enzymes for plant hormone production and

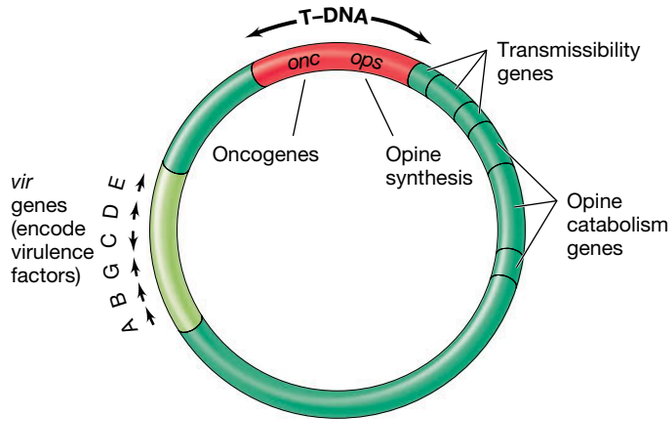



**Figure 23.24 Structure of the Ti plasmid of *Agrobacterium tumefaciens*.** T-DNA is the region transferred to the plant. Arrows indicate the direction of transcription of each gene. The entire Ti plasmid is about 200 kilobase pairs (kbp) of DNA and the T-DNA is about 20 kbp.

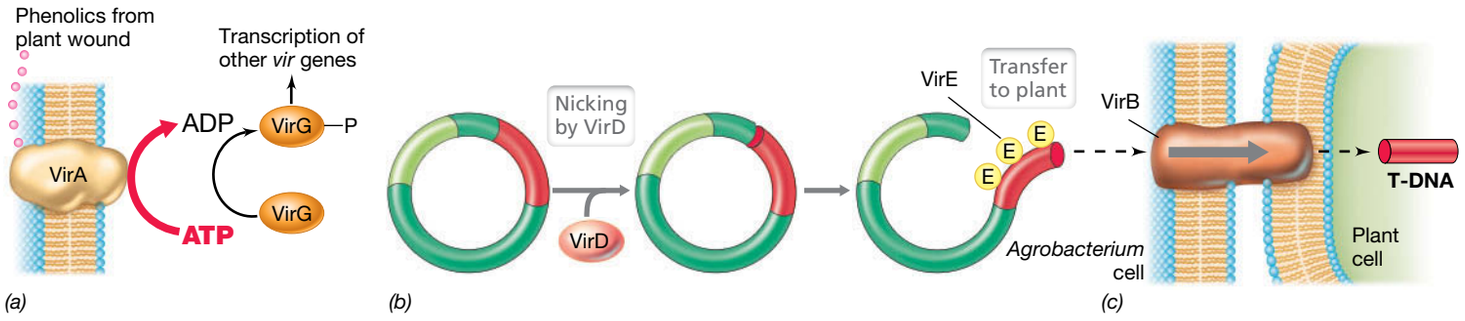



**Figure 23.25 Mechanism of transfer of T-DNA to the plant cell by *Agrobacterium tumefaciens*.**
*(a)* VirA activates VirG by phosphorylation and VirG activates transcription of other *vir* genes. *(b)* VirD is an endonuclease that nicks the Ti plasmid, exposing the T-DNA. *(c)* VirB functions as a conjugation bridge between the *A. tumefaciens* cell and the plant cell, and VirE is a single-strand binding protein that assists in T-DNA transfer. Plant DNA polymerase produces the complementary strand to the transferred single strand of T-DNA.

at least one key enzyme of opine biosynthesis. Expression of these genes leads to tumor formation and opine production. The Ri plasmid responsible for hairy root disease also contains *onc* genes. However, in this case the genes confer increased auxin responsiveness on the plant, and this promotes overproduction of root tissue and the symptoms of the disease. The Ri plasmid also encodes several opine biosynthetic enzymes.

### Genetic Engineering with the Ti Plasmid

From the standpoint of microbiology and plant pathology, crown gall disease and hairy root disease both require intimate interactions that lead to genetic exchange from bacterium to plant. In other words, tumor induction in these diseases is the result of a natural plant-transformation system. Because of this, recent interest in the Ti–crown gall system has shifted away from the disease itself toward applications of this natural genetic exchange process in plant genetic engineering and biotechnology.

Several modified Ti plasmids that lack disease genes but that can still transfer DNA to plants have been developed by genetic engineering. These have been used for the construction of genetically modified (transgenic) plants. Many transgenic plants have been constructed thus far, including crop plants carrying genes for resistance to herbicides, insect attack, and drought. We discuss the use of the Ti plasmid as a vector in plant biotechnology in Section 12.7.

**MINIQUIZ**

- What are opines and whom do they benefit?
- How do the *vir* genes differ from T-DNA in the Ti plasmid?
- How has an understanding of crown gall disease benefited plant agriculture?

# III • Insects as Microbial Habitats

Insects are the most abundant class of animals living today, with over 1 million species known. As many as 20% of all insects are thought to support symbiotic microbes in a mutually beneficial way. The symbioses contribute to the insects' ecological success by providing them either nutritional advantages or protection from parasites (see page 732 for an example of how the gut microbiota of bees benefits them in these ways). Some symbionts are found on insects' outer surfaces or in their digestive tracts. *Endosymbionts* are intracellular bacteria and are typically localized at specialized organs within the insect.

## 23.6 Heritable Symbionts of Insects

How symbionts are transferred from one generation to the next determines how a mutualism functions and how stable it is. Microbial symbionts can either be acquired by a host from an environmental reservoir (*horizontal* transmission) or be transferred directly from the parent to the next generation (*heritable* or *vertical* transmission). The mode of symbiont transmission is related to the specificity and persistence of an association. In general, less specificity is associated with horizontal transmission. In this section we focus only on mutualisms in which the microbial symbiont has no free-living form; that is, the symbionts are transmitted in a vertical fashion.

### Types of Heritable Symbionts

All known heritable symbionts of insects lack a free-living replicative stage. Thus, they are *obligate* symbionts. However, although these bacteria require the host for replication, not all hosts are dependent upon the symbiont. Relative to host dependence, heritable symbionts are either *primary* or *secondary symbionts*. Primary symbionts are required for host reproduction. They are restricted to a specialized region called the **bacteriome** present in several insect groups; within the bacteriome the bacterial cells reside in specialized cells called **bacteriocytes**. Secondary symbionts are not required for host reproduction. Unlike primary symbionts, secondary symbionts are not always present in every individual of a species and are not restricted to particular host tissues.

Secondary symbionts are broadly distributed among insect groups. Like pathogens, they invade different cell types and may live extracellularly within the insect's *hemolymph* (the fluid bathing the body cavity). In insects with bacteriomes, secondary symbionts can invade the bacteriocytes, co-residing with or sometimes

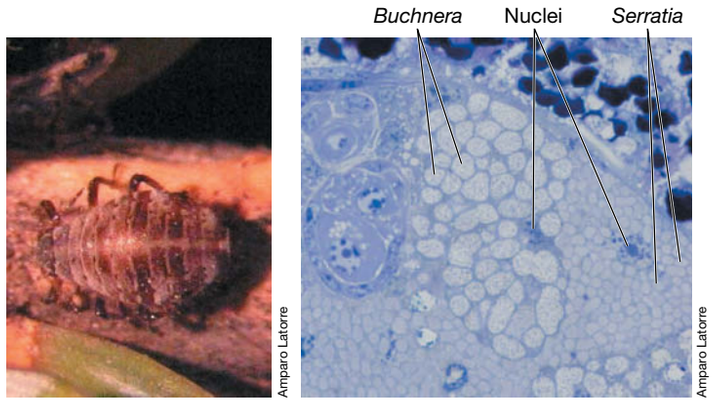



**Figure 23.26 Primary and secondary symbionts of an aphid.** *(a)* The cedar aphid *Cinara cedri*, a model organism for studies of symbioses. *(b)* Transmission electron micrograph of the bacteriome of *C. cedri* showing two bacteriocytes. Packed within each bacteriocyte are cells of *Buchnera aphidicola* (the primary symbiont) or *Serratia symbiotica*, the smaller, secondary symbiont. The nucleus of each bacteriocyte is identified. The bacteriocyte containing *Buchnera* cells is about 40 µm wide.

displacing the primary symbionts (**Figure 23.26*b***). However, in order to persist in the insect host, the secondary symbiont must confer some advantage, such as a nutritional advantage or protection from environmental stresses such as heat. For example, whiteflies infected with *Rickettsia* bacteria (Section 16.1) produce offspring at about twice the rate of uninfected flies, and more offspring survive to adulthood. Secondary symbionts may also provide protection against invasion by pathogens or predators. A *Spiroplasma* species (Section 16.9), which was first observed in *Drosophila neotestacea* in the 1980s, provides protection against a parasitic nematode worm. In most instances the basis for increased fitness or protection is unknown, but in one case a toxin encoded by a lysogenic bacteriophage (Section 8.7) carried by the symbiont is known to confer protection on the insect from infection by a parasitic wasp.

There are heritable parasitic symbionts that manipulate the host's reproductive system, increasing the frequency of female progeny (sex-ratio skewing, Figure 16.27). Because most heritable symbionts are transmitted maternally, the suppression of male progeny serves to expand the number of infected individuals and increase the rate of spread through an insect population. Since symbiont-conferred functions can spread rapidly within a population, acquisition of symbiont-encoded traits provides a mechanism for much more rapid adaptation than is possible through mutations in insect genes. *Rickettsia* infection of the whitefly population provides one example of how rapidly symbiont-conferred traits can spread through a population. Only 1% of whiteflies in the southwestern United States were infected with *Rickettsia* in 2000. In 2006, 97% of flies were infected. In another example, a strain of *Wolbachia* (Section 16.1) swept through populations of *Drosophila simulans* in California in only 3 years.

An important applied benefit of improved basic understanding of insect symbionts is the increased use of symbionts in insect pest management and the control of vectorborne diseases, such as malaria and filariasis in humans (Sections 33.5 and 33.7). For example, symbiotic *Wolbachia*, which are reproduction manipulators, are widely distributed among insect species (possibly infecting as many as 60–70% of all insect species). The sperm of *Wolbachia*-infected males can sterilize uninfected females. Although the mechanism for sterilization is not fully understood, the phenomenon is being tested as a means to suppress disease transmission. Release of a large number of *Wolbachia*-infected male *Culex quinquefasciatus* mosquitoes, the vector of the filarial nematode causing elephantiasis (Section 33.7), in Myanmar (Burma) effectively eliminated the local mosquito population.

In some cases, the presence of the symbiont decreases insect transmission of disease. For instance, *Aedes aegypti* mosquitoes infected with *Wolbachia* are less likely to transmit the virus causing dengue fever (Section 31.5). However, in some other cases, the presence of the symbiont *increases* disease transmission. For example, whiteflies infected with *Hamiltonella* bacteria (a symbiont affiliated with the *Enterobacteriaceae*) are more likely than uninfected flies to transmit tomato yellow leaf curl virus.

## Nutritional Significance of Obligate Intracellular Symbionts of Insects

The association of bacteria and insects has allowed many insects to use food resources that are rich in some nutrients, but poor in others. To achieve adequate nutrition, some insects exploit the metabolic potential of their symbionts. For instance, aphids feed on the carbohydrate-rich but otherwise nutrient-poor sap of phloem vessels in plants. Early on it was suspected that obligate symbionts might benefit the insect by providing nutrients not provided by their primary diet, and this is now known to be true.

Molecular analyses have shown that most families of aphids harbor the bacterium *Buchnera* in their bacteriomes. The role of *Buchnera* in host nutrition was first indicated by experiments using defined diets to examine the nutrient requirements of aphids. Compared with infected controls, symbiont-free aphids required a diet containing all amino acids that are either lacking or rare in phloem sap. Subsequent genomic studies documented the presence in *Buchnera* of genes encoding the biosynthesis of nine amino acids missing from the sap. There are also examples of synergy between host and symbiont where the synthesis of certain amino acids becomes a joint venture. For example, *Buchnera* lacks the enzyme needed for the last step in leucine biosynthesis, but the necessary gene is present in the aphid's genome. Presumably, this enzyme is made by the aphid and participates in the leucine biosynthetic pathway along with the bacterial enzymes.

A secondary symbiont can also contribute to a joint venture. For example, the *Buchnera* symbiont of the cedar aphid is unable to supply tryptophan to the aphid. Two genes in the tryptophan biosynthetic pathway are present in *Buchnera*, but the remaining genes for the pathway are located on the chromosome of a secondary endosymbiont (Figure 23.26). Thus, different parts of a required metabolic pathway can be encoded by different endosymbionts present in the same insect. The fungus-cultivating ants provide yet another example of a complex symbiosis that has formed between an insect and multiple microorganisms (see Explore the Microbial World, "The Symbiotic Organ of The Bean Bug").

## THE SYMBIOTIC ORGAN OF THE BEAN BUG

The bean bug *Riptortus pedestris* (**Figure 1*a***) is a common pest affecting leguminous crops, such as soybean, in East Asia. They feed on pods or seeds and can cause significant damage to crops. These insects are an example of an intimate symbiotic association with bacteria. The bean bugs have developed a facultative, but exclusive, relationship with bacteria of the species *Burkholderia*. Unlike most insects, which acquire their specific symbionts via vertical transmission (i.e. from mother to offspring), *R. pedestris* acquires *Burkholderia* symbionts from the environment at each generation (also known as horizontal transmission). Soon after hatching, second instar *R. pedestris* larvae ingest bacteria from the surrounding soil. The bacteria colonize crypts of the posterior region of the mid-gut (**Figure 1*b*, *c***). Once established, this simple gut symbiosis is stable throughout the insect's life.

The guts of bean bugs reared in a sterile environment are not colonized by the bacteria. However, uninfected insects are noticeably smaller than symbiotic ones and exhibit slower development. The *Burkholderia* symbionts also increase the reproductive success of the host because uninfected females produce approximately 50% fewer eggs than females colonized with *Burkholderia*. The specific function of the *Burkholderia* symbiont is unknown, but it is hypothesized that the bacteria contribute nutrients to the host or participate in the detoxification of plant metabolites. The detoxification hypothesis is further supported by the fact that *Burkholderia* isolated from agricultural soil polluted with the organophosphate fenitrothion confer resistance to this insecticide to their bean bug hosts.

Remarkably, the association is highly specific, as bean bugs are consistently colonized by a single species of the genus *Burkholderia* in a way that is reminiscent of the symbiosis between bobtail squids and *Aliivibrio fischeri* (Section 23.8) or legumes and *Rhizobium* (Section 23.3). As in these other examples of horizontally transmitted symbioses, the specificity of the association between the bean bug and *Burkholderia* is enforced by a severe infection bottleneck.

Symbiotically competent bacteria are effectively winnowed from the millions of other bacterial species present in the soil inoculum via a sorting organ located at the entrance of the posterior midgut. A constriction in the gut, filled with mucus and microvilli, "guards" the entrance of the symbiotic region (**Figure 1*d***). Only symbiotically competent *Burkholderia* are allowed through the constricted region while other bacteria and food particles are restricted to the anterior sections of the midgut. The molecular mechanisms allowing the recognition of the symbiotic bacteria are so far unknown, but flagellar motility has been shown to be essential for passage through the sorting organ. This active and specific colonization of the symbiont, together with the many vertically transmitted symbioses of insects, illustrates the many mechanisms animals use to select beneficial symbionts.

Takeshita, K., Kikuchi, Y. (2017) Riptortus pedestris and Burkholderia symbiont: an ideal model system for insect–microbe symbiotic associations. Res. Microbiol. 168(3), 175–87, Doi: 10.1016/j.resmic.2016.11.005.

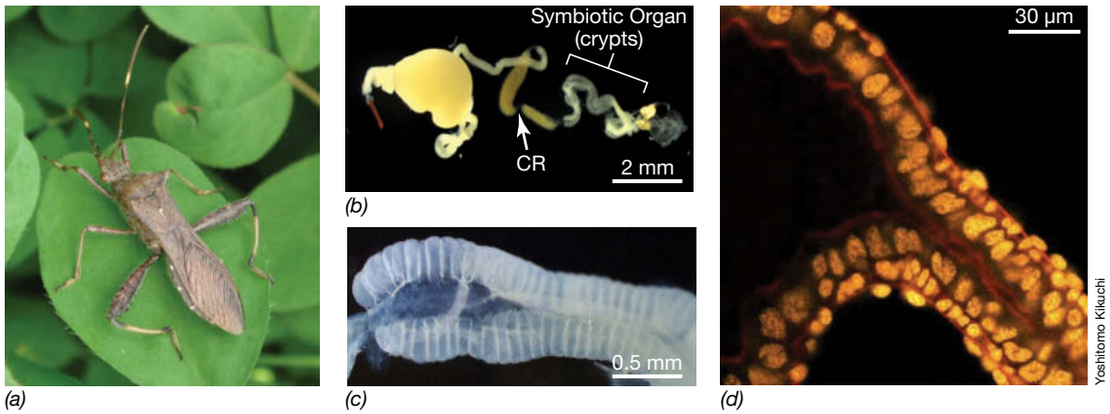



**Figure 1 The bean bug *Riptortus pedestris*.** *(a)* Adult female of *R. pedestris*. *(b)* Dissected digestive tract. The posterior section is the symbiotic organ. The arrow indicates the constricted region (CR). *(c)* Crypts of the midgut's posterior section. *(d)* A section image of the constricted region involved in symbiont sorting.



Mealybugs (*Planococcus citri*) present one of the most unusual examples of a partnership between two symbionts infecting the same insect. Mealybugs have two stable bacterial symbionts, "*Candidatus* Tremblaya princeps" (a *Betaproteobacterium*) and "*Candidatus* Moranella endobia" (a *Gammaproteobacterium*) (the term "*Candidatus*" means that these organisms are not yet in pure culture, Section 13.10). These symbionts cooperate in providing their host with essential amino acids missing in its diet, as is true for the symbionts of many sap-feeding insects. However, the Moranella bacterium actually lives *inside of* Tremblaya, the only known example of a bacterium-within-a-bacterium symbiosis. The highly reduced Tremblaya genome has lost all genes for tRNA synthetases, an essential function supplied either by the host or by the Moranella residing within the cytoplasm of Tremblaya.

### Genome Reduction and Gene Transfer Events

Common features of primary symbionts are extreme genome reduction (Table 9.1), high adenine plus thymine content, and accelerated rates of mutation. Genomes of most insect symbionts

**TABLE 23.2 Genome features of some endosymbiotic *Bacteria* of animals[a]**

| Host | Symbiont (genus) | Genome size (Mbp) | G+C (%) | Genes |
|---|---|---|---|---|
| Aphid | Heterotroph (*Buchnera*) | 0.42–0.62 | 20–26 | 362–574 |
| Tsetse fly | Heterotroph (*Wigglesworthia*) | 0.70 | 22 | 617 |
| Carpenter ant | Heterotroph (*Blochmannia*) | 0.71–0.79 | 27–30 | 583–610 |
| Sharpshooter | Heterotroph (*Sulcia*) | 0.25 | 22 | 227 |
| Mealybug | Heterotroph ("*Candidatus* Moranella endobia" *Gammaproteobacteria*) | 0.54 | 43.5 | 406 |
| Mealybug | Heterotroph ("*Candidatus* Tremblaya princeps" *Betaproteobacteria*) | 0.14 | 58.8 | 121 |
| Clam (*Calyptogena okutanii*) | Sulfur oxidizer (unnamed) | 1.0 | 32 | 975 |
| Clam (*Calyptogena magnifica*) | Sulfur oxidizer (*Ruthia*) | 1.2 | 34 | 1248 |
| Tube worm (*Riftia pachyptila*) | Sulfur oxidizer (unnamed) | 3.3[b] | NA | NA |

[a]All listed symbionts are obligately associated with their hosts, with the exception of the symbiont of *Riftia*, which also has a free-living stage. For a comparison with the genomes of free-living *Bacteria*, see Table 9.1.
[b]The free-living sulfur-oxidizing bacterium *Thiomicrospira crunogena* has a genome significantly smaller (2.4 Mb) than this symbiont.

fall within a range from 0.14 to 0.80 megabase pairs (Mbp) and 16.5 to 33% G+C (**Table 23.2**). The 0.14-Mbp (140 kilobase-pair) genome of "*Candidatus* Tremblaya princeps" is among the smallest genome known for any cell. In contrast, the genomes of related free-living bacteria range from 2 to 8 Mbp with a base composition closer to 50% G+C. Two common types of spontaneous mutation, cytosine deamination and the oxidation of guanosine, if not repaired, change a GC pair to an AT pair (↔ Section 11.4). Symbionts with reduced genomes have fewer DNA repair enzymes (↔ Section 11.4), and this likely facilitates a shift over time to genomes of lower G+C content.

The streamlined genomes of insect symbionts have lost genes from most functional categories (Chapter 9) and tend to retain only genes required for host fitness and essential molecular processes, such as translation, replication, and transcription. Genome reduction implies that the symbionts are reliant on the host for many functions no longer encoded in the symbiont genome (↔ Section 9.4). For example, in many cases genes needed for the biosynthesis of cell wall components are missing, including lipid A and peptidoglycan, suggesting that the host supplies these functions or that the structures are not required to form stable cells within the bacteriocyte.

There is an interesting genomic contrast between primary symbionts and typical disease-causing bacteria (pathogens). While primary symbionts tend to lose genes encoding proteins required in *catabolic* pathways, pathogenic bacteria typically retain these, but lose genes for *anabolic* pathways. This reflects their differing relationships with their hosts; the insect symbiont provides the host with essential biosynthetic nutrients while the pathogen obtains important biosynthetic nutrients from the host.

Because genome sequences for a large number of insects and their symbionts are now appearing, microbiologists can begin to evaluate the frequency of gene transfer between them. Horizontal gene transfer is the movement of genetic information across normal mating barriers (Chapters 11 and 13). Although early research demonstrated that DNA of *Wolbachia* bacteria has been transferred to the nuclear genomes of their insect and nematode hosts, inspection of other insect mutualisms for which both host and symbiont genome sequences have become available (e.g., aphid and body louse) indicate that DNA transfer is very rare. This suggests that horizontal transfer is highly variable for reasons yet to be determined.

**MINIQUIZ**

- What factors stabilize the presence of a secondary insect symbiont?
- What are the consequences of symbiont genome reduction?
- How could it be determined if a symbiont and its host have experienced a long period of coevolution?

## 23.7 Termites

Microorganisms are primarily responsible for the degradation of wood and cellulose in natural environments. However, the activities of free-living microbial species have been exploited by certain groups of insects that have established microbial symbioses in order to digest lignocellulosic materials. Like the rumen of herbivorous animals (see Section 23.13), the insect gut provides a protective niche for microbial symbionts, and in return, the insect gains access to nutrients derived from an otherwise indigestible carbon source. Termites are among the most abundant representatives of this type of symbiotic alliance.

### Termite Natural History and Biochemistry

Microbial symbionts in termites decompose the greater part of cellulose (74–99%) and hemicellulose (65–87%) in the plant material termites ingest. In contrast to the insect examples discussed in the previous section, most termites do not harbor *intracellular* bacteria. Instead, the symbiotic bacteria are present in digestive organs (guts) as in the case of mammals. Termite diets include lignocellulosic plant materials (either intact or at various stages of decay), dung, and soil organic matter (humus). About two-thirds of the terrestrial environment supports one or more termite species, with the greatest representation in tropical and subtropical regions, where termites may constitute as much as 10% of all animal biomass and 95% of soil insect biomass. In savannas, their numbers sometimes exceed 4000/$m^2$, and their biomass density (1–10 g/$m^2$) may be higher than that of grazing mammalian herbivores.

Termites are categorized as higher or lower based on their phylogeny, and this classification correlates with different symbiotic strategies. The posterior alimentary tract of *higher* termites (family *Termitidae*, comprising about three-fourths of termite species) contains a dense and diverse community of mostly anaerobic bacteria, including cellulolytic species. In contrast, the *lower* termites harbor diverse populations of both anaerobic bacteria and cellulolytic

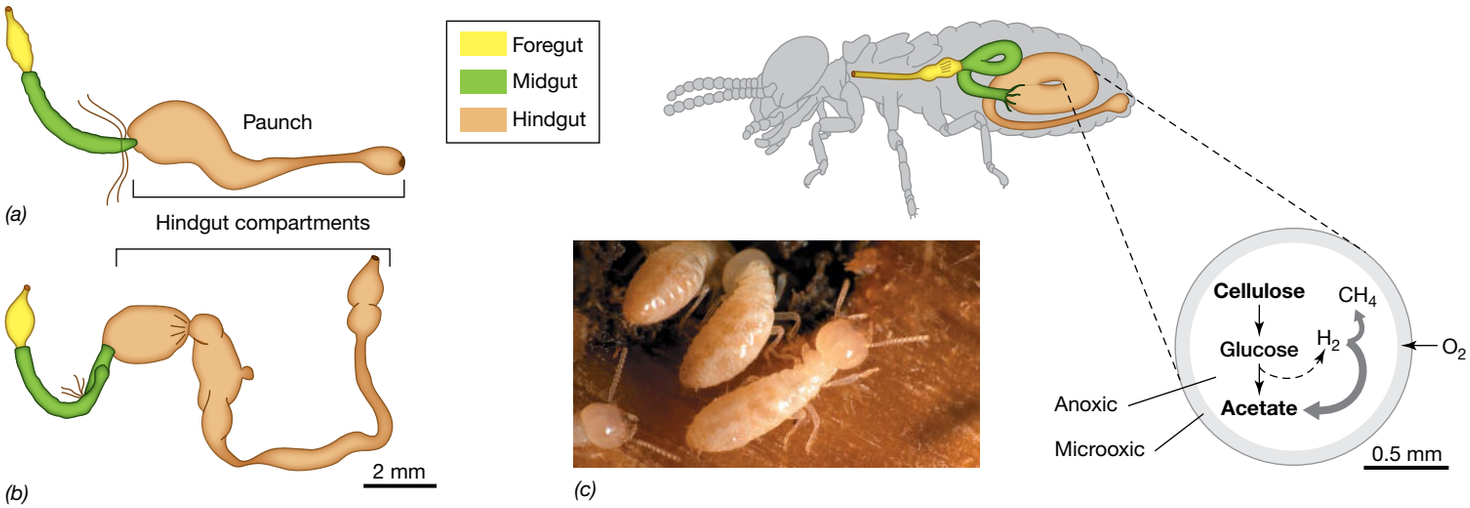



**Figure 23.27 Termite gut anatomy and function.** Gut architecture of lower *(a)* and higher *(b)* termites, showing the foregut, midgut, and differing complexity of the hindgut compartments. *(c)* Photo of workers, gut architecture, and biochemical activities of the lower termite *Coptotermes formosanus*. Microbial fermentation products, particularly acetate, are assimilated by the termite. Hydrogen produced by fermentation is consumed primarily by $CO_2$-reducing acetogens, with a smaller amount going to hydrogenotrophic methanogens. Acetogenesis and methanogenesis are discussed in Sections 14.16 and 14.17, respectively.

protists. Bacteria of lower termites participate little or not at all in cellulose digestion; only the protists phagocytize and degrade the wood particles ingested by the termites. The termite itself produces cellulases in its salivary glands or the midgut epithelium, but the relative contributions of microbial and termite enzymes to lignocellulosic breakdown are unclear.

The termite gut consists of a foregut (including the crop and muscular gizzard), a tubular midgut (site of secretion of digestive enzymes and absorption of soluble nutrients), and a relatively large hindgut of about 1 microliter volume (**Figure 23.27**). In lower termites, the hindgut consists primarily of a single chamber, the *paunch* (Figure 23.27*a*). The hindgut of most higher termites is more complex, being divided into several compartments (Figure 23.27*b*). For both higher and lower termites, the hindgut harbors a dense and diverse microbial community and is a major site of nutrient absorption. Acetate and other organic acids are produced during microbial fermentation of carbohydrate in the hindgut, and these products are primary carbon and energy sources for the termite (Figure 23.27*c*). High $O_2$ consumption by bacteria near the gut wall keeps the interior of the hindgut anoxic. However, microsensor measurements (↔ Section 19.9) have shown that $O_2$ can penetrate up to 200 µm into the gut before it is completely removed by microbial respiration (Figure 23.27*c*). Thus, this tiny gut compartment offers distinct microbial niches with respect to $O_2$ and can support diverse microbial activities.

## Bacterial Diversity and Lignocellulose Digestion in Higher Termites

In termites of different genera, the microbial gut communities differ significantly. Analysis of 16S rRNA gene sequences from hindgut contents of three genera (*Nasutitermes, Reticulitermes*, and *Microcerotermes*) of higher termites revealed a high diversity of microbial species from 12 phyla of *Bacteria*, but few *Archaea* (**Figure 23.28**). Spirochetes of the genus *Treponema* (↔ Section 15.19) dominated, with a lesser contribution from thus far uncultured organisms distantly related to the phylum *Fibrobacteres* (↔ Section 16.21), a group also present in the rumen (Figure 23.40). Metagenomic analysis (↔ Section 9.8) of the *Nasutitermes* hindgut microbial community has revealed bacterial genes encoding glycosyl hydrolases that hydrolyze cellulose and hemicelluloses. These metagenomic data clearly implicate spirochetes and *Fibrobacteres* in the digestion of lignocellulose, although the corresponding cellulolytic bacteria have not yet been isolated from the higher termites. At every molting of an individual termite, gut symbionts are lost, yet there is good conservation of the gut community within each termite species. Stable horizontal transmission of gut symbionts likely occurs as a result of the intimate social behavior and close contact characteristic of termites.

## Acetogenesis and Nitrogen Fixation in the Termite Gut

Genes encoding enzymes of the acetyl-CoA pathway are highly represented in the spirochetes of the *Nasutitermes* hindgut, consistent with their function as the major $CO_2$-reducing acetogens (↔ Section 14.16). The termite gut microbial communities have long been recognized as important to host nitrogen metabolism, providing new fixed nitrogen through nitrogen fixation (↔ Section 14.6) and helping to conserve nitrogen by recycling excretory nitrogen back to the insect for biosynthesis. Consistent with this, metagenomic analyses reveal that many bacteria, including *Fibrobacteres* and treponeme spirochetes, contain genes encoding nitrogenase, the enzyme required to fix $N_2$.

From a simple energetic viewpoint, methanogenesis from $H_2$ and $CO_2$ is more favorable than acetogenesis from the same substrates (−34 kJ/mol of $H_2$ versus −26 kJ/mol of $H_2$, respectively), and thus methanogens should have a competitive advantage in all habitats in which the two processes compete (↔ Sections 14.16–14.17).

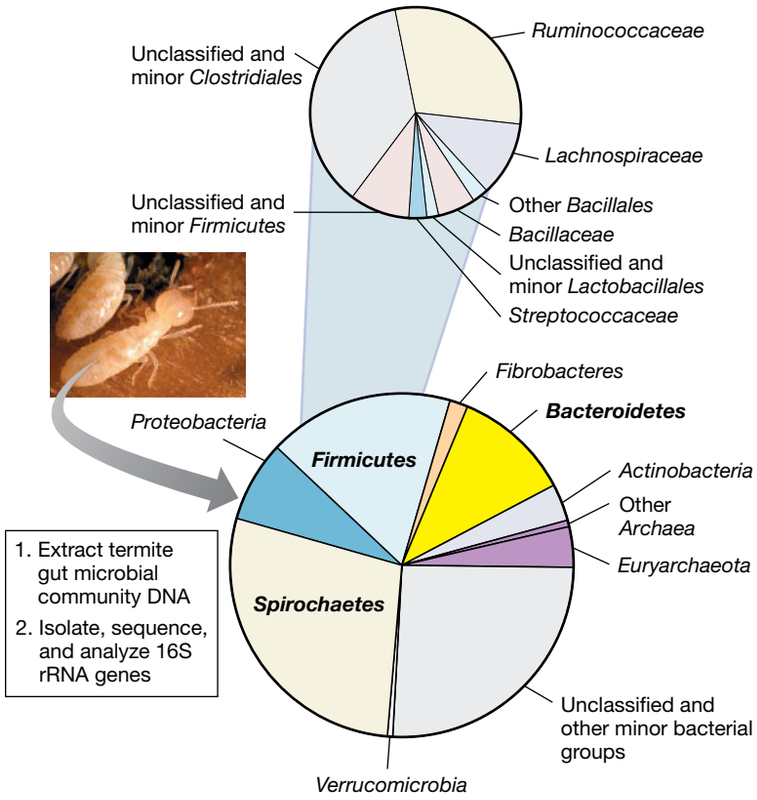



**Figure 23.28 Microbial composition of termite hindgut inferred from 16S rRNA sequences.** The results are pooled analyses of 5075 sequences from amplified or metagenomic sequencing studies of three genera of wood-feeding higher termites, *Nasutitermes, Reticulitermes*, and *Microcerotermes*. The data provide information primarily of diversity, not relative abundance. Data assembled and analyzed by Nicolas Pinel.

However, in termites they do not. There are at least two reasons for this. First, unlike methanogens, acetogens are able to use other substrates such as sugars or methyl groups from lignin degradation as electron donors for energy metabolism. Second, termite acetogens (which seem to consist mostly of spirochetes) can for some reason better colonize the $H_2$-rich termite gut center, whereas methanogens are largely restricted to the gut wall. On the gut wall, methanogens are located downstream of the $H_2$ gradient and thus receive only a fraction of the $H_2$ flux. In addition, the wall likely contains higher $O_2$ tensions, which may negatively affect the physiology of methanogens. So, despite the fact that termites are methanogenic, producing up to 150 teragrams of $CH_4$ per year on a global basis (1 teragram = $10^{12}$ grams), carbon and electron flow in the termite gut favor acetogenesis in this interesting anoxic microbial habitat.

**MINIQUIZ**

- How are anoxic conditions maintained in the termite hindgut?
- Why does reductive acetogenesis predominate over methanogenesis in many termites?
- Which group of morphologically unusual bacteria, absent from molecular surveys of prokaryotic cells in the rumen, seem to dominate activities in the termite hindgut?

# IV • Other Invertebrates as Microbial Habitats

Thus far in this chapter we have discussed how certain macroorganisms that live in terrestrial environments provide habitats for microbial symbionts. We now consider some additional symbioses occurring in both terrestrial and aquatic environments. Aquatic environments—especially marine environments—impose different constraints on symbioses and offer different opportunities and challenges for the evolution of symbioses between macroorganisms and microorganisms. Nevertheless, microbial symbioses with marine animals, especially with invertebrates, are common. By finding habitats in marine invertebrates, microorganisms establish a safe residence in a nutritionally rich environment. The invertebrates benefit, too, as exemplified by the squid and the hydrothermal vent animal symbioses we cover here.

## 23.8 Hawaiian Bobtail Squid

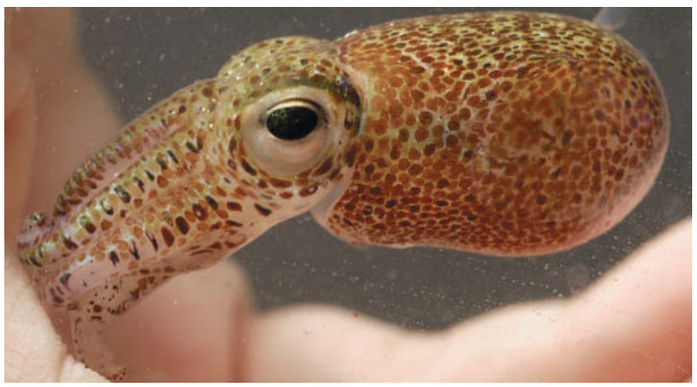
The Hawaiian bobtail squid, *Euprymna scolopes*, is a small marine invertebrate (**Figure 23.29*a***) that harbors a large population of the bioluminescent gram-negative gammaproteobacterium *Aliivibrio fischeri* (↔ Section 16.4) in a light organ located on its ventral side. Squid and bacterium are partners in a mutualism. The bacteria emit light that resembles moonlight penetrating marine waters, and this is thought to camouflage the squid from predators that strike from beneath. Several other species of *Euprymna* inhabit marine waters near Japan and Australia and in the Mediterranean, and these contain *Aliivibrio* symbionts as well.

### The Squid–*Aliivibrio* System as a Model Symbiosis

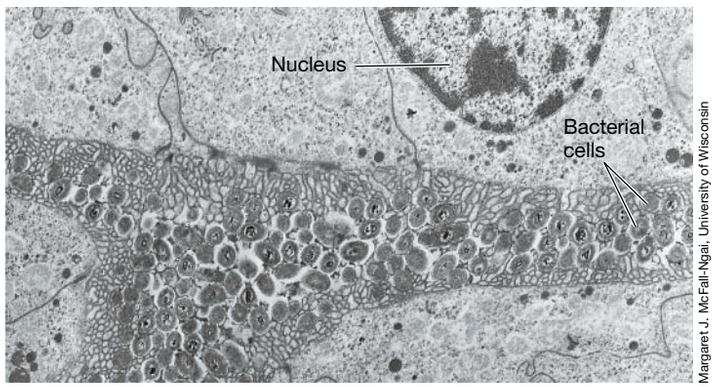
Many features of the *E. scolopes*–*A. fischeri* symbiosis have made it an important model for studies of animal–bacterial symbioses. These include the facts that the animals can be grown in the laboratory and that there is only a single bacterial species in the symbiosis in contrast to the huge number of species in symbioses such as those of the termite (Figure 23.28) or the mammalian large intestine (Chapter 24). In addition, the symbiosis is not an essential one; both the squid and its bacterial partner can be cultured apart from each other in the laboratory. This allows juvenile squid to be grown without bacterial symbionts and then experimentally colonized. Experiments can be done to study specificity in the symbiosis, the number of bacterial cells needed to initiate an infection, the capacity of genetically defined mutants of *A. fischeri* to initiate infection of the squid, and many other aspects of the relationship. Moreover, because the genome of *A. fischeri* has been sequenced, the powerful techniques of microbial genomics may be employed.

### Establishing the Squid–*Aliivibrio* Symbiosis

Juvenile squid just hatched from eggs do not contain cells of *A. fischeri*. Thus, transmission of bacterial cells to juvenile squid is a horizontal (environmental) rather than a vertical (parent to offspring) event. Almost immediately after juveniles emerge from eggs, cells of *A. fischeri* in surrounding seawater begin to colonize them, entering through ciliated ducts that end in the immature light organ. Amazingly, the light organ becomes colonized specifically with




(a)




(b)

**Figure 23.29 Squid–*Aliivibrio* symbiosis.** *(a)* An adult Hawaiian bobtail squid, *Euprymna scolopes*, is about 4 cm long. (b) Thin-sectioned transmission electron micrograph through the *E. scolopes* light organ shows a dense population of bioluminescent *Aliivibrio fischeri* cells.

*A. fischeri* and not with any of the many other species of gram-negative bacteria present in the seawater. Even if large numbers of other species of bioluminescent bacteria are offered to juvenile squid along with low numbers of *A. fischeri*, only *A. fischeri* establishes residence in the light organ. This implies that the animal in some way recognizes and accepts *A. fischeri* cells and excludes those of other species.

The squid–*Aliivibrio* symbiosis develops in several stages. Contact of the squid with any bacterial cells triggers recognition in a very general way. Upon contact with peptidoglycan (a component of the cell wall of *Bacteria*, Section 2.4), the young squid secretes mucus from its developing light organ. The mucus is the first layer of specificity in the symbiosis, as it makes gram-negative but not gram-positive bacteria aggregate. Within the aggregates of gram-negative cells that may contain only low numbers of *A. fischeri*, this bacterium outcompetes the other gram-negative bacteria to form a monoculture. Formation of the monoculture is aided by antimicrobial substances including the gas nitric oxide (NO, see below) in the mucus outside the light organ and is established quickly, within 2 h of a juvenile's hatching from an egg. The highly motile *A. fischeri* cells present in the aggregate migrate up the ducts and into the light organ tissues. Once there, they lose their flagella, become nonmotile, divide to form dense populations (Figure 23.29*b*), and trigger developmental events that lead to maturation of the host light organ. The light organ in a mature *E. scolopes* contains between $10^8$ and $10^9$ *A. fischeri* cells.

Colonization of *A. fischeri* by the squid is assisted by nitric oxide (NO). Nitric oxide is a well-known defense response of animal cells to attack by bacterial pathogens; the gas is a strong oxidant and causes sufficient oxidative damage to bacterial cells to kill them (Section 26.7). Nitric oxide produced by the squid is incorporated into the aggregates in the mucus and is present in the light organ itself. As *A. fischeri* colonizes the light organ, NO levels diminish rapidly. It appears that cells of *A. fischeri* can tolerate exposure to NO and consume it through the activity of NO-inactivating enzymes. The inability of other gram-negative bacteria in the aggregates to detoxify NO helps explain the sudden enrichment of *A. fischeri* in the ducts even before the actual colonization of the light organ. Then, after establishment, continued production of NO in the light organ prevents colonization by other bacterial species.

## Propagating the Symbiosis

The squid matures into an adult in about 2 months and then lives a strictly nocturnal existence in which it feeds mostly on small crustaceans. During the day, the animal buries itself and remains quiescent in the sand. Each morning at dawn the squid nearly empties its light organ of *A. fischeri* cells and begins to grow a new population of the bacterium. The bacterial cells grow rapidly in the light organ; by midafternoon, the structure contains the dense populations of *A. fischeri* cells required for the production of visible light. The actual emission of light requires a certain density of cells and is controlled by the regulatory mechanism called *quorum sensing* (Section 6.8). The daily expulsion of bacterial cells is thought to be a mechanism for seeding the environment with cells of the bacterial symbionts. This, of course, increases the chances that the next generation of juvenile squid will be colonized.

*A. fischeri* grows much faster in the light organ than in the open ocean, presumably because it is supplied with nutrients by the squid. Thus *A. fischeri* benefits from the symbiosis by having an alternative habitat to seawater in which rapid growth and dense populations are possible. Isolation studies have shown that *A. fischeri* is not a particularly abundant marine bacterium. Daily expulsion of *A. fischeri* cells from the light organ increases the bacterium's numbers in the microbial community. Thus, the symbiotic relationship of the bacterium with the squid probably helps maintain larger *A. fischeri* populations than would exist if all cells were free-living. Because the competitive success of a microbial species is to some degree a function of population size (Section 20.1), this boost in cell numbers may confer an important ecological advantage on *A. fischeri* in its marine habitat.

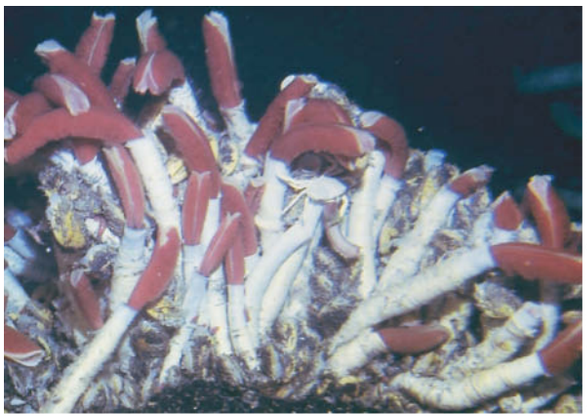
### MINIQUIZ

- Of what value is the squid–*Aliivibrio* symbiosis to the squid? To the bacterium?
- What features of the squid–*Aliivibrio* symbiosis make it an ideal model for studying animal–bacterial symbioses?

## 23.9 Marine Invertebrates at Hydrothermal Vents and Cold Seeps

Diverse invertebrate communities develop near undersea hot springs called *hydrothermal vents*. We covered the geochemistry and microbiology of hydrothermal vents and cold seeps of natural gas in Sections 20.14 and 21.1. Here we focus on hydrothermal vent animals and their microbial symbionts.

Macroinvertebrates, including tube worms over 2 m in length and large clams and mussels, are present near these vents (**Figure 23.30**). Photosynthesis cannot support these invertebrate communities because they exist below the photic zone. However, hydrothermal fluids contain large amounts of reduced inorganic materials, including $H_2S$, $Mn^{2+}$, $H_2$, and CO (carbon monoxide), and some vents contain high levels of ammonium ($NH_4^+$) instead of $H_2S$; all of these are good electron donors for chemolithotrophs, *Bacteria* and *Archaea* that use inorganic compounds as electron donors and fix $CO_2$ as their carbon source (Chapter 14). Thus, hydrothermal vent invertebrates are able to exist in permanent darkness because they receive nourishment through a symbiotic association with these autotrophic bacteria.




(a)


(b)

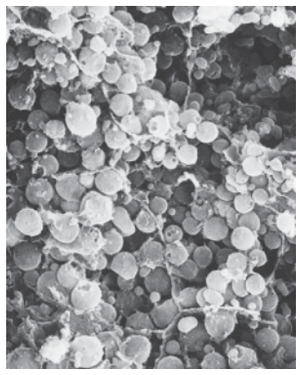
**Figure 23.30 Invertebrates living near deep-sea thermal vents.** *(a) Riftia* (tube worms, phylum *Annelida*), showing the sheath (white) and plume (red) of the worm bodies. *(b)* Mussel bed in vicinity of a warm vent. Note yellow deposition of elemental sulfur from the oxidation of $H_2S$ emitted from the vents.

### Tube Worms, Mussels, and Giant Clams

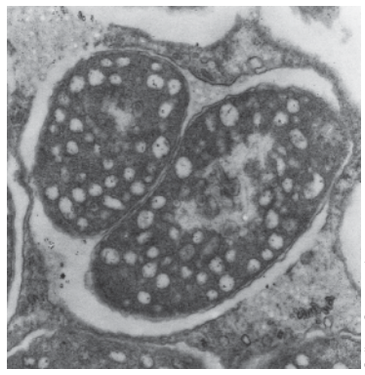
Hydrothermal vent–associated animals either feed directly on cells of free-living chemolithotrophs or have formed tight symbiotic associations with them. Mutualistic chemolithotrophs are either tightly attached to the animal surface (that is, as *epibionts*) or actually live within the animal tissues, supplying organic compounds to the animals in exchange for a safe residence and ready access to the electron donors needed for their energy metabolism. For example, the 2-m-long tube worms (Figure 23.30*a*) lack a mouth, gut, and anus, but contain an organ consisting primarily of spongy tissue called the *trophosome*. This structure, which constitutes half the worm's weight, is filled with sulfur granules and large populations of spherical sulfur-oxidizing bacteria (**Figure 23.31**). Bacterial cells taken from trophosome tissue show activity of enzymes of the Calvin cycle, a major pathway for autotrophy (↔ Section 14.5), but interestingly, they also contain enzymes of the reverse citric acid cycle, a second autotrophic pathway (↔ Section 14.5). In addition, they show a suite of sulfur-oxidizing enzymes necessary to obtain energy from reduced sulfur compounds (↔ Sections 14.9 and 15.11). The tube worms are thus nourished by organic compounds produced from $CO_2$ and secreted by the sulfur chemolithotrophs.

Along with tube worms, giant clams and mussels (Figure 23.30*b*) are also common near hydrothermal vents, and sulfur-oxidizing bacterial symbionts have been found in the gill tissues of these animals. Phylogenetic analyses have shown that each individual animal harbors one or more different strains of bacterial symbiont and that a variety of species of bacterial symbionts inhabit different species of vent animal. With the exception of the bacterial symbiont of *Riftia* (tube worms), which also has a free-living stage (Table 23.2), none of the bacterial symbionts of hydrothermal vent animals have yet been obtained in laboratory culture, even though they are fairly closely related to free-living sulfur chemolithotrophs (↔ Sections 14.9, 15.11, and 16.5).

The red plume of the tube worm (Figure 23.30*a*) is rich in blood vessels and is used to trap and transport inorganic substrates to the




(a)




(b)

**Figure 23.31 Chemolithotrophic sulfur-oxidizing bacteria associated with the trophosome tissue of tube worms from hydrothermal vents.** *(a)* Scanning electron micrograph of trophosome tissue showing spherical chemolithotrophic sulfur-oxidizing bacteria. Cells are 3–5 μm in diameter. *(b)* Transmission electron micrograph of bacteria in sectioned trophosome tissue. The cells are frequently enclosed in pairs by an outer membrane of unknown origin. Reprinted with permission from *Science 213:* 340–342 (1981), © AAAS.

bacterial symbionts. Tube worms contain unusual hemoglobins that bind $H_2S$ and $O_2$; these are then transported to the trophosome where they are released to the bacterial symbionts. The $CO_2$ content of tube-worm blood is also high, about 25 mM, and presumably this is released in the trophosome as a carbon source for the symbionts. In addition, stable isotope analyses (Section 19.10) of elemental sulfur from the trophosome have shown that its $^{34}S/^{32}S$ composition is the same as that of the sulfide emitted from the vent. This ratio is distinct from that of seawater sulfate and is further proof that geothermal sulfide is actually entering the worm in large amounts.

Other marine invertebrates have coevolved bacterial symbioses that supply their nutrition as well (Table 23.3). For example, methanotrophic ($CH_4$-consuming) symbionts are present in giant clams that live near cold seeps of natural gas at relatively shallow depths in the Gulf of Mexico. Although not autotrophs ($CH_4$ is an organic compound), the methanotrophs do provide nutrition to the clams; the methanotrophs use $CH_4$ as their electron donor and carbon source and secrete organic carbon to the clams. Molecular hydrogen ($H_2$) is used as an electron donor by the mussel *Bathymodiolus puteoserpentis*, the most abundant macrofauna in vent fields associated with the peridotite-hosted vent systems of the Mid-Atlantic Ridge (Section 20.14). These systems release extremely high levels of $H_2$ and $CH_4$, with measured $H_2$ concentrations as high as 19 mM. This mussel was previously shown to live in a dual symbiosis with methane-oxidizing bacteria and chemolithotrophic sulfur-oxidizing bacteria localized to the gill tissue. Remarkably, the sulfur-oxidizing symbiont of *B. puteoserpentis* also has the capacity to use $H_2$ as an electron donor, making this mussel one of the most versatile of vent macrofauna.

**TABLE 23.3 Marine animals with chemolithotrophic or methanotrophic endosymbiotic *Bacteria***

| *Host phylum (genus or order)* | *Common name* | *Habitat* | *Symbiont metabolic type* |
|---|---|---|---|
| *Porifera* (*Demospongiae*) | Sponge | Seeps | Methanotrophs |
| *Platyhelminthes* (*Catenulida*) | Flatworm | Shallow water | Sulfur chemolithotrophs |
| *Nematoda* (*Monhysterida*) | Mouthless nematode | Shallow water | Sulfur chemolithotrophs |
| *Mollusca* (*Solemya*, *Lucina*) | Clam | Vents, seeps, shallow water | Sulfur chemolithotrophs |
| *Mollusca* (*Calyptogena*) | Clam | Vents, seeps, whale falls[a] | Sulfur chemolithotrophs |
| *Mollusca* (*Bathymodiolus*) | Mussel | Vents, seeps, whale and wood falls[a] | Sulfur and $H_2$ chemolithotrophs, methanotrophs |
| *Mollusca* (*Alviniconcha*) | Snail | Vents | Sulfur chemolithotrophs |
| *Annelida* (*Riftia*) | Tube worm | Vents, seeps, whale and wood falls[a] | Sulfur chemolithotrophs |

[a]Whale and wood falls are sunken whale carcasses and wood, respectively.

### Genomics and Hydrothermal Vent Symbioses

Genome sequencing is revealing additional features of the metabolic interaction and coevolution of marine invertebrates and their bacterial symbionts. The genome sequence of the gill endosymbiont of the giant vent clam *Calyptogena magnifica* offers direct evidence for carbon fixation via the Calvin cycle; the genome encodes the key enzymes of the Calvin cycle, ribulose bisphosphate carboxylase (RubisCO) and phosphoribulokinase (Section 14.5), and contains genes encoding key sulfur oxidation processes. The genome of this symbiont also encodes the biosynthesis of most vitamins and cofactors and all 20 amino acids needed to support the host. However, because few substrate-specific transporters are encoded by the symbiont genome, it is suspected that the clam actually digests symbiont cells for nutrition, as do mussels (Table 23.3).

Like the obligate symbionts of insects, most symbionts of marine invertebrates have small genomes (Table 23.2), indicating reduced function and an obligate association with their host. The bacterial symbiont of the giant tube worm *Riftia pachyptila* is an exception, having a genome larger than some free-living sulfur-oxidizing chemolithotrophs (Table 23.2). The *R. pachyptila* symbiont is acquired by uninfected juvenile animals from the environment (horizontal transmission), and its larger genome is likely important for survival as a free-living bacterium.

**MINIQUIZ**

- How do giant tube worms receive their nutrition?
- What are the similarities of the obligate symbioses of insects and hydrothermal vent invertebrates?
- What factors determine the genome size of the symbionts of marine invertebrates?

## 23.10 Entomopathogenic Nematodes

Here we consider two families of nematodes (the *Heterorhabditidae* and *Steinernematidae*) that are obligate pathogens of insects; together, they constitute a group of entomopathogenic (insect-killing) nematodes that are globally distributed and have a wide range of insect hosts. The basis for their insect lethality is a specific association between the species of nematode and its bacterial symbionts that produce a variety of insecticidal compounds.

### Specificity of Entomopathogenic Nematodes for Their Symbionts and Insect Hosts

Entomopathogenic nematodes have been extensively studied as biocontrol agents, providing an alternative to broad-spectrum chemical pesticides in the control of insect pests. In addition to alleviating possible human health effects of chemical pesticides, biologically based insect control strategies can be highly specific, targeting only the pest species and not other, co-resident insects. This helps maintain the natural insect biodiversity in environments that require pest species management, most commonly in agricultural settings. Apart from their demonstrated utility in pest management, this symbiosis has also provided a powerful model system for developing a detailed understanding of the evolution, physiology, and genetics of beneficial host–microbe interactions.

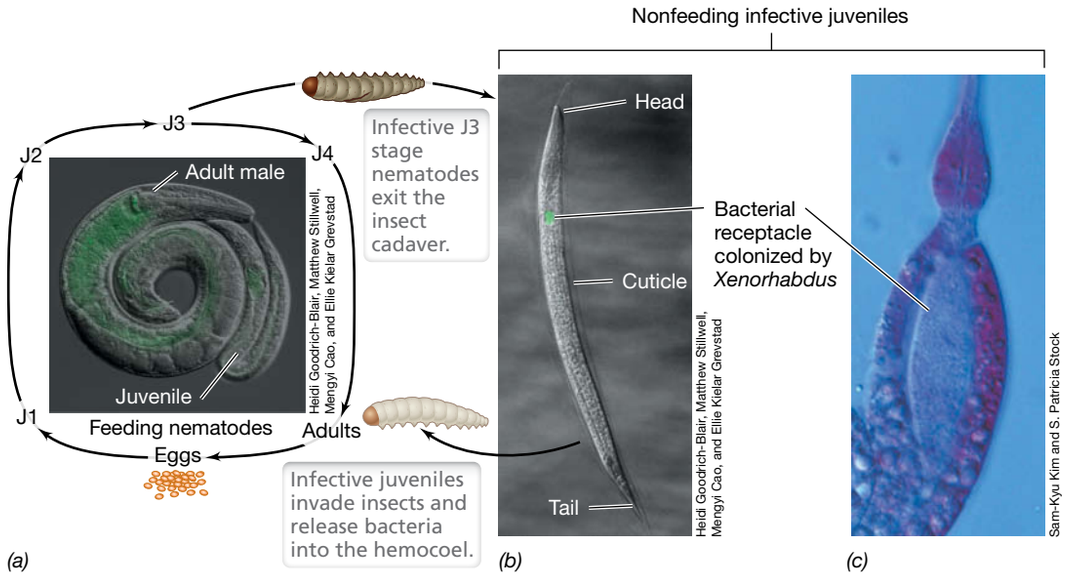



**Figure 23.32 Nematode–*Xenorhabdus* life cycle.** *(a)* In the presence of nutrients released in the infected insect, the nematodes develop through four juvenile stages (J1–J4), molting between stages, to egg-laying adults. When nutrients become limiting, the nematodes develop into an alternative nonfeeding J3 stage (known as the infective juvenile) that closes off most of its intestine and forms a receptacle in the gut where symbiotic bacteria localize. The infective J3 stage nematodes are then released into the environment to infect other insects. *(b) Xenorhabdus* localized to the bacterial receptacle of the infective juvenile. *(c)* Bacteria-filled receptacle (br) imaged by differential interference contrast microscopy (Section 1.6). Nematodes shown in *a* and *b* were genetically engineered to express the green fluorescent protein (Section 7.1) for imaging by fluorescence microscopy. Nematodes are approximately 50 μm in diameter.

Species of the gram-negative bacteria *Photorhabdus* and *Xenorhabdus* are the primary bacterial symbionts of the entomopathogenic nematodes. Comparative 16S rRNA sequence analysis (Section 13.7) has shown very high specificity of association between bacterial and nematode species. Species of *Photorhabdus* specifically associate with nematode species within the *Heterorhabditidae*, whereas *Xenorhabdus* species selectively associate with *Steinernematidae* nematodes. Each *Steinernema* species is thought to associate specifically with only one *Xenorhabdus* species. However, a single *Xenorhabdus* species may be associated with more than one nematode species. In turn, the nematodes comprising these two families can be separated into several phylogenetic groups that correspond to the phylogenetic relationships of their insect hosts. Together these data point to a history of cospeciation (coevolution) between nematodes and their bacterial symbionts that has resulted in the emergence of entomopathogenic nematodes having different insect host specificities.

### The Nematode Life Cycle and Lethality

All entomopathogenic nematodes have a similar life cycle in which only one stage, the nonfeeding third-stage infective juvenile, survives outside the host (**Figure 23.32**). The long association between nematode and bacterial symbiont has also resulted in modification of the anterior part of the infective juvenile nematode's intestine. This region forms a discrete chamber in the infective juvenile, called the *bacterial receptacle* (Figure 23.32*b* and *c*). This vessel becomes filled with bacteria by growth of the symbiont during maturation of the infective juvenile, protecting the symbiont for the next infective cycle.

When the infective juvenile encounters an appropriate insect host, it invades the insect's hemocoel (the body cavity containing the bloodlike hemolymph of the insect's circulatory system) through a natural opening such as the mouth or anus. The bacteria are then released from the bacterial receptacle into the hemolymph and rapidly multiply. They are able to thwart the insect's natural immune system in part by producing a variety of hemolysins, toxins, and digestive enzymes (e.g., proteases, lipases, chitinases) that promote the release of nutrients from host tissues. The bacterial symbionts also produce antibiotics to inhibit competitive colonization by other microbes. Both the multiplying bacteria and digested host tissues nourish the multiplying nematodes, and this results in a slow insect death, taking anywhere from 1 to 22 days. As the nutrients in the host become depleted, the adult nematodes produce new infective nonfeeding juveniles that are adapted to withstand the outside environment (Figure 23.32*a*), and the life cycle repeats.

**MINIQUIZ**

- What evidence suggest that the nematodes and their bacterial symbionts have coevolved?
- What prevents other bacteria from colonizing the dead insect and competing with the nematode and *Xenorhabdus* for nutrients?

## 23.11 Reef-Building Corals

Coral reef ecosystems are the products of mutualistic associations between microscopic algae and simple marine animals. The extensive ecosystems associated with the worldwide distribution of these mutualisms support tens of thousands of species.

### Phototrophic Symbioses with Animals

We began this chapter exploring lichens—the mutualism between a fungus and a phototrophic partner, either an alga or cyanobacterium. Like lichen fungi, some animals establish mutualistic associations with algae or cyanobacteria (**Table 23.4**). The animals in most of these associations are in phyla that display very simple body plans, for example, the *Porifera* (sponges) and *Cnidaria* (corals, sea anemones, and hydroids). These mutualistic animal–bacterial associations live in clear tropical waters where nutrients for the

**TABLE 23.4 Symbioses between animals and phototrophic symbionts**

| Host | Common name | Symbionts[a] |
|---|---|---|
| *Porifera* | Sponge | Cyanobacteria, *Chlorella*, *Symbiodinium* |
| *Cnidaria* | Coral, sea anemone | *Symbiodinium*, *Chlorella* |
| *Platyhelminthes* | Flatworm | Diatoms, primitive chlorophytes |
| *Mollusca* | Snail, clam | *Symbiodinium*, *Chlorella* |
| *Ascidia* | Sea squirt | Cyanobacteria |

[a]Cyanobacteria are *Bacteria*; all others are eukaryotic phototrophs.

animals are scarce, and the animal body typically has a large surface area relative to its volume and is thus well suited for capturing light.

There are only a few instances of algae forming associations with more complex animals, such as those in the phyla *Platyhelminthes* (flatworms), *Mollusca* (snails and clams), and *Urochordata* (sea squirts). In these cases either the animal has a suitable surface-to-volume ratio or has evolved specific light-gathering surfaces. The unicellular phototrophic symbionts are phylogenetically diverse and include cyanobacteria (Section 15.3), and red and green algae, diatoms, and dinoflagellates (Chapter 18). Most common are the green algae *Chlorella* (associating with sponges and freshwater hydras), cyanobacteria (associating with marine sponges), and species of the dinoflagellate genus *Symbiodinium*. Dinoflagellates and other alveolates comprise eight genera and around 2000 extant species (Section 18.4). Although dinoflagellate mutualisms are common, most are between species of *Symbiodinium* and marine invertebrates or protists. We focus here on the symbiotic association between the dinoflagellate *Symbiodinium* and the stony coral cnidarians.

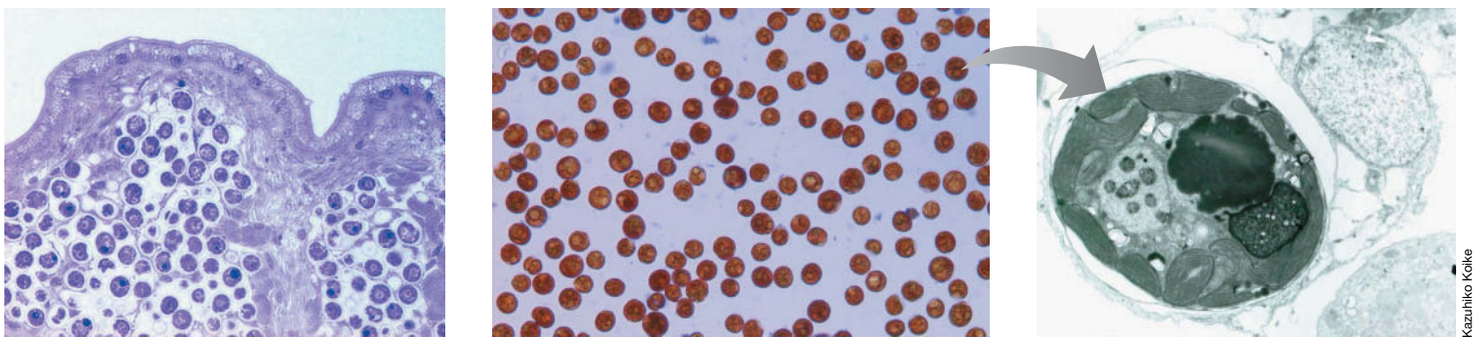
The mutualisms between the cnidarian stony corals (order *Scleractinia*) and *Symbiodinium* are among the most spectacular and ecologically significant animal–phototroph associations (**Figure 23.33**). Together the corals and dinoflagellates form the trophic and structural foundation of the coral reef ecosystem. The cnidarians possess a very simple two-tissue-layer body plan (ectoderm and gastroderm) and harbor the dinoflagellate symbiont intracellularly in membrane-bound vesicles (symbiosomes) within cells of the inner (gastrodermal) tissue layer (Figure 23.33*c*). The coral symbiosomes are analogous to the bacteroid-filled vesicles that develop in plant cells of the legume root nodules (Section 23.3). The coral skeleton is an extremely efficient light-gathering structure that greatly enhances light harvesting by the *Symbiodinium*. The algae receive key inorganic nutrients from host metabolism and pass photosynthetically produced organic compounds to the corals. This mutualism has allowed coral reefs to develop in large expanses of nutrient-poor ocean waters.

## Transmission, Specificity, and Benefits of the *Symbiodinium*–Coral Association

Reef-building corals reproduce sexually by releasing gametes into the seawater (broadcast spawning). A male and a female gamete fuse to form a free-swimming larva that later settles on a surface, where it may initiate a new coral colony. Algal symbionts are typically present in the egg before it is released from the parent (vertical transmission), although free-living *Symbiodinium* cells can also be ingested by juvenile corals (horizontal transmission). A developing coral that ingests dinoflagellates digests all of them except the particular *Symbiodinium* of its mutualism. After establishing an association, the coral controls the growth of *Symbiodinium* via chemical signaling and, following each cell division, each *Symbiodinium* daughter cell is allocated to a new symbiosome.

Both partners in the cnidarian–dinoflagellate mutualism have evolved adaptations for nutritional exchange. The dinoflagellates donate most of their photosynthetically fixed carbon (in the form of small molecules such as sugars, glycerol, and amino acids) to the cnidarian in exchange for inorganic nitrogen, phosphorus, and inorganic carbon from the host. The cnidarian is thought to control division of *Symbiodinium* through nitrogen limitation. Moreover, in addition to providing protection and inorganic nutrients, the calcium carbonate skeleton of corals is one of the most efficient collectors of solar radiation in nature, amplifying the incident light field for the symbionts by as much as fivefold; this benefits the symbiont in carrying out photosynthesis under a light-absorbing water column.






(a) (b) (c)

**Figure 23.33 *Symbiodinium* symbiont of marine invertebrates.** *(a)* Thin-section micrograph of *Symbiodinium* in the mantle tissue of a giant clam. *(b) Symbiodinium* cells recovered from a soft coral. *(c)* Transmission electron micrograph of a *Symbiodinium* cell within a vacuole of a cell of the stony coral *Ctenactis echinata*. The *Symbiodinium* cell is about 10 μm in diameter.


(a)


(b)

**Figure 23.34 Coral bleaching.** *(a)* Two colonies of the brain coral *Colpophyllia natans*. The coral on the left is a healthy brown color, whereas the coral on the right is fully bleached. *(b)* A large colony of partially bleached mountainous star coral (*Orbicella faveolata*).

## Coral Bleaching—The Risk of Harboring a Phototrophic Symbiont in a Changing World

Many of the extensive coral reef systems in the oceans worldwide are now threatened with extinction, primarily as a consequence of human activities. Ongoing loss of these beautiful and productive ecosystems is thought to be the result of elevated atmospheric $CO_2$; namely, increased sea surface temperature, rising sea levels, and ocean acidification (Sections 21.6 and 21.8). Coastal development also threatens reef systems, contributing to pollution from sewage discharge, eutrophication from nutrient runoff, and overfishing. These environmental changes are contributing to high mortality through disease, loss of coral structure from reduced calcification caused by acidification, and bleaching. Healthy corals harbor millions of cells of *Symbiodinium* per square centimeter of tissue. Coral bleaching is the loss of color from host tissues caused by the lysis of these symbionts, revealing the underlying white limestone skeleton (**Figure 23.34**).

Coral reefs live close to their optimum temperature, and it is the synergistic effect of increased sea surface temperature and irradiance that causes massive bleaching. Elevated temperature and high irradiance impair the photosynthetic apparatus of the dinoflagellates, resulting in the production of reactive oxygen species (for example, singlet oxygen and superoxide, Section 5.14) that cause damage to both host and symbiont. Bleaching is thought to be caused by a protective immune response of the host that destroys compromised symbionts. Increases in sea surface temperatures as small as 0.5–1.5°C above the local maximum, if sustained for several weeks, can induce rapid coral bleaching. A significant decrease in temperature below the optimum range for coral growth can have a similar effect. Thermal stress, accentuated by seasonal increases in electromagnetic radiation of ultraviolet and some visible wavelengths, has resulted in bleaching of huge expanses of coral reefs.

Although coral reefs are clearly threatened, there is much uncertainty in projecting their future. The more ominous projections, based on projected increases in sea temperature, point to a collapse of Indian Ocean coral reef systems within only a few years and a possible global collapse of coral reefs by the middle of this century. Moreover, the Great Barrier Reef off the northeast coast of Australia in the Coral Sea—the world's largest coral reef system—has experienced significant coral bleaching in recent years; nearly half the reef has been bleached. However, these observations and future projections lack much basic knowledge on the vulnerability of individual coral species and the adaptive capacity of individual coral–symbiont mutualisms. For example,

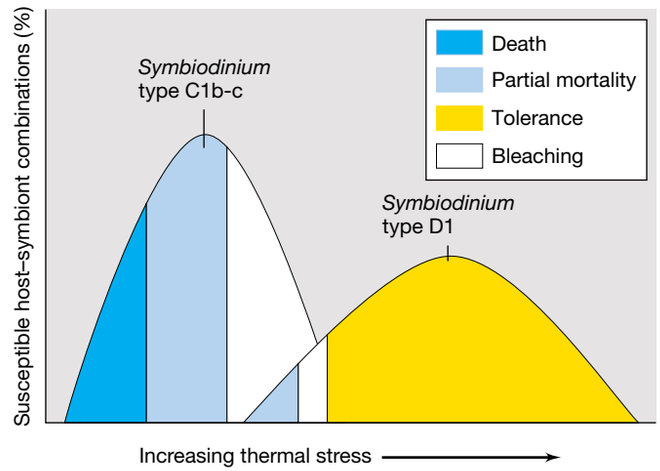



**Figure 23.35 Differential stress tolerance of a coral species associated with different *Symbiodinium* phylotypes.** *Pocillopora* corals symbiotically associated with *Symbiodinium* type C1b-c were much more sensitive to a thermal stress event than the same coral species associated with *Symbiodinium* type D1. The more tolerant *Symbiodinium* type D1–*Pocillopora* association suffered very low mortality. The response also suggested additional genetic variation within each *Symbiodinium* type, since the two mutualisms displayed a range of sensitivity to increasing thermal stress.

thermal tolerance is in part conferred by the species or strain of *Symbiodinium*, and following a bleaching event the mutualism can shift to a more thermally tolerant symbiont (Figure 23.35). Nevertheless, coral bleaching stands as additional evidence that climate change is upon us and that microbially based ecosystems are responding.

Molecular results have indicated that there are over 150 different *Symbiodinium* phylotypes, each possibly representing a distinct species with different stress tolerance. Both symbiont swapping and symbiont switching have been proposed as the underlying mechanism for shifting between symbionts. In switching, the symbiont is taken up from a water column population. In swapping, the shift results from differential growth of a genetic variant already associated with the coral, but in very low numbers, thereby swapping places with the previously dominant mutualist following the bleaching event. Most studies indicate that swapping is the more common adaptive mechanism, but uncertainty remains. Because the type of symbiont influences the ability of the coral to adapt to stresses associated with climate change, a more complete understanding of the alternative mechanisms of adaptive response, including possible symbiont switching, is essential to predicting the future health of corals, their symbionts, and the reefs they build.

**MINIQUIZ**

- What gives corals their spectacular colors?
- What are the two mechanisms of *Symbiodinium* transfer to developing corals?
- What are the major environmental factors contributing to coral bleaching?

# V • Mammalian Gut Systems as Microbial Habitats

The evolution of animals has been shaped in part by a long history of symbiotic associations with microorganisms and includes vertebrates as well as the invertebrate systems we just explored. We end this chapter by considering microbial symbioses with mammals other than humans. In the next chapter, we focus specifically on the microbiota of humans. Microbes inhabit all sites on mammalian bodies, but the greatest diversity and density of microbes are found in the mammalian gut, and so we center our discussion there.

## 23.12 Alternative Mammalian Gut Systems

Some mammals are *herbivores*, consuming only plant materials, whereas others are *carnivores*, eating primarily the flesh of other animals. *Omnivores* eat both plants and animals. As Figure 23.36 indicates, closely related mammals have evolved adaptations for differing diets. Notice that mammals of different lineages independently evolved the herbivorous lifestyle, mostly during the Jurassic period, an era in Earth's history of roughly 60 million years beginning about 200 million years ago.

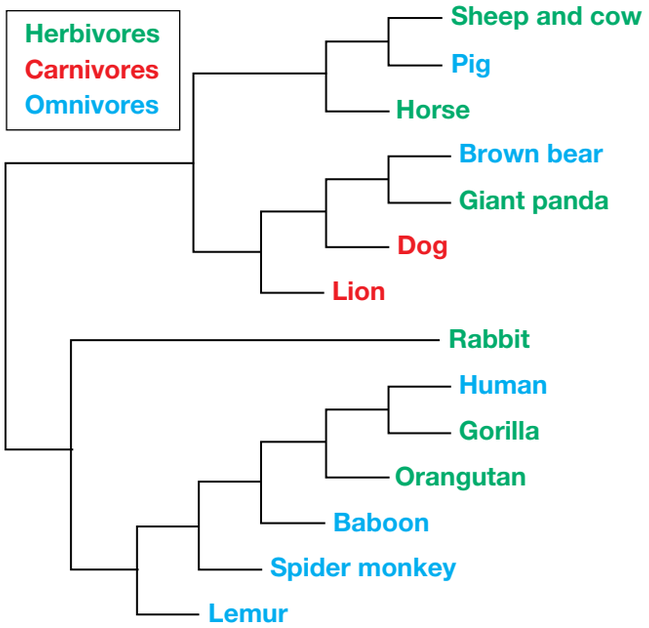


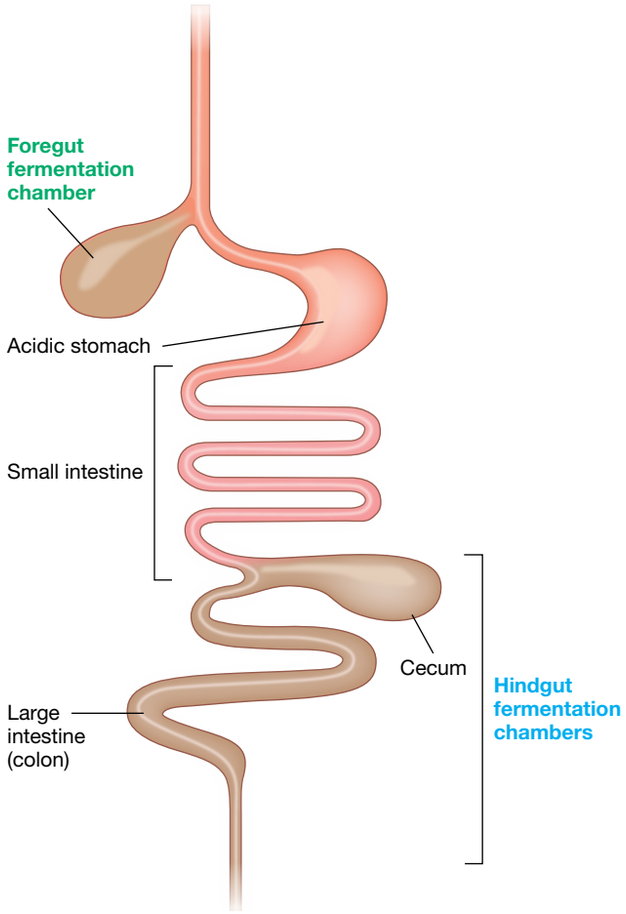

**Figure 23.36 Phylogenetic tree showing multiple origins of herbivory among mammals.** Some of the herbivores listed are foregut fermenters, while others are hindgut fermenters (see Figure 23.37). Instead of animal flesh, some mammalian carnivores eat only insects (the *insectivores*, such as bats), or fish (the *piscivores*, such as the river otter).

The massive evolutionary radiation of mammals during the Jurassic led to the evolution of several feeding strategies. Most mammalian species evolved gut structures that foster mutualistic associations with microorganisms. As anatomical differences evolved, microbial fermentation remained important or essential in mammalian digestion. *Monogastric* mammals, such as humans, have a single compartment, the stomach, positioned before the intestine. Such animals may get a substantial part of their energy requirement from microbial fermentation of otherwise indigestible foods, while herbivores are totally dependent on such fermentations.

### Plant Substrates

Microbial associations with various mammalian species led to the capacity to catabolize plant fiber, the structural component of plant cell walls. Fiber is composed primarily of insoluble polysaccharides of which cellulose is the most abundant component. Mammals—and indeed virtually all animals—lack the enzymes necessary to digest cellulose and certain other plant polysaccharides. However, many microbes have genes encoding the glycoside hydrolases and polysaccharide lyases required to decompose these polysaccharides.

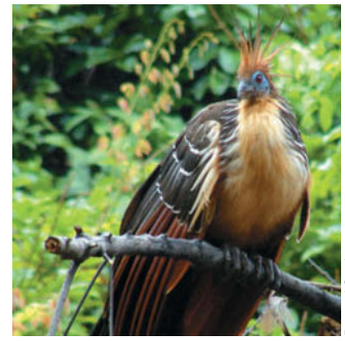
As the most abundant organic compound on Earth and one composed exclusively of glucose, *cellulose* offers a rich source of carbon and energy for animals that can digest it. The two primary traits that evolved to support herbivory are (1) an enlarged anoxic fermentation chamber for holding ingested plant material and (2) an extended retention time—the time that ingested material remains in the gut. A longer retention time allows for a longer association of microorganisms with the ingested material and thus a more complete degradation of the plant polymers.




**Foregut fermenters** Examples: Ruminants (photo 1), colobine monkeys, macropod marsupials, hoatzin (photo 2)

1.

2.



Bernard Swain

**Hindgut fermenters** Examples: Cecal animals (photos 3 and 4), primates, some rodents, some reptiles

3.

Nancy L. Spear

4.

**Figure 23.37 Variations on vertebrate gut architecture.** All vertebrates have a small intestine, but vary in other gut structures. Most host absorption of dietary nutrients occurs in the small intestine, whereas microbial fermentation can occur in the forestomach, cecum, or large intestine (colon). Foregut fermentation is found in four major clades of mammals and one avian species (the hoatzin). Hindgut fermentation, either in the cecum or large intestine/colon, is common to many clades of mammals (including humans), birds, and reptiles. Compare with Figure 23.36.



### Foregut versus Hindgut Fermenters

Two digestive patterns have evolved in herbivorous mammals. In herbivores with a *foregut* fermentation, the microbial fermentation chamber *precedes* the small intestine. This gut architecture originated independently in ruminants, colobine monkeys, sloths, and macropod marsupials (Figure 23.37). These all share the common feature that ingested nutrients are degraded by the gut microbiota *before* reaching the acidic stomach and small intestine. We examine the digestive processes of ruminants, as examples of foregut fermenters, in the next section.

Horses and rabbits are herbivorous mammals, but they are not foregut fermenters. Instead, these animals are *hindgut* fermenters. They have only one stomach but use an organ called the *cecum*, a digestive organ located between the small and large intestines, as their fermentation vessel (Figure 23.37). The cecum contains fiber- and cellulose-digesting (cellulolytic) microorganisms. Mammals, such as the rabbit, that rely primarily on microbial breakdown of plant fiber in the cecum are called *cecal fermenters*. In other hindgut fermenters, both the cecum and colon are major sites of fiber breakdown by microorganisms.

Anatomical differences among monogastric mammals, foregut fermenters, and hindgut fermenters are summarized in Figure 23.37. Nutritionally, foregut fermenters have an advantage over hindgut fermenters in that the cellulolytic microbial community of the foregut eventually passes through an acidic stomach. As this occurs, most microbial cells are killed by the acidity and become a protein source for the animal. By contrast, in animals such as horses and rabbits, the remains of the cellulolytic community pass out of the animal in the feces because of its position posterior to the acidic stomach.

**MINIQUIZ**

- How do animals with foregut and hindgut fermentation differ in recovery of nutrients from plants?
- How does retention time affect microbial digestion of food in a gut compartment?

## 23.13 The Rumen and Ruminant Animals

A very successful group of foregut fermenters are *ruminants*, herbivorous mammals that possess a special digestive organ, the **rumen**, within which cellulose and other plant polysaccharides are digested by microorganisms. Some of the most important

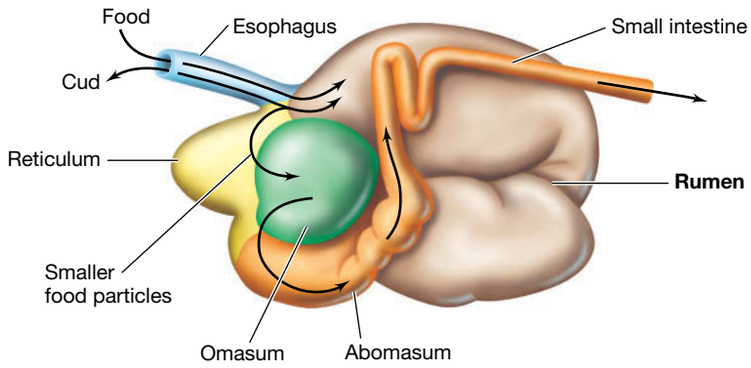




**Figure 23.38 The rumen.** *(a)* Schematic diagram of the rumen and gastrointestinal system of a cow. Food travels from the esophagus into the reticulo-rumen, consisting of the reticulum and rumen. Cud is regurgitated and chewed until food particles are small enough to pass from the reticulum into the omasum, abomasum, and intestines, in that order. The abomasum is an acidic vessel, analogous to the stomach of monogastric animals like pigs and humans. *(b)* Photo of a fistulated Holstein cow. The fistula, shown unplugged, is a sampling port that allows access to the rumen.

domesticated animals—cows, sheep, and goats—are ruminants. Camels, buffalo, deer, reindeer, caribou, and elk are also ruminants. Indeed, ruminants are Earth's dominant herbivores. Because the human food economy depends to a great extent on ruminant animals, rumen microbiology is of considerable economic significance and importance.

## Rumen Anatomy and Activity

Unique features of the rumen as a site of cellulose digestion are its relatively large size (capable of holding 100–150 liters in a cow, 6 liters in a sheep) and its position in the gastrointestinal system *before* the acidic stomach. The rumen's warm and constant temperature (39°C), narrow pH range (5.5–7, depending on when the animal was last fed), and anoxic environment are also important factors in overall rumen function. Figure 23.38*a* shows the relationship of the rumen to other parts of the ruminant digestive system. The digestive processes and microbiology of the rumen have been well studied, in part because it is possible to remove samples for analysis by way of a sampling port, called a *fistula*, implanted into the rumen of a cow (Figure 23.38*b*) or a sheep.

After a cow swallows its food, the food enters the first chamber of the four-compartment stomach, the reticulum. Partially digested plant materials flow freely between the rumen and reticulum, sometimes referred to together as the *reticulo-rumen*. The main function of the reticulum is to collect smaller food particles and move them to the omasum. Larger food particles (called *cud*) are regurgitated, chewed, mixed with saliva containing bicarbonate, and returned to the reticulo-rumen where they are digested by rumen bacteria. Solids may remain in the rumen for more than a day during digestion. Eventually, small and more thoroughly digested food particles are passed to the omasum and from there to the abomasum, an organ similar to a true, acidic stomach. In the abomasum, chemical digestive processes begin that continue in the small and large intestine.

## Microbial Fermentation in the Rumen

Food remains in the rumen for 20–50 h depending on the feeding schedule and other factors. During this relatively long retention time, cellulolytic microorganisms hydrolyze cellulose, which frees glucose. The glucose then undergoes bacterial fermentation with the production of **volatile fatty acids (VFAs)**, primarily *acetic, propionic*, and *butyric* acids, and the gases carbon dioxide ($CO_2$) and methane ($CH_4$) (Figure 23.39). The VFAs pass through the

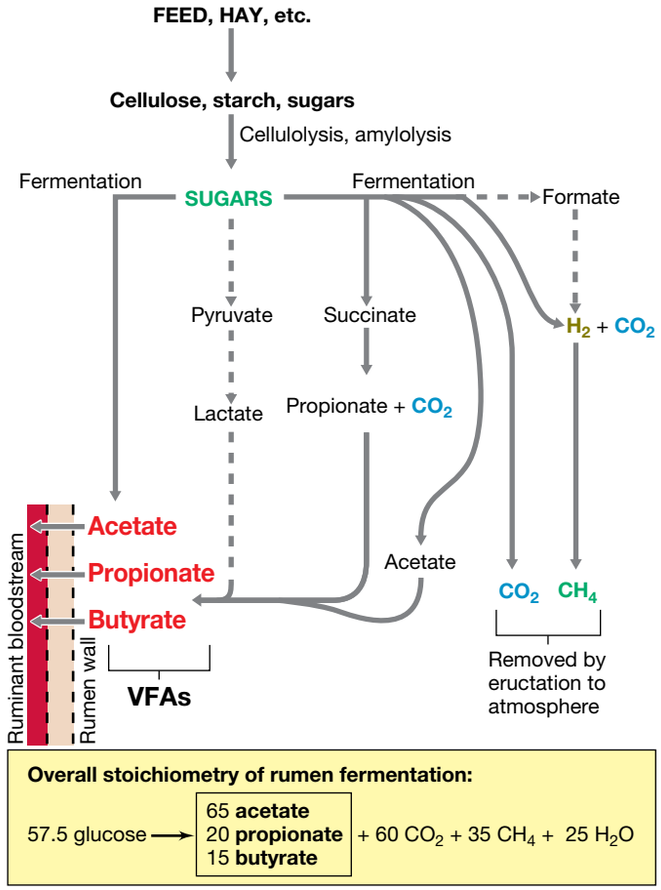



**Figure 23.39 Biochemical reactions in the rumen.** The major pathways are solid lines; dashed lines indicate minor pathways. Approximate steady-state rumen levels of volatile fatty acids (VFAs) are acetate, 60 mM; propionate, 20 mM; butyrate, 10 mM.

rumen wall into the bloodstream and are oxidized by the animal as its main source of energy. The gaseous fermentation products $CO_2$ and $CH_4$ are released by eructation (belching).

The rumen contains enormous numbers of bacteria ($10^{10}$–$10^{11}$ cells/g of rumen contents). Most of the bacteria adhere tightly to food particles. These particles proceed through the gastrointestinal tract of the animal where they undergo further digestive processes similar to those of nonruminant animals. Bacterial cells that digested plant fiber in the rumen are themselves digested in the acidic abomasum. Because bacteria living in the rumen biosynthesize amino acids and vitamins, the digested bacterial cells are a major source of protein and vitamins for the animal.

## Rumen Bacteria

Anaerobic bacteria dominate in the rumen because it is a strictly anoxic compartment, and some anaerobic microbial eukaryotes are also present. Cellulose is converted to fatty acids, $CO_2$, and $CH_4$ (Figure 23.39) in a multistep microbial food chain, with several different anaerobes participating in the process. Recent estimates of rumen microbial diversity from analysis of 16S rRNA gene sequences suggest that the typical rumen contains 300–400 bacterial "species" (defined as "operational taxonomic units" sharing less than 97% sequence identity, ↔ Section 13.8) (**Figure 23.40**). This is more than 10 times higher than culture-based diversity estimates. Molecular surveys show that species of *Firmicutes* and *Bacteroidetes* dominate the *Bacteria* in the rumen, while methanogens make up virtually the entire archaeal population (Figure 23.40).

A number of rumen anaerobes have been cultured and their physiology characterized (**Table 23.5**). Several different rumen bacteria hydrolyze cellulose to sugars and ferment the sugars to VFAs. *Fibrobacter succinogenes* and *Ruminococcus albus* are the two most abundant cellulolytic rumen anaerobes. Although both organisms produce cellulases, *Fibrobacter*, a gram-negative bacterium, produces enzymes localized to its outer membrane. *Ruminococcus*, a gram-positive bacterium (and therefore lacking an outer membrane) produces a cellulose-degrading protein complex stabilized by scaffold proteins and bound to its cell wall. Thus, cells of both *Fibrobacter* and *Ruminococcus* need to bind to cellulose particles in order to degrade them.

If a ruminant is gradually switched from cellulose to a diet high in starch (grain, for instance), the starch-digesting bacteria *Ruminobacter amylophilus* and *Succinomonas amylolytica* grow to high numbers in the rumen. On a low-starch diet these organisms are typically minor constituents. If an animal is fed legume hay, which is high in pectin, a complex polysaccharide containing both hexose and pentose sugars, then the pectin-digesting bacterium *Lachnospira multipara* (Table 23.5) becomes an abundant member of the rumen microbial community. Some of the fermentation products of these rumen bacteria are used as energy sources by secondary fermenters in the rumen. For example, succinate is fermented to propionate plus $CO_2$ (Figure 23.39) by the bacterium *Schwartzia*, and lactate is fermented to acetate and other fatty acids by *Selenomonas* and *Megasphaera* (Table 23.5). Hydrogen ($H_2$) produced in the rumen by fermentative processes never accumulates because it is quickly consumed by methanogens for the reduction of $CO_2$ to $CH_4$. $H_2$ removal facilitates greater fermentative activity because $H_2$ accumulation negatively affects the energetics of fermentative reactions that produce $H_2$ (↔ Section 14.23).

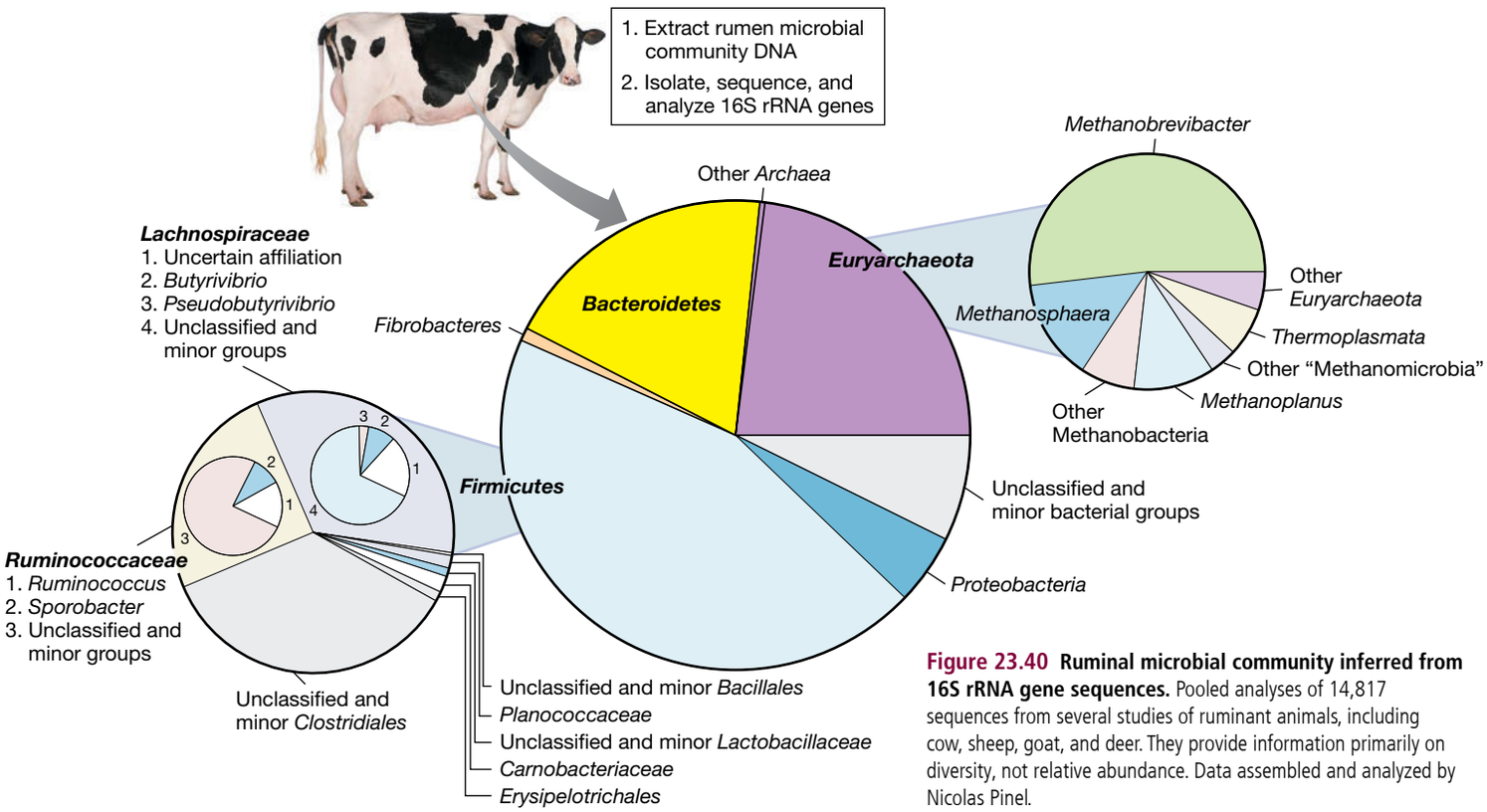



**Figure 23.40 Ruminal microbial community inferred from 16S rRNA gene sequences.** Pooled analyses of 14,817 sequences from several studies of ruminant animals, including cow, sheep, goat, and deer. They provide information primarily on diversity, not relative abundance. Data assembled and analyzed by Nicolas Pinel.

**TABLE 23.5 Characteristics of some rumen *Bacteria* and *Archaea***

| *Organism*[a] | *Morphology* | *Fermentation products* |
|---|---|---|
| ***Cellulose decomposers*** | | |
| ***Gram-negative*** | | |
| *Fibrobacter succinogenes*[b] | Rod | Succinate, acetate, formate |
| *Butyrivibrio fibrisolvens*[c] | Curved rod | Acetate, formate, lactate, butyrate, $H_2$, $CO_2$ |
| ***Gram-positive*** | | |
| *Ruminococcus albus*[c] | Coccus | Acetate, formate, $H_2$, $CO_2$ |
| *"Clostridium lochheadii"* | Rod (endospores) | Acetate, formate, butyrate, $H_2$, $CO_2$ |
| ***Starch decomposers*** | | |
| ***Gram-negative*** | | |
| *Prevotella ruminicola*[d] | Rod | Formate, acetate, succinate |
| *Ruminobacter amylophilus* | Rod | Formate, acetate, succinate |
| *Selenomonas ruminantium* | Curved rod | Acetate, propionate, lactate |
| *Succinomonas amylolytica* | Oval | Acetate, propionate, succinate |
| ***Gram-positive*** | | |
| *Streptococcus bovis* | Coccus | Lactate |
| ***Lactate decomposers*** | | |
| ***Gram-negative*** | | |
| *Selenomonas ruminantium* subsp. *lactilytica* | Curved rod | Acetate, succinate |
| *Megasphaera elsdenii* | Coccus | Acetate, propionate, butyrate, valerate, caproate, $H_2$, $CO_2$ |
| ***Succinate decomposer*** | | |
| ***Gram-negative*** | | |
| *Schwartzia succinovorans* | Rod | Propionate, $CO_2$ |
| ***Pectin decomposer*** | | |
| ***Gram-positive*** | | |
| *Lachnospira multipara* | Curved rod | Acetate, formate, lactate, $H_2$, $CO_2$ |
| ***Methanogens*** | | |
| *Methanobrevibacter ruminantium* | Rod | $CH_4$ (from $H_2 + CO_2$ or formate) |
| *Methanomicrobium mobile* | Rod | $CH_4$ (from $H_2 + CO_2$ or formate) |

[a]Except for the methanogens, which are *Archaea*, all organisms listed are *Bacteria*.
[b]These species also degrade xylan, a major plant cell wall polysaccharide.
[c]Also degrades starch.
[d]Also ferments amino acids, producing $NH_3$. Several other rumen bacteria ferment amino acids as well, including *Peptostreptococcus anaerobius* and *Clostridium sticklandii*.

## Dangerous Changes in the Rumen Microbial Community

Significant changes in the microbial composition of the rumen can cause illness or even death of the animal. For example, if a cow is changed abruptly from forage to a grain diet, the gram-positive bacterium *Streptococcus bovis* grows rapidly in the rumen. The normal level of *S. bovis*, about $10^7$ cells/g, is an insignificant fraction of total rumen bacterial numbers. But if large amounts of grain are fed abruptly, numbers of *S. bovis* can quickly rise to over $10^{10}$ cells/g and dominate the rumen microbial community. This occurs because grasses contain mainly cellulose, which does not support growth of *S. bovis*, while grain contains high levels of starch, on which *S. bovis* grows rapidly.

Because *S. bovis* is a lactic acid bacterium (Sections 14.20 and 16.6), large populations are capable of producing large amounts of its fermentation product, lactic acid. Lactic acid is a much stronger acid than the VFAs produced during normal rumen function. Lactate production thus acidifies the rumen below its lower functional limit of about pH 5.5, thereby disrupting the activities of normal rumen bacteria. Rumen acidification, a condition called *acidosis*, causes inflammation of the rumen epithelium, and severe acidosis can cause hemorrhaging in the rumen, acidification of the blood, and death of the animal.

Despite the activities of *S. bovis*, ruminants such as cattle can be fed a diet exclusively of grain. However, to avoid acidosis, they must be switched from forage to grain *gradually* over a period of many days. The slow introduction of starch selects for VFA-producing, starch-degrading bacteria (Table 23.5) instead of *S. bovis*, and thus normal rumen functions continue and the animal remains healthy.

## Protective Changes in the Rumen Microbial Community

The overgrowth of *S. bovis* is an example of how a single microbial species can have a deleterious effect on animal health. There is also at least one well-studied example of how a single bacterial species can *enhance* the health of ruminant animals; in this case, animals fed the tropical legume *Leucaena leucocephala*. This plant has a very high nutritional value, but contains an amino acid–like compound called *mimosine* that is converted to toxic 3-hydroxy-4(1H)-pyridone (3,4-DHP) and 2,3-dihydroxypyridine (2,3-DHP) by rumen microorganisms (**Figure 23.41**). The observation that ruminants in

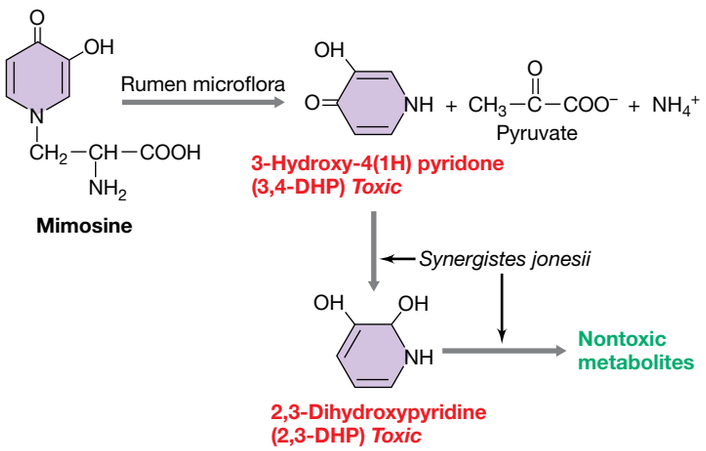



**Figure 23.41 Conversion of mimosine to toxic pyridine and pyridone metabolites by ruminal microorganisms.** Mimosine is converted to toxic 3,4-DHP by normal ruminal microbiota. *Synergistes jonesii* converts 3,4-DHP to nontoxic metabolites through a 2,3-DHP intermediate, preventing buildup of toxic metabolites of mimosine.

UNIT 5

Hawaii, but not Australia, could feed on *Leucaena* without toxic effect led investigators to hypothesize that further metabolism of DHP by bacteria present in Hawaiian ruminants alleviated DHP toxicity. This was subsequently confirmed by the isolation of the bacterium *Synergistes jonesii*, a unique anaerobe related to the *Deferribacter* group (Section 16.21) and not closely related to any other rumen bacteria. Inoculation of Australian ruminants with cells of *S. jonesii* conferred resistance to mimosine by-products, allowing the animals to feed on *Leucaena* without ill effect.

The success of this single-organism modification of the rumen microbial community has encouraged further studies of this sort, including genetic engineering of bacteria to improve their ability to utilize available nutrients or to detoxify toxic substances. A notable success has been inoculation of the rumen of sheep with genetically engineered cells of *Butyrivibrio fibrisolvens* (Table 23.5) containing a gene encoding the enzyme fluoroacetate dehalogenase; this successfully prevented fluoroacetate poisoning of sheep fed plants containing high levels of this highly toxic inhibitor of the citric acid cycle.

### Rumen Protists and Fungi

In addition to huge populations of *Bacteria* and *Archaea*, the rumen has characteristic populations of ciliated protists (Chapter 18) present at a density of about $10^6$ cells/ml. Many of these protists are obligate anaerobes, a physiology that is rare among eukaryotes. Although these protists are not essential for rumen fermentation, they contribute to the overall process. In fact, some protists are able to hydrolyze cellulose and starch and ferment glucose with the production of the same VFAs formed by cellulose-fermenting bacteria (Figure 23.39 and Table 23.5). Rumen protists also consume rumen bacteria and smaller rumen protists and are likely to play a role in controlling bacterial densities in the rumen. An interesting commensal interaction has been observed between rumen protists that produce VFAs and $H_2$ as products and methanogenic bacteria that consume the $H_2$, producing $CH_4$. Because their cells autofluoresce (Section 14.17), methanogens are easily observed in rumen fluid bound to the surface of $H_2$-producing protists.

Anaerobic fungi also inhabit the rumen and play a role in its digestive processes. Rumen fungi are typically species that alternate between a flagellated and a thallus form, and studies with pure cultures have shown that they can ferment cellulose to VFAs. *Neocallimastix*, for example, is an obligately anaerobic fungus that ferments glucose to formate, acetate, lactate, ethanol, $CO_2$, and $H_2$. Although a eukaryote, this fungus lacks mitochondria and cytochromes and thus lives an obligately fermentative existence. However, *Neocallimastix* cells contain a redox organelle called the *hydrogenosome*; this mitochondrial analog evolves $H_2$ and has otherwise been found only in certain anaerobic protists (Section 2.15).

Rumen fungi play an important role in the degradation of polysaccharides other than cellulose, including a partial solubilization of lignin (the strengthening agent in the cell walls of woody plants), hemicellulose (a derivative of cellulose that contains pentoses and other sugars), and pectin.

We now move on to Chapter 24 where the microbial world that populates humans will be center stage and considered in some detail.

**MINIQUIZ**

- What physical and chemical conditions prevail in the rumen?
- What are VFAs and of what value are they to the ruminant?
- Why is the metabolism of *Streptococcus bovis* of special concern for ruminant nutrition?





# Chapter Review

## I • Symbioses between Microorganisms

**23.1** Lichens are a mutualistic association between one or two species of fungi and an oxygenic phototroph, either an alga or a cyanobacterium.

**Q How does a phototroph benefit from associating with a fungus in lichen symbiosis?**

**23.2** The consortium "*Chlorochromatium aggregatum*" is a mutualism between a phototrophic green sulfur bacterium and a motile heterotrophic bacterium. Mutual benefit is based on the phototroph supplying organic matter to the heterotroph in exchange for motility that permits rapid repositioning in stratified lakes to obtain optimal light and nutrients.

**Q Considering its metabolic properties, what advantage might the phototroph in the "*Chlorochromatium aggregatum*" consortium gain from rapid repositioning?**

## II • Plants as Microbial Habitats

**23.3** One of the most agriculturally important plant–microbial symbioses is that between legumes and nitrogen-fixing bacteria. The bacteria induce the formation of root nodules within which nitrogen fixation occurs. The plant provides the energy needed by the root nodule bacteria, and the bacteria provide fixed nitrogen for the plant.

**Q Describe the steps in the development of root nodules on a leguminous plant. What is the nature of**

**the recognition between plant and bacterium and how do Nod factors help control this? How does this compare with recognition in the *Agrobacterium*–plant system (Section 23.5)?**

**23.4** Mycorrhizae are mutualistic associations between fungi and the roots of plants that allow the plant to extend its root system via intimate interaction with an extensive network of fungal mycelia. Both ectomycorrhizae and endomycorrhizae are known. The mycelial network provides the plant with essential inorganic nutrients, and the plant, in turn, supplies organic compounds to the fungus.

**Q How do mycorrhizae improve the growth of trees? In what way(s) are the root nodule and mycorrhizal symbioses similar? In what major way do they differ?**

**23.5** The crown gall bacterium *Agrobacterium* enters into a unique relationship with plants. Part of the Ti plasmid in the bacterium can be transferred into the genome of the plant, initiating crown gall disease. The Ti plasmid has also been used for the genetic engineering of crop plants.

**Q Compare and contrast the production of a plant tumor by *Agrobacterium tumefaciens* and a root nodule by a *Rhizobium* species. In what ways are these structures similar? In what ways are they different? Of what importance are plasmids to the development of both structures?**

## III • Insects as Microbial Habitats

**23.6** A large proportion of insects have established obligate mutualisms with bacteria, the basis of the mutualism often being bacterial biosynthesis of nutrients such as amino acids that are absent from the food the insect feeds on. Long-established obligate mutualisms are marked by extreme genome reduction of the symbiont, with retention of only those genes essential for the mutualism.

**Q How is it possible for aphids to feed only on the carbohydrate-rich but nutrient-poor sap of phloem vessels in plants? Why do symbionts that are transmitted horizontally show less genome reduction, as opposed to the significant genome reduction observed in heritable symbionts?**

**23.7** Termites associate symbiotically with bacteria and protists capable of digesting plant cell walls. The unique termite gut configuration and the hindgut microbial community composed largely of cellulolytic bacteria and protists and acetogenic bacteria result in high levels of acetate, the primary source of carbon and energy for the termite.

**Q How do the microbial communities of higher and lower termite guts differ in composition and degradation of cellulose?**

## IV • Other Invertebrates as Microbial Habitats

**23.8** A light-emitting organ on the underside of the Hawaiian bobtail squid provides a habitat for bioluminescent cells of the bacterium *Aliivibrio fischeri*. From the mutualism in the light organ, the squid gains protection from predators while the bacterium benefits from a habitat in which it grows quickly and contributes cells to its free-living population.

**Q How is the correct bacterial symbiont selected in the squid–*Aliivibrio* symbiosis?**

**23.9** Most invertebrates living on the seafloor near regions receiving hydrothermal fluids have established obligate mutualisms with chemolithotrophic bacteria. These mutualisms are nutritional, allowing the invertebrates to thrive in an environment enriched in reduced inorganic materials, such as $H_2S$, that are abundant in vent fluids. The invertebrates provide the symbionts an ideal nutritional environment in exchange for organic nutrients.

**Q Why is the genome of the tube worm symbiont thought to be so much larger than the genomes of insect symbionts?**

**23.10** Entomopathogenic nematodes have established symbiotic associations with species of *Photorhabdus* and *Xenorhabdus* bacteria. Following invasion of the insect by the nematode, bacterial symbionts are released into the insect's hemolymph and multiply rapidly by thwarting the insect's immune system, killing the insect by the release of toxins and digestive enzymes. When nutrients are depleted, the nematodes then transition to a nonfeeding juvenile form, harboring the symbiont in a specialized receptacle that will go on to infect other insects.

**Q Why are entomopathogenic nematodes so attractive for the biocontrol of insect pest species?**

**23.11** The mutualism between the dinoflagellate *Symbiodinium* and the stony corals produces the extensive worldwide coral reef ecosystems that sustain a tremendous diversity of marine life. Coral bleaching caused by climate change threatens these ecosystems.

**Q How have both partners in the cnidarian–dinoflagellate mutualism evolved adaptations for nutritional exchange?**

## V • Mammalian Gut Systems as Microbial Habitats

**23.12** Microbial fermentation is important for digestion in all mammals. Several microbial mutualisms have evolved in different mammals that allow for the digestion of different types of food. Herbivores derive almost all of their carbon and energy from plant fiber.

**Q What are the major benefits and the disadvantages of a rumen system? How does a cecal animal compare with a ruminant?**

**23.13** The rumen, the digestive organ of ruminant animals, specializes in cellulose digestion, which is carried out by microorganisms. Bacteria, protists, and fungi in the rumen produce volatile fatty acids that provide energy for the ruminant. Rumen microorganisms synthesize vitamins and amino acids and are also a major source of protein—all used by the ruminant.

**Q Give an example of a single microbial species contributing to herbivore nutrition. What is an example of a single microbial species that can contribute to herbivore pathology?**

## Application Questions

1. Imagine that you have discovered a new animal that consumes only grass in its diet. You suspect it to be a ruminant and have available a specimen for anatomical inspection. If this animal is a ruminant, describe the position and basic components of the digestive tract you would expect to find and any key microorganisms and substances you might look for. What metabolic types of microorganisms or specific genes would you predict would be present?
2. Why would you be very surprised to find the exact same microbial symbionts inhabiting a lichen and the rumen of a cow? Consider both the physical and chemical conditions of the habitat and the requirement for specific microbes to be present in order for the symbiosis to be successful.

## Chapter Glossary

**Arbuscule** branched or coiled hyphal structure within cells of the inner cortex of plants with a mycorrhizal infection

**Bacteriocyte** a specialized insect cell in which bacterial symbionts reside

**Bacteriome** a specialized region in several insect groups that contains insect bacteriocyte cells packed with bacterial symbionts

**Bacteroid** the misshapen cells of rhizobia inside a leguminous plant root nodule; can fix $N_2$

**Coevolution** evolution that proceeds jointly in a pair of intimately associated species owing to the effects each has on the other

**Consortium** a mutualism between bacteria, for example, a phototrophic green sulfur bacterium and a motile nonphototrophic bacterium

**Infection thread** in the formation of root nodules, a cellulosic tube through which *Rhizobium* cells can travel to reach and infect root cells

**Leghemoglobin** an $O_2$-binding protein found in root nodules

**Lichen** one or two species of fungi and an alga (or cyanobacterium) living in symbiotic association

**Mutualism** a symbiosis in which both partners benefit

**Myc factors** lipochitin oligosaccharides produced by mycorrhizal fungi to initiate symbiosis with a plant

**Mycorrhizae** a symbiotic association between a fungus and the roots of a plant

**Nod factors** lipochitin oligosaccharides produced by root nodule bacteria that help initiate the plant–bacterial symbiosis

**Root nodule** a tumorlike growth on plant roots that contains symbiotic nitrogen-fixing bacteria

**Rumen** the major fermentation vessel in the multichambered gut of ruminant animals, where most cellulose digestion occurs

**Symbiosis** an intimate relationship between organisms, often developed through prolonged association and coevolution

**Ti plasmid** a conjugative plasmid in the bacterium *Agrobacterium tumefaciens* that can transfer genes into plants

**Volatile fatty acids (VFAs)** the major fatty acids (acetate, propionate, and butyrate) produced during fermentation in the rumen

# 24

# Microbial Symbioses with Humans

## microbiologynow

### Frozen in Time: The Iceman Microbiome

Humans and their microbial associates—collectively called the *human microbiome*—have coevolved for millennia. As we will see in this chapter, the human microbiome influences a person's health, disease, and predisposition to disease. Among our intimate microbial associates, the pathogenic bacterium *Helicobacter pylori* is known to have developed a close relationship with humans in the distant past and to have coevolved with humans. *H. pylori* colonizes the stomachs of about half the human race. Although this bacterium generally does not cause overt disease, it is a major risk factor for the development of ulcers and stomach cancer. Moreover, because *H. pylori* is transmitted primarily by contact within families, the distribution of genetic variants of this bacterium may yield clues to past human migrations.

Unraveling the details of the *H. pylori* ancestry is complicated by the ability of different strains of this bacterium to recombine their genetic information. Because the DNA of various strains has mixed over long periods, the reconstruction of population movement inferred from genome sequences of modern *H. pylori* strains is incomplete. One of the biggest unanswered questions was the origin of strains now common among modern Europeans, which appear to be hybrids of strains originating in Asia and Africa. Unfortunately, the sequence data did not point to a reliable time interval in which that mingling of human populations occurred—an important period of human migration that was estimated to have occurred 10,000–50,000 years ago.

This estimate has now been greatly refined following the remarkable discovery of a well-preserved 5300-year-old European Copper Age mummy frozen in the Italian Alps. Using the newest methods for DNA sequencing, it was possible to reconstruct the genome of *H. pylori* preserved in the stomach of the "Iceman" (see photo), the corpse discovered when melting ice revealed the human remains on the side of a mountain. The Iceman *H. pylori* genome sequence turned out to be an almost pure representative of the Asian population, which means this *H. pylori* strain was present in Europe before hybridization of African and Asian strains produced the modern European variant. Thus, by employing historical biogeography, we now know this important period of human migration was much more recent than previously thought.

**Source:** Maixner, F., et al. 2016. The 5300-year-old *Helicobacter pylori* genome of the Iceman. *Science 351:* 162–165.