GROUND-WATER MICROBIOLOGY AND GEOCHEMISTRY

GROUND-WATER MICROBIOLOGY AND GEOCHEMISTRY

Second Edition

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United States Geological Survey

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PREFACE

This book is about microbial processes and how they affect the chemical composition of ground water. Ten or fifteen years ago, this topic was considered to be merely an interesting novelty. Since then it has become clear that microbial processes do more to abate and detoxify human-induced contamination of ground-water systems than any other natural or human-contrived mechanism. Furthermore, it has become clear that the chemistry and water-quality of pristine ground-water systems—which produce about half of the world's drinking water—is largely determined by microbial processes. For these reasons, understanding the unique ecology of ground-water systems has gone beyond being a novelty to become an important component of water-resources engineering and management.

But understanding the microbiology of ground-water systems, and using this understanding to solve practical water-quality problems, is not easy or straightforward. Microbiologists are trained to understand the complexity and diversity of microbial processes, but seldom have a background in geology or hydrology. Thus it is often difficult for them to appreciate how geologic and hydrologic factors mold and shape the expression of microbial processes in subsurface environments. Geoscientists, on the other hand, understand the complexities and uncertainties inherent to ground-water systems, but generally lack formal training in microbiology. Thus it is often difficult for them to appreciate how the metabolism and physiology of microorganisms can affect hydrogeologic systems. This book is specifically designed to bridge this gap between applied microbiology and applied hydrogeology.

This book is also designed for civil and environmental engineers. Engineers are trained to solve problems, and thus are often called upon to find practical solutions to human-induced and natural water-quality problems. By giving an overview of how geologic and microbial processes interact, and by giving numerous examples of how water-quality problems can be addressed using these principles, this book can serve as a resource for engineers dealing with ground-water quality issues.

This book is divided into three parts. Part I is an overview of basic microbiology. Many geoscientists and engineers have not had formal training in microbiology and often are not familar with microbiologic techniques and nomenclature. Although not intended as an exhaustive treatise or as a substitute for formal training, this lays the groundwork for how the growth and metabolism of microorganisms affects mineral dissolution processes, redox geochemistry, and contaminant biodegradation. In particular, this section shows how new methods of molecular ecology can be applied to ground-water systems.

Part II focuses specifically on microbial processes in pristine ground-water systems, and shows how these processes are shaped by geologic and hydrologic factors. In particular, this section shows how the microbial ecology of subsurface environments—and particularly how carbon, oxygen, hydrogen, sulfur, and iron cycling—affect ground-water chemistry. Because these well-known biogeochemical cycles are often truncated by isolation from solar energy, it is often possible to predict certain water-quality trends in subsurface environments. In addition, this part contains a systematic evaluation of how microbial processes affect redox geochemistry, and provides methods for identifying the distribution of microbial redox processes in ground-water systems.

Part III deals with the biodegradation and bioremediation of human-introduced contaminants in ground-water systems. There have been rapid advances in our understanding of how microbial processes contribute to the degradation of contaminants in ground-water systems in the last ten years. This section gives an up-to-date overview of the physiology and biochemistry of important biodegradation processes. The treatment of chlorinated ethenes shows how redox conditions affect biodegradation efficiency, and includes an overview of how reductive, oxidative, and cometabolic processes contribute to biodegradation. This section gives several examples of how biodegradation processes affect the mobility of petroleum hydrocarbons, chlorinated solvents, pesticides, and herbicides in different ground-water systems.

There are many reasons for studying the microbiology of ground-water systems. One reason is that they are unique ecosystems containing unique microorganisms and microbial processes. Thus, understanding these systems will contribute our overall understanding of microbial life on earth and possibly on other planets as well. Another reason is that naturally occurring water-quality problems—such as excessive concentrations of dissolved sulfide or well clogging by iron-oxidizing bacteria—can be solved or mitigated by understanding the underlying microbial processes. Finally, subsurface microorganisms can help abate many kinds of environmental pollution. The common ground for each of these issues is the chemical quality of ground water. This, therefore, is the focus of this book.

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PART I

OVERVIEW OF MICROBIOLOGY

CHAPTER 1

HISTORY, GEOLOGY, AND MICROBIOLOGY

History is filled with unlikely coincidences, but perhaps one of the oddest is the early association of microbiology with the study of ground water. The scientific investigation of microorganisms can be traced to about 1677 and the studies of Anton van Leeuwenhoek. Leeuwenhoek was a Dutch merchant and amateur scientist who had an amazing talent for grinding fine optical lenses. The microscopes that he contrived with his lenses (Fig. 1.1) could magnify objects as much as 300 times. This gave him the ability, for the first time in human history, to observe directly bacteria-sized microorganisms.

Leeuwenhoek was an extremely curious individual, and he immediately began to examine all sorts of materials, including tooth scrapings, blood, and semen. To his amazement, just about everything he examined was teeming with various microscopic creatures. He was especially interested in examining water samples from various sources. In one of many letters to the Royal Society of London, he described the presence of bacteria-like microorganisms in water drawn from a well. Thus, the study of microorganisms associated with ground water can be traced back to the very beginnings of microbiology.

This is an odd coincidence mainly because the development of microbiology and geology have followed such different paths. Table 1.1 shows a parallel chronology of some milestones in the development of these two disciplines. Note that the majority of milestones in the development of geology relate directly to unraveling the history of the earth and reflect an observational approach. The milestones in microbiology, on the other hand, relate to solving practical medical, industrial, and agricultural problems, and reflect an experimental approach.

It is important that these differences in approach, which have left such a strong imprint on each discipline, be explicitly understood. Geoscientists are often reluctant to rely solely on experimental methods to elucidate geologic phenomena. This reluctance is firmly grounded in the realization that complex geologic systems resist simulation under laboratory conditions. Microbiologists, on the other hand, are equally reluctant to accept observational evidence as being conclusive. This reflects

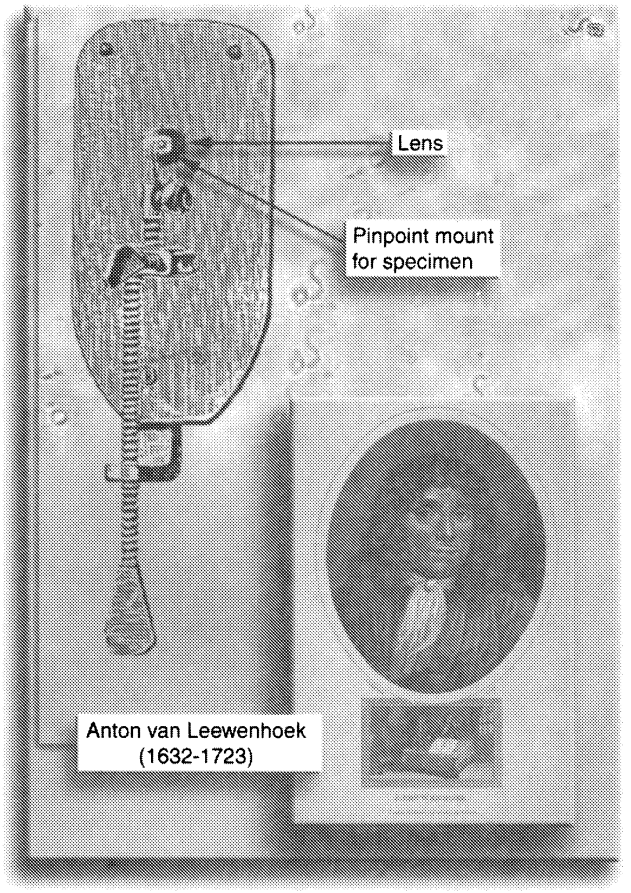


Figure 1.1. Anton van Leewenhoek was the first person able to resolve bacteria-sized microorganisms with his early microscope.

accumulated years of experience that appearances are often deceptive. Effective cooperation between geoscientists and microbiologists, which is critical for studying microbial processes in ground-water systems, requires that each understand the other's point of view. This, in turn, is most readily appreciated by considering the history of each discipline.

1.1 GEOLOGY—AN OBSERVATIONAL SCIENCE

From its beginnings, the primary emphasis of geology has been to unravel the history of the earth and to understand the nature of such puzzling phenomena as earthquakes, volcanos, and fossils. Early peoples developed elaborate mythologies dealing with geologic phenomena. To the Achaean Greeks of the Late Bronze Age (c. 1200 B.C.), for example, earthquakes were associated with the whims of the god Poseidon. When angry, he would crush cities by shaking the earth. He could also summon earthquakes to help the causes of those he favored.

TABLE 1.1 Milestones in the Development of Geology and Microbiology

Date	Geology	Date	Microbiology
Pre-1 A.D.	Geologic phenomena are attributed to actions of gods. Poseidon is thought to cause earthquakes when angered. Volcanoes are interpreted as the workshops of Hephaestus, the god of fire and metal working.	Pre-1 A.D.	Fermentations are used at least as early as 2400 B.C. in making beer and bread.
B.C. 500–1500	Fossils observed in rocks are attributed to Noah's flood.		
1500	Leonardo da Vinci observes fossiliferous rocks interbedded with fossil-free rocks and concludes that they could not have been formed by a single flood.		
		1546	Girolamo Fracastoro hypothesizes that disease is caused by germs being passed from one person to another.
1670	Nicholas Steno formulates the principles of superposition and original horizontality. Robert Hook suggests fossils could be used to establish chronology.	1670	Anton van Leeuwenhoek build usable microscope and makes first observation of bacteria-sized microorganisms
1749	Georges deBuffon questions literal significance of the Six Days of creation and suggests the earth is as old as 3 million years.	1770	Lazzarro Spallanzani disputes theory of spontaneous generation and demonstrates that putrefaction is caused by microorganisms.
1787	A. G. Werner founds Neptunist school that regarded granites and basalts as chemical precipitates from seawater. The neptunists are opposed by vulcanists who propose an igneous origin for granites and basalts.		
1795	James Hutton publishes "Theory of the Earth" in which he states that earth's history cycles between periods of deposition and periods of uplift and erosion.		

TABLE 1.1 ***(Continued)***

Date	Geology	Date	Microbiology
		1810	Chemist Jons Jakob Berzelius proclaims fermentation to be a strictly inorganic phenomenon that occurs without microorganisms.
1811	Georges Cuvier and William Smith, working independently, show a relationship between fossil assemblages and successions of sedimentary rocks. Cuvier espouses a "catastrophic" origin, such as Noah's flood, for the observed sequences.		
1830	Charles Lyell publishes *Principles of Geology,* a compendium of observations showing that geologic process observed today can be assumed to have been operating in the past. This view is termed "uniformitarianism" and directly opposes the "catastrophic" view of Cuvier.	1830	Charles Cagniard de Latour performs experiment showing fermentation to be caused by microorganisms. This view was criticized by chemists on the grounds that heat sterilization changes some essential component in air.
		1845	M. J. Berkley shows that Irish potato blight is caused by a mold. This is the first clear demonstration that microorganisms can cause disease.
1850	Henry Clifton Sorby uses a petrographic microscope to observe sedimentary rocks.		
1859	Charles Darwin publishes *The Origin of Species,* proposing that species are not immutable. This provides a frameword for understanding why fossil assemblages change with time. Edwin Drake's oil well drilled in Pennsylvania.	1859	Charles Darwin publishes *The Origin of Species* and establishes evolution as a basic concept in biology.
1860	James Hall proposes that accumulation of sediments depresses the earth's crust, forming basins of great depth.		

1861 Louis Pasteur shows that fermentations are caused by microorganisms and ends the controversy concerning spontaneous generation.

1867 "Pasteurization," or heating, to kill microorganisms in the wine, beer, and milk is used for the first time.

1873 James D. Dana coins the term "geosyncline" for thick sequences of sediments. He rejects Hall's sediment-loading hypothesis and proposes that geosyncline are a result, not a cause, of basin down warping.

1876 Robert Koch enumerates "Koch's postulates" and firmly establishes the germ theory of disease.

1881 Joseph Lister and Robert Koch develop methods for isolation of bacteria in pure cultures.

1885 Louis Pasteur develops a rabies vaccine.

1890 Sergei Winogradsky isolates autotrophic nitrifying bacteria and shows that they transform ammonia to nitrate in soils and manure. Martinus Beijerinck describes symbiotic and nonsymbiotic nitrogen fixation by bacteria, and isolates sulfate-reducing bacteria.

1905 Physicist Ernest Rutherford suggests using radioactive minerals to obtain absolute age of rocks.

1910 Paul Ehrlich establishes chemotherapy as a tool for treating bacterial infections, such as syphilis.

1912 Alfred Wegener proposes that continents are slowly drifting relative to each other and that they split off from a single "supercontinent."

1913 Frederick Soddy clarifies transformation of radioisotopes. This leads to reliable radiometric dating methods that demonstrate the earth to be billions of years old.

TABLE 1.1 (*Continued*)

Date	Geology	Date	Microbiology
1929	Arther Holmes invokes the mechanism of thermal convection in the mantle as the driving force for "continental drift." He envisions "spreading centers" where new crust is formed and "regions of compression" where crust is returned to the mantle.	1929	Alexander Fleming reports antibacterial action of *Penicillium,* a species of mold.
		1935	Wendell Stanley crystallizes tobacco mosaic virus and shows it is largely composed of protein.
1940	The discovery is made of bands of positive and negative magnetic anomalies on either side the Mid Atlantic ridge.	1940	The electron microscope is invented, antibiotics are first used in medicine.
1953	Evidence accumulates that the earth's magnetic field has reversed polarity periodically through geologic time.	1953	Structure of DNA is discovered by J. Watson and F. Crick. Hans Krebs receives the Nobel Prize for elucidation of the tricarboxylic acid cycle.
		1960	Francis Jacob and Jacques Monad propose the operon theory to explain how genetic information controls protein synthesis.
		1961	Peter Mitchell proposes the chemiosmotic model for ATP synthesis.
1963	F. J. Vine and D. H. Mathews in England, and L. Morley and A. Lorochelle in Canada independently propose that the Mid-Atlantic magnetic anomalies correspond to bands of rock that were magnetized during episodes of reversed polarity. This evidence supports the concept of sea-floor spreading and leads to wide acceptance of Plate Tectonics.		
1970	Plate tectonics is widely used to explain distribution of mountain ranges and deep-sea trenches.	1970	Harold Boyer and Stanley Cohen use plasmids and recombinant DNA methodology to clone genes.

It is no accident that, after ten unsuccessful years of the Trojan War, the Achaeans built the fabled Trojan Horse in a last-ditch effort to defeat the city of Troy. The horse was the symbol of Poisidon and was meant as an offering to the god. Whether or not the Achaeans actually hid inside it in order to sneak into the city is immaterial. The fact is, as has been documented by archaeological excavation, the destruction of Troy at the end of the Bronze Age (c. 1270 B.C.) coincided with a major earthquake. This earthquake, which enabled the Achaeans to sack and burn the city, was interpreted by them as being the intervention of Poseidon.

Interestingly, the biblical city of Jericho was also destroyed by earthquake at about the same time. The destruction of Jericho, an event that removed a formidable obstacle to the Israelite invasion of Canaan, was also attributed to divine intervention, although from a different deity.

The presence of fossilized clams, snails, and other obviously marine creatures in rocks hundreds of meters above sea level had been considered a mystery from very early times. Early Christian observers generally attributed them to the effects of Noah's flood. This interpretation was first seriously questioned by Leonardo da Vinci around 1500 A.D. Leonardo was fascinated by water and spent a lifetime carefully observing the action of flowing water in his native Tuscany. These observations are beautifully recorded in the waterfalls, rivers, and springs incorporated into most of his paintings. In addition to springs and rivers, the hills of Tuscany are characterized by the abundant occurrence of marine fossils in limestone. By comparing his own observations of how floods carried debris to what he observed in alternating beds of sedimentary rocks, he became convinced that there were numerous flooding episodes recorded in the rocks, not just the single event mentioned in the Bible.

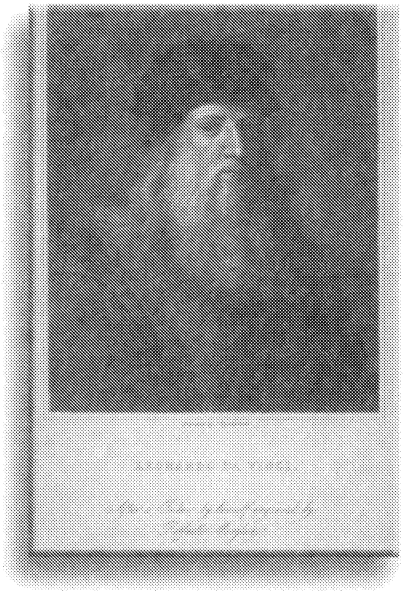


Figure 1.2. Leonardo da Vinci's study of water and floods led him to question whether sedimentary rocks could have been formed by a single (Noah's) flood.

Leonardo da Vinci was a secretive man who disliked sharing his observations with other people. More than 150 years passed before Nicholas Steno repeated Leonardo's observations and came to very similar conclusions. Steno recognized that the shell-bearing sedimentary rocks underlying the site of ancient Rome—rocks that were used in the city's construction—had to be older than the city itself (~3,000 years). However, Steno couldn't shake the biblical notion that the earth was only as old as written human history, or about 4,000 years. This obviously didn't leave much time for the rocks to form. If one accepted this time scale, one was forced to the conclusion that the rocks must have formed catastrophically. This view was roughly consistent with the biblical story of creation, and thus catastrophism came to guide much geologic thought over the next couple of centuries.

Unlike Leonardo, Steno published his observations in 1669. The principle of superposition (younger sediments overlie older sediments) is first attributed to Steno. Similarly, the principle of original horizontality (deformed sedimentary rocks must have been originally deposited in flat-lying beds) and original lateral continuity (faulted sedimentary rocks were originally deposited in continuity) are first attributed to Steno. These principles are the basis of modern stratigraphy.

A hundred years after Steno, the Frenchman Georges de Buffon realized that the thick sections of sedimentary rocks observed in the Paris Basin could not have been deposited in a few thousand years. Daring greatly, in 1749 he publicly suggested that the earth was as old as 75,000 years (his private writings indicate that he thought that the real age of the earth was more like 3 million years). This suggestion took some courage, because he was, in effect, challenging the 4,000-year-old age given in the Bible. The church was not broaching any opposition, however, and he was forced to retract his statements publicly. Buffon represented a major turning point in geologic thought, however, because he planted the idea that the Bible was not necessarily a reliable source of insight into geologic phenomena.

About the same time that Buffon was thinking about the age of the earth, two major schools of geologic thought arose in Europe. The "Vulcanists," principally Italians, proposed that the oldest rocks on earth (which were called the "primitive") were ultimately of volcanic origin. The "Neptunists," on the other hand, who were principally Germans, viewed the primitive rocks as being chemical precipitates from a primeval ocean. For a while, the Neptunists held sway, largely because of the persuasive teaching of their leader, A. Gottlieb Werner. Werner, who taught at the Freiburg Mining Academy in Saxony, was not a field geologist. Instead of collecting samples himself, he preferred to send students to do this work and confined himself to looking at rocks in his laboratory. This reluctance to do fieldwork seriously biased many of his observations. Werner himself never gave up his Neptunist views. However, because several Italian geologists actually witnessed the formation of igneous rocks from active volcanos, the Vulcanist view gradually replaced the Neptunist view.

The Neptunist position also came under attack from the writings of the Scottish naturalist James Hutton, who published his *Theory of the Earth* in 1795. Hutton, unlike Werner, believed in going into the field, carefully observing the rocks, and forming conclusions based solely on those observations. The Neptunists' view of the formation of the earth's original crust was largely one of quiescence. They envisioned a vast ocean with granites and basalts gradually precipitating from sea-

Figure 1.3. James Hutton's observations in the field led him to believe that the earth's crust was subject to cycles of sedimentation, uplift, and erosion.

water. In the hills around Edinburgh, however, Hutton could see clear evidence that "primitive" rocks had been deformed and thrust above the level of the sea. Rather than a static earth, where rocks precipitated out of seawater and remained largely unaltered thereafter, Hutton envisioned cycles of sediment accumulation, deformation, uplift, and subsequent erosion. Hutton's greatest contribution was the idea that the earth is internally dynamic and ever changing. This view clearly implied that the earth had to be very old, certainly older than a few thousand years.

Hutton also questioned the view that the earth had been largely shaped by single "catastrophic" events such as the biblical flood. Hutton reasoned that the processes of erosion and deposition that could be presently observed must have occurred in the past. Thus, the best guide to understanding the geologic record was to understand the nature of geologic process that could be presently observed. This view came to be called uniformitarianism—a view that was most eloquently developed by a British lawyer turned geologist named Charles Lyell.

Charles Lyell had little patience with the attitude that earth history reflected a series of mysterious catastrophes, such as Noah's flood or the disappearance of mysterious continents (i.e., Atlantis). He preferred a knowable, predictable earth, in which processes proceeded at a slow but steady pace. Given enough time, he reasoned, even gently flowing streams could erode deeply into the landscape. Lyell's extreme uniformitarianism was largely a reaction to the biblical catastrophists and greatly appealed to geologists of the nineteenth century. However, in the twentieth century, it has become apparent that certain catastrophic events (asteroid impacts, for instance) *have* occurred periodically throughout geologic time and that they have greatly influenced the history of the earth.

Meanwhile, in the early nineteenth century, it became clear that different sedimentary strata were characterized by different fossil assemblages. In England, this was the period when great networks of canals were constructed to facilitate moving coal, iron ore, and other commodities. One of the men involved in this huge effort, an engineer named William Smith, developed a deep interest in the fossils exposed by canal construction. Smith realized that rocks could be correlated from place to place based solely on fossil content. He wrote of the "wonderful order and regularity with which nature has disposed of these singular productions (fossils) and assigned to each its class and its peculiar Stratum." Eventually, Smith got to the point where he could recognize most of the strata in the English countryside and correlate them over long distances. In 1815, Smith completed publication of the first comprehensive geologic map of England. This map is still recognized as a masterpiece of cartography.

At the same time that Smith was working in England, two Frenchmen, Georges Cuvier and Alexandre Brongniart, made similar observations in the vicinity of Paris. Like Smith, they compiled and published a geologic map based upon the fossils in the rocks. The ability to distinguish strata on the basis of fossils, coupled with the realization that older rocks had to be overlaid by younger rocks, allowed geologists, for the first time, to establish relative chronologies. Two English geologists, Adam Sedgwick and Roderick Murchison, proceeded to construct such a chronology for the sedimentary rocks exposed in Wales. By 1835, Sedgwick had named the oldest fossil-bearing rocks Cambrian (for the ancient roman name for Wales) and Murchison had named the rocks immediately above Silurian (after an ancient Welch tribe).

Unfortunately, Murchison and Sedgwick could not agree on the boundary between the two units. Murchison suspected Sedgwick of trying to enlarge the Cambrian at the expense of the Silurian, and Sedgwick had similar suspicions about Murchison. The resulting row between the two men was finally resolved by naming the disputed interval the Ordovician. Successively younger rocks were named Devonian (after Devonshire) and Carboniferous (due to the presence of coal beds). Later, Murchison recognized rocks in Russia that were younger than the Carboniferous and named them Permian.

By the late nineteenth century, the relative ages of major sedimentary rock units in Europe were well known. No one, however, had much of an idea of their absolute ages. In 1854, the German thermodynamicist Herman von Helmholtz inferred that the earth could be no more than 20 to 40 million years old based on the luminosity of the sun. The English physicist William Thomson, better known as Lord Kelvin, embellished von Helmholtz's arguments with estimates of cooling rates of the primitive earth and came up with ages ranging from 20 to 75 million years.

The discovery of radioactivity in 1895 changed the debate considerably. Since radioactivity could heat the earth well beyond the length of time estimated by Lord Kelvin, it was quickly realized that the earth must be much older than formerly thought. In 1905, Ernest Rutherford, who first proposed a structure of the atom, suggested that rates of radioactive decay could be used to date rocks. By the 1920s, when the full series of uranium decay products had been worked out by the chemist Frederick Soddy, it became possible to assign absolute ages to rock units in addition to relative ages. Isotopic dating made it clear that the earth was not just millions of years old, but several billion years old.

Meanwhile, geologists had other knotty problems to worry about. By the mid–nineteenth century, it was clear that many mountain chains, the Alps being an obvious example, consisted of sedimentary rocks that were thousands of meters thick. How could such thick sediment sequences, which were thicker than the deepest part of the oceans, form? Furthermore, what forces could result in the uplift and deformation of such huge masses of rock? In 1860, the American geologist James Hall proposed that, as sediments accumulate in sedimentary basins, the increased weight causes downwarping of the crust, allowing even more sediments to accumulate. This hypothesis, termed isostasy, appealed to many geologists because it neatly explained away the sediment thickness problem. Unfortunately, it couldn't explain the uplift and deformation of the sediments. In 1873, the American geologist James Dana coined the term geosyncline to describe the massive accumulations of sediment associated with basins. He rejected Hall's isostasy hypothesis and argued instead that the basins were the result and not the cause of crustal downwarping. However, Dana offered no mechanism to account for the inferred downwarping. The origin of mountain building events was clearly related in some fashion to the accumulation of sediments in geosynclines. This was evident from ongoing efforts to map the geology of Europe and the United States. Furthermore, these mapping efforts showed that there had been, not one, but several episodes of mountain building throughout geologic time. What could explain these findings?

As early as 1620, Francis Bacon had remarked on the parallel configuration of the continental margins on each side of the Atlantic Ocean. This seemed to imply that the continents had at one time been contiguous and then had drifted apart. In 1858, a geologist named Antonio Snider actually published maps depicting the continents as drifting apart. In 1912, a German meteorologist named Alfred Wegener cited the remarkable similarity between rocks, geological structures, and fossils on opposite sides of the Atlantic Ocean as evidence for continental drift. For the next several years, Wegener doggedly made the case for the continental drift hypothesis in the face of severe criticism from most geologists. Wegener's argument suffered first from the fact that he himself had no credentials in geology. Second, Wegener could not put forward an adequate explanation as to the forces driving the continents apart.

A major contribution was made in 1928 by the British geologist Arthur Holmes. Holmes suggested that convection currents in the semisolid mantle, which were driven by heat from the decay of radioactive elements, could move continents and create deep-sea trenches (Fig. 1.5). This model is remarkably similar to our modern concept of plate tectonics. Holmes was a widely respected geologist, and his ideas lent considerable credibility to the idea of continental drift. Holmes himself, however, considered his paper to be merely an interesting idea and admonished his readers to remember that "purely speculative ideas of this kind, specially invented to match the requirements, can have no scientific value until they acquire support from independent evidence."

The independent evidence needed to establish this concept was a long time in coming. During World War II, the British and U.S. navies had developed extremely sensitive airborne magnetometers to detect German submarines. After the war, oceanographers adapted these magnetometers to be towed behind their research ships. By repeatedly steaming back and forth over the ocean, it gradually became clear that there were broad bands of positive and negative magnetic anomalies.

Figure 1.4. Alfred Wegener was an early advocate of the idea that continents moved relative to each other.

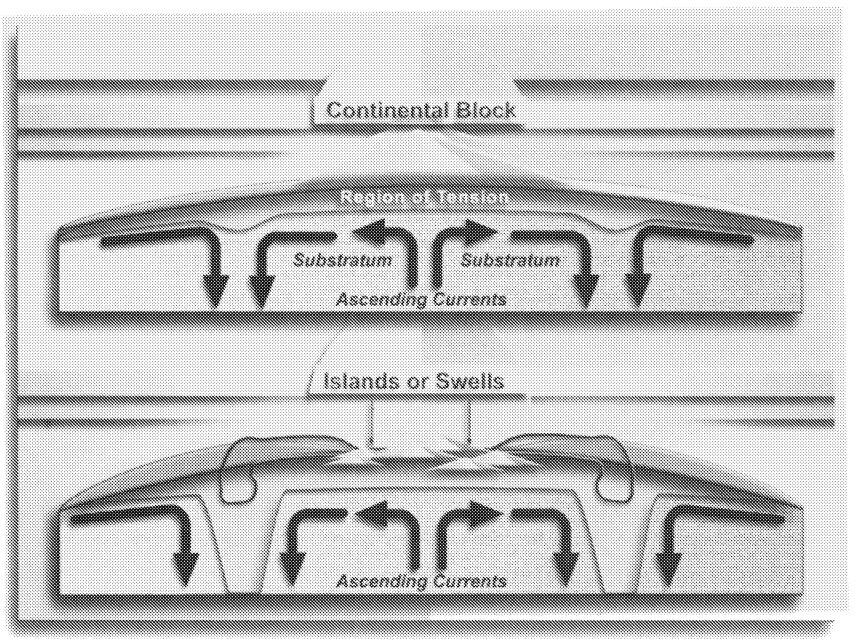


Figure 1.5. Holmes' 1928 model of mantle convection and continental drift.

Curiously, these bands were symmetrical around the newly discovered mid-oceanic ridge. What could possibly explain these findings?

In the 1950s, it became evident that the polarization of the earth's magnetic field had changed repeatedly over geologic time. In 1963, two Englishmen, F. J. Vine and D. H. Mathews, and two Canadians, L. Morley and A. Larochelle, independently made a connection between the magnetic anomalies on the seafloor and magnetic polarization reversals. Specifically, they proposed that the positive and negative magnetic zones corresponded to bands of oceanic basalt magnetized during ancient episodes of reversed polarity in the earth's magnetic field. This, in turn, implied that the ocean floor was progressively spreading at the mid-oceanic ridge, a process remarkably similar to the suggestions of Arthur Holmes. These findings were later confirmed by dating the rocks on the seafloor. The youngest rocks were at the mid-oceanic ridge and became progressively older away from the ridge. The demonstration of seafloor spreading firmly established the reality of continental drift.

With the general acceptance of plate tectonics, the model that accounted for the phenomenon of continental drift, numerous puzzling geologic questions were suddenly answered. Deep oceanic trenches, a phenomenon first observed in the nineteenth century, were areas where oceanic crust was being thrust underneath lighter continental crust. Sediments filling such trenches could be thousands of meters thick, explaining the presence of geosynclines. The collision of plate boundaries was a ready mechanism for deforming these sediments and thrusting mountains high above sea level. Finally, the motions of the earth at plate boundaries could explain the occurrence and distribution of earthquakes and volcanos. As a unifying model, plate tectonics has been remarkably successful in tying together numerous observations that otherwise seemed unrelated.

The point of this brief history is to emphasize the observational nature of geology. The early geoscientists who made significant contributions—DaVinci, Steno, Buffon, Cuvier, Hutton, Smith, and others—all had one thing in common. They strongly believed that the only way to understand the earth was by careful observation, followed by careful interpretation. The establishment of the most important unifying concept in geology—plate tectonics—was based entirely on observational evidence. Experimental evidence, indicating that rocks were insufficiently deformable for continents to move at all, largely muddied the issues involved. Other instances in which the progress of geology has faltered—such as the Neptunist teachings of Werner—can largely be attributed to sloppy observation. As a direct result of this history, the belief that accurate observation is the principal means for understanding geologic phenomena is deeply rooted in the earth sciences.

1.2 MICROBIOLOGY—AN EXPERIMENTAL SCIENCE

Human beings have an interesting ability to make use of phenomena that they do not understand. The classic example of this is the use that ancient peoples made of microorganisms in order to manufacture yogurt, bread, beer, and wine. The ancients had no idea that these useful processes were microbially mediated, and yet they developed quite sophisticated techniques for using them. Early Egyptian brewers, for example, originally used yeasts on wheat husks to carry out beer

fermentation. However, by about 2400 B.C., they were using virtually pure cultures of yeast in order to produce beer of predictable quality. This was all done by trial and error, and the brewers were certainly unaware that they were culturing microorganisms. It does seem, however, that they knew that their brewing made use of some "living" force. Similarly, people used salting and drying techniques to preserve food long before the role of microorganisms in food spoilage was understood.

With our modern understanding of medicine, it's difficult to imagine just how terrifying infectious diseases must have been to early peoples. Not only were the consequences of disease to be feared, but also the fact that no one knew their causes meant that people were largely helpless to protect themselves. Nevertheless, again by trial and error, effective means of dealing with infectious diseases were developed. The practice of "quarantine" for people with certain diseases dates to biblical times. For some diseases, such as cholera, quarantine could be effective. Ironically, however, the diseases to which this technique was most commonly applied—leprosy and the bubonic plague—are not easy to contain with quarantine. The reason for this is that the bacteria that cause both leprosy and plague can be carried and transmitted by small mammals such as rats.

The advent of the black plague, in the mid–fourteenth century, had an enormous impact on medical thinking in Europe. From its outbreak in early 1348 in Marseilles, France, the disease spread northward, reaching Paris on June 30. Later that year, it appeared in Bristol, England, and by 1349 had engulfed all of Europe. The systematic nature of the plague's spread was not lost on the horrified Europeans. It followed trade routes on both land and sea. Clearly, the plague was caused by some communicable agent. This observation was put to immediate use by the inhabitants of fourteenth-century Sicily who required the men in arriving ships to stay at anchor for 40 days before coming ashore. Unfortunately, this quarantine was ineffective against the rats that apparently swam ashore at their leisure, bringing disease-harboring fleas with them.

Two centuries later, in 1546, an Italian physician named Girolamo Fracastoro published a treatise on contagious diseases titled *De Contagione*. In his book, Fracastoro hypothesized, largely on philosophical grounds, that disease could be caused by "germs" being passed from one person to another. Furthermore, Fracastoro recognized that contagious diseases were similar to the putrefaction of wine. This was the first hint that the two processes might have a common cause. Fracastoro also seemed to understand that certain germs caused only certain diseases and that different diseases could be spread by different means.

The actual study of microorganisms began in 1665 with the publication of Robert Hook's *Micrographia or Some Physiological Descriptions of Minute Bodies Made by Magnifying Glasses with Observations and Inquiries Thereupon.* In this work, Hook provided drawings of various species of fungi. However, Hook's crude magnifying glass could only resolve fairly large microorganisms.

Anton van Leeuwenhoek was able to grind much better lenses than were available to Hook and was able to construct a microscope capable of resolving bacteria. It's interesting to note that van Leeuwenhoek was a close friend of the great Dutch physicist Christian Huygens. Huygens was deeply interested in the behavior of light and developed a wave theory to account for its behavior. It is probable that the quality of Leeuwenhoek's lenses benefited substantially from the theoretical insights of Huygens.

The discoveries of Hook and van Leeuwenhoek opened the way for investigation of the nature of microorganisms. One controversy that emerged and that lasted throughout the eighteenth and into the nineteenth century concerned the origin of microorganisms. Aristotle (384–322 B.C.) had taught that animals could originate spontaneously from soil, plants, or even other unlike animals. To many, it seemed perfectly logical that microorganisms could be generated spontaneously. For example, freshly killed meat was apparently free of microorganisms. However, after a couple of days, microorganisms could be observed growing on the meat. Where did they come from? Most people thought that they simply appeared spontaneously.

The modern debate on spontaneous generation of microorganisms began when in 1749 an Englishman named John Needham experimented with meat exposed to hot ashes. Needham observed that bacteria that apparently were not present at the beginning of the experiment later appeared. He concluded that the bacteria must have been generated by the meat. A contemporary of Needham named Lazzarro Spallanzani observed something quite different. When Spallanzani boiled beef broth for an hour and then sealed the flask, no microbes appeared. When informed of Spallanzani's results, Needham was unmoved. Needham insisted that air was essential to the spontaneous generation of microbes and by sealing the flasks, Spallanzani was simply excluding a necessary ingredient to spontaneous generation.

An argument closely related to the spontaneous generation debate was whether or not microorganisms were responsible for "putrefaction" (fermentation) of organic compounds, such as sugars or proteins. Several otherwise quite capable chemists, including Justus von Liebig, Jons Jakob Berzelius, and Friedrich Wohler, were convinced that fermentations were chemical phenomena that had nothing to do with microorganisms.

The idea of air being necessary for spontaneous generation, as opposed to being a medium carrying microorganisms, was a particularly difficult problem to address. In the nineteenth century, this problem was taken up by Franz Schulze and Theodor Schwann. Schulze passed air through strong acid solutions and into boiled broth, and microbes failed to appear. Schwann passed air through red-hot tubing into broth and again microbes failed to appear. Advocates of spontaneous generation were unmoved. The acid and the heat was changing something in the air needed for growth, they argued. About 1850, two German scientists named Schroeder and von Dusch performed an even more convincing experiment by passing air through cotton filters into boiled broth. Amazingly, however, the proponents of spontaneous generation and chemical fermentation remained unconvinced.

In 1859, a Frenchman named Pouchet published an extensive study "proving" the existence of spontaneous generation. In some ways, Pouchet's paper proved to be pivotal, because the data and the logic irritated a fellow Frenchman, Louis Pasteur. Pasteur had been trained as a chemist and was adept at experimental design. He prepared a flask with a long, narrow gooseneck opening that allowed air into the flask but excluded airborne particles. When Pasteur reported his results in 1864, he exhibited the flourish for which he became famous, saying:

> For I have kept from them, and am still keeping from them, that one thing which is above the power of man to make; I have kept from them the germs that float in the air, I have kept from them life.

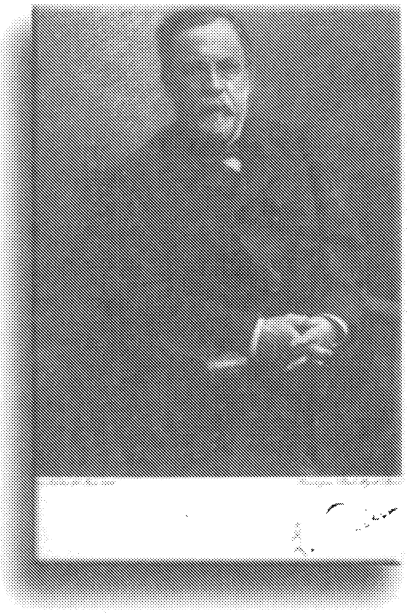


Figure 1.6. Louis Pasteur was instrumental in establishing deductive experimentation as the basis for modern microbiology.

Pasteur went on to chide the supporters of spontaneous generation:

> They who allege it [spontaneous generation] have been the sport of illusions, of ill-made experiments, vitiated by errors which they have not been able to perceive and have not known how to avoid.

It's worth noting that the essence of Pasteur's criticism was that his opponents were poor experimentalists. Pasteur recognized that the first requirement of proper experimentation was to have a testable hypothesis. Given a hypothesis, one could design experiments to test its validity. Pasteur followed this same procedure throughout his long and productive career.

Pasteur's work on spontaneous generation occurred some years after he became actively involved in microbiological studies. In 1854, while he was a professor at the University of Lille, a local industrialist approached him concerning a problem in local wine production. It seemed that, when grape juice was being fermented in vats, some of the vats produced good wine, whereas others produced sour wine. Pasteur observed wine samples from both kinds of vats under the microscope and saw that the good ones were characterized by a single kind of budding yeast cell. In the bad vats, on the other hand, there were rod-shaped bacteria. Furthermore, these bacteria produced lactic acid from grape sugar, accounting for the sour taste of the wine.

From these studies, Pasteur reasoned that the quality of the wine could be controlled by seeding the fermentation vats only with the proper kind of microorganism. To do this, however, it was first necessary to kill or inactivate the

microorganisms naturally present in the juice. Boiling, the accepted method of sterilization at the time, was not possible as this would change the character of the grape juice. Pasteur found that heating the juice at 145°F for half an hour removed the undesirable bacteria without hurting the flavor of the grape juice. This heating process, which we still refer to as pasteurization, greatly increased the productivity and quality of the local wine industry.

In the 1870s, Pasteur turned his attention to studying the cause of anthrax, a deadly disease of sheep, cattle, and sometimes people. Coincidentally, a German country physician named Robert Koch became interested in this disease at about the same time. Koch was a quiet, meticulous man who became fascinated with manipulating bacterial cultures in the laboratory. It was Koch who first discovered the rather large bacilli present in the blood of cattle that had died of anthrax. Koch carefully transferred these cultures, examining them periodically under the microscope, until he was sure that there was only one kind of microorganism present. He then injected cultures of the bacilli into healthy animals and observed that they too became sick and died. Lastly, Koch was able to again isolate the bacilli from the blood of the dead animal. This constituted the first rigorous demonstration that a bacterium could cause disease in animals. Koch published these results in 1876.

The steps that Koch followed in demonstrating the causative agent of anthrax have come to be known as "Koch's postulates." They are:

1. The organism should be present in all animals suffering from the disease and should be absent from all healthy animals.
2. The organism must be grown in pure culture outside the diseased animal.

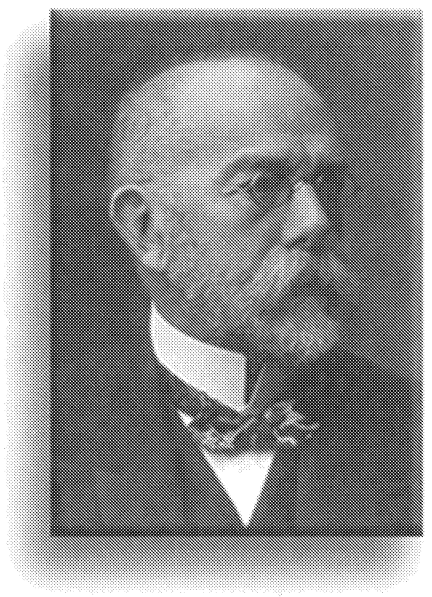


Figure 1.7. Robert Koch showed the importance of isolating pure cultures of bacteria in microbiology.

3. When the pure culture is used to inoculate a healthy animal, the animal must develop the symptoms of the disease.
4. The organism must again be isolated from the experimentally infected animal and shown to be identical to the original isolate.

Koch's work was the final confirmation of the germ theory of disease. This theory, at least in rudimentary form, can be traced back to the writings of Fracastoro in the sixteenth century. The theory was formally proposed in 1762 by Anton von Plenciz of Vienna. Plenciz stated that living agents were the cause of disease and went on to state that particular germs caused particular diseases. The establishment of the germ theory of disease by Koch was a milestone in the development of microbiology.

Robert Koch understood at a very early stage in his research that being able to grow and to manipulate bacteria in the laboratory was crucial. Being a born tinkerer who was very clever with his hands, Koch was able to refine techniques for studying bacteria. For example, he found that by smearing bacteria on a glass slide and adding certain dyes, individual bacterial cells were much easier to observe. It was Koch (or possibly his female laboratory assistant, Hesse) who learned how to add gelatin and agar as solidifying materials to culture media in order to grow isolated colonies of bacteria. These colonies could then be sequentially transferred to fresh media until a pure culture was obtained. The development of solid media made it much easier to study bacteria in the laboratory. Using these techniques, Koch isolated the bacteria that caused pulmonary tuberculosis in 1884.

Louis Pasteur, while begrudging Koch in particular, and Germans in general, a share of the limelight, immediately understood the importance of Koch's techniques and began applying them in his own research. In 1880 Pasteur isolated the organism that caused cholera in chickens. In order to prove to a skeptical public that this organism really was the cause of the disease, Pasteur arranged for a public demonstration of an experiment that he had performed many times. He inoculated healthy chickens with cultures of the bacteria, but to his chagrin, the chickens failed to get sick.

Going back over his notes, he discovered that, instead of being injected with freshly grown bacteria, the chickens had been inoculated with a culture that was several weeks old. Evidently, only freshly grown bacteria had the ability to cause the disease. The real surprise came when, a few weeks later, Pasteur injected freshly grown inoculum into a new group of chickens, as well as chickens that had had the old inoculum. Those chickens that had previously been exposed to the old inoculum remained healthy whereas unexposed chickens promptly developed cholera.

This was a sensational discovery for two reasons. First, it explained the phenomenon of immunization that had been discovered in 1798 by Edward Jenner. Jenner had found that inoculating people with material from cowpox boils could provide immunity against smallpox. Pasteur realized that the chicken cholera experiment explained how immunization worked. Pasteur acknowledged this in naming the new procedure "vaccination," which was based on *vacca,* the Latin word for "cow."

Second, this discovery suggested a means for providing immunity against other diseases. Working with old or "attenuated" cultures, Pasteur soon developed an immunization for anthrax. The crowning achievement of Pasteur's career, however, was the development of a vaccine for the viral disease rabies. Pasteur knew that rabies was not caused by a bacterium, as it could not be observed under the microscope. However, he found that injecting an extract of the dried brains of infected animals provided immunity to dogs. This remained untested in humans until a young boy named Joseph Meister was bitten by a rabid wolf in 1885. The boy's parents brought him to Pasteur who injected Meister with successively more virulent pieces of ground rabbit spinal cord. To Pasteur's delight, and possibly to his surprise, Meister did not develop rabies.

The triumph of the rabies vaccine led to widespread financial support for Pasteur's research and the establishment of the Pasteur Institute. The establishment of this institute led to the widespread production and utilization of vaccines in medicine and agriculture. Just as important, however, it provided the means for training students in Pasteur's techniques.

One of the students who spent time at the Pasteur Institute was a young Russian named Sergei Winogradsky. Winogradsky took the pure culture techniques developed for investigating infectious diseases and applied them to the investigation of other bacterially mediated processes. As early as 1877, it had been proposed that the process of nitrification—forming nitrate ions from ammonia—was bacterially mediated. Winogradsky took up this problem and succeeded in isolating pure cultures of nitrifying bacteria. This, in turn, showed for the first time that microorganisms could obtain energy for growing by oxidizing inorganic (in this case, ammonia) as well as organic compounds. Winogradsky later described the microbial oxidation of sulfur, hydrogen sulfide, and ferrous iron.

The early twentieth century was a period of explosive growth in the application of microbiology to medical, industrial, and agricultural problems. During this period, literally thousands of microbes were described and cataloged. This created, as one might suspect, a nightmare of microbial taxonomy (classification). In 1894, the Gram staining procedure was incorporated as a key feature of bacterial classification systems. Beginning in 1915, Robert Buchanan proposed a classification system that was fundamentally different from zoological or botanical systems. Buchanan's system incorporated morphological, biochemical, and pathogenic characteristics in order to classify bacteria. Buchanan's efforts led to the establishment of a Committee on Characterization and Classification of Bacterial Types, of which the first chairman was David Bergey. The first edition of *Bergey's Manual of Determinative Bacteriology* was published in 1923. Subsequent editions of *Bergey's Manual* have been the basis of bacterial taxonomy ever since.

It was during the mid–twentieth century that the basics of bacterial metabolism and physiology were worked out. Albert Kluyver, who was director of the Delft Polytechnical School (a legacy of Anton van Leeuwenhoek), examined microbial metabolism, recognizing the importance of intermediate products in transferring energy. He established that hydrogen transfer was the basis of all metabolic processes. In addition, Kluyver recognized the importance of central metabolic pathways in microbial metabolism. The most important of these pathways, the Tricarboxylic Acid (TCA) cycle and the Calvin cycle, were worked out in the 1930s and 1940s. Interestingly, the Nobel Prizes for discovery of the TCA and Calvin

cycles were both awarded in chemistry. This is indicative of the critical role played by biochemistry in understanding microbial processes.

The discovery of the double helix structure of DNA by James Watson and Francis Crick in 1953 set the stage for new studies into bacterial genetics. In 1960, the operon theory for explaining how genetic information controls protein synthesis was proposed by François Jacob and Jacques Monad. The genetic code, the cell's "dictionary" for how DNA base sequences specify amino acid sequences in proteins, was broken by Robert Holley, H. G. Khorana, and Marshal Nirenberg in the early 1960s. In the 1970s, advances in microbial genetics led to "genetic engineering" technology, in which genes for specific proteins could be inserted into bacteria. An early use of this technology was the production of human insulin from bacteria.

This brief overview of the history of microbiology does scant justice to the important fields of virology, phycology (study of algae), mycology (study of fungi), protozoology (study of protozoa), or immunology, all of which are important subdisciplines of microbiology. In fact, modern microbiology is best thought of as a collection of specialized subdisciplines unified mainly by the microscopic nature of the organisms being studied.

The point of this history is to emphasize the experimental nature of microbiology. Observations of microorganisms had been made for 150 years before the rigorous experimental methods of Pasteur and Koch were introduced. With experimental methods replacing simple observations, the plodding pace of advancement that had dominated for so long was replaced by an explosion of information. Microbiology went from a virtual non science in 1850 to a mature, highly productive science in 1900. As a direct result of this history, the belief that experimentation is the principal means for understanding microbial phenomena is deeply rooted in the practice of microbiology.

1.3 GROUND-WATER CHEMISTRY AND SUBSURFACE MICROBIOLOGY

Given the very different histories and different traditions of geology and microbiology, it's not particularly surprising that they have stayed largely separate during much of the twentieth century. As the scopes of each discipline enlarged over time, however, it was inevitable that they would eventually reach a point of intersection. The issue that provided this point of intersection was the chemical quality of ground water.

Characteristically, however, each discipline approached the ground-water quality issue from different viewpoints. Geoscientists were initially concerned with understanding the natural water chemistry of pristine aquifer systems. It was observations associated with oil field brine geochemistry that first suggested that microbial processes in deep subsurface environments could be of major importance. This, of course, is perfectly in keeping with the observational tradition of the geosciences.

Microbiologists were also drawn into the study of subsurface environments by water-quality issues. In this case, however, concern about chemical contamination due to waste-disposal practices was the primary motivating factor. Furthermore, development of asceptic drilling techniques and experimental evidence for the presence and activity of subsurface microorganisms was required before the presence

of subsurface microorganisms were widely accepted in the microbiological community. This, in turn is in keeping with the experimental nature of the microbiological sciences.

1.3.1 Subsurface Microbiology and the Geosciences

In 1919, a geologist named Sherburne Rogers, working for the U.S. Geological Survey, published a comprehensive study of the Sunset-Midway oil field in California. As part of this study, analyses of the brines associated with the hydrocarbons were made. These analyses pointed out that the oil field brines had a significantly different composition than shallower ground water in the area. Specifically, shallow ground waters were characterized by relatively high sulfate and relatively low bicarbonate concentrations. The brines associated with oil, on the other hand, showed high concentrations of bicarbonate but an almost total absence of sulfate.

Rogers interpreted this as evidence that, where ground water and hydrocarbons coexisted, the oxidation of hydrocarbons resulted in the reduction of sulfate with the production of bicarbonate. Furthermore, Rogers suggested that sulfate-reducing bacteria were involved in these reduction processes. At the time, however, there was no direct evidence that such microorganisms were actually present in hydrocarbon reservoirs.

This evidence was provided by a geologist named Edson S. Bastin from the University of Chicago who took up the problem of sulfate reduction associated with oil field brines. The first thing Bastin did was to test the hypothesis that oil could abiotically reduce sulfate at low temperatures. Previous investigators had attempted to reduce sulfate at 300°C, using glucose and starch as a reducing agent, without success. However, Bastin methodically went about duplicating these results. He set up several experiments in which different salts of sulfate were combined with crude oil, coal, and oil shales under asceptic conditions. After incubations that were allowed to proceed for up to 170 days, no production of hydrogen sulfide or bicarbonate was detected.

The absence of evidence for the abiotic reduction of sulfate by crude oil obviously implied that microorganisms were involved in the sulfate reduction observed in oil field brines. To test this possibility, Bastin enlisted the aid of a bacteriologist, Frank Greer, who was also at the University of Chicago. Greer prepared a magnesium sulfate–lactate medium and adjusted the salt content to that of oil field brines found in the Illinois Basin. These brines were in many ways similar to those of the Sunset-Midway field, being characterized by low sulfate content. Next, water from 19 oil wells was collected and used to inoculate the media. In ten days, 17 of the 19 samples showed the active growth of sulfate-reducing bacteria.

In 1926, Bastin reported his findings in the journal *Science,* observing:

> From the work here reported it is evident that anaerobic bacteria of the sulphate-reducing type are present in abundance in some of the waters associated with oil in productive fields and it is very probable that they are responsible for the low sulphate content of these waters.

Over the next 50 years or so in the United States, the idea that bacteria are present in deep subsurface environments and that they have important effects on

ground-water chemistry was gradually broadened. D. J. Cedarstrom (1946), following Bastin's lead, suggested that bacterial sulfate reduction was a principal cause of high-bicarbonate ground water in the Atlantic Coastal Plain. Unlike Bastin, however, Cedarstrom did not produce any direct evidence that bacteria were actually present in the systems they were studying. While conducting studies on the origins of petroleum and associated pore fluids, Claude Zobell described microbial processes in deeply buried marine sediments (Zobell, 1947).

Around this time, it became common for geoscientists—Cedarstrom, for example—to attribute ground-water chemistry changes to microbial processes—a practice that brought a rebuke from Zobell, who insisted that actual evidence of microorganisms was also necessary. The unfortunate practice of attributing ground-water chemistry changes to the activity of microorganisms, without any evidence that such microorganisms were even present, eventually provoked a backlash. Margaret Foster, a chemist working for the U.S. Geological Survey, carefully considered Cedarstrom's hypothesis. She noted that the bicarbonate concentration increases documented by Cedarstrom were not accompanied by decreases in sulfate concentrations and were therefore unlikely to have been caused by sulfate reduction. Foster performed a series of experiments, showing that such water chemistry patterns could be reproduced without sulfate-reducing bacteria (Foster, 1950). Rather, she suggested that sedimentary organic material subjected to "dynamo-chemical processes" (i.e., increased temperature and pressure) would progressively eliminate carboxyl groups and produce carbon dioxide abiotically. This carbon dioxide could then react with carbonate minerals to form the observed high-bicarbonate ground water. Foster's paper was highly cited in the geochemical literature and was enormously influential. Consequently, the previous tradition that microbial processes were important in ground-water chemistry began to be replaced by a conviction that inorganic processes were predominant.

Curiously, at about the time when consideration of microbial processes in ground-water chemistry was falling out of favor in the United States, Russian scientists were coming to very different conclusions. In 1962, a Russian scientist, M. I. Gurevich, wrote:

> A study of the microflora of the ground waters of the artesian basins in western Siberia lowland has shown that sulfur oxidizing and denitrifying bacteria that oxidize sulfur are widespread in these deep artesian aquifers, even at depths exceeding 1.8 km.

Gurevich, as did Bastin, made the connection between the presence of viable bacteria and the chemistry of ground water:

> There is now no doubt that the chemical composition of ground water may change not only by inorganic processes, but also through the activity of microorganisms.

One reason that consideration of microbial processes fell out of favor in the United States during the 1960s and 1970s was that ground-water chemists focused on other equally important lines of inquiry. The concept of hydrogeochemical facies, as outlined by William Back (1966), showed that water chemistry evolution followed regular, predictable patterns that depended on ground-water flow paths. Back's observations were crucial because, for the first time, it could be shown that the chem-

Figure 1.8. William Back showed that water chemistry evolution followed regular, predictable patterns that he termed *hydrochemical facies*.

ical composition of ground water was not random (as it had often seemed) but that it followed regular, predictable patterns. Subsequent studies by Garrels and MacKenzie (1967) showed that basic concepts of inorganic chemistry and phase equilibria could explain many of the patterns that were observed.

The equilibrium approach was especially important because it provided a theoretical basis for understanding ground-chemistry patterns. This approach was also emphasized by John Hem (1959) in his studies of iron and manganese geochemistry. However, in the 1960s, the cumbersome algebra and arithmetic associated with equilibrium calculations limited the use of this approach by geochemists performing field studies. With the introduction of computers, however, these calculations could be quickly and efficiently performed (Truesdell and Jones, 1974). As a consequence, the equilibrium approach became the basis of most subsequent ground-water chemistry studies. At about the same time, advances in mass spectrometry technology began to enable ground-water geochemists to use radioisotopes (Pearson and White, 1967) and stable isotopes (Pearson and Hanshaw, 1970) in hydrochemical studies.

The use of these new quantitative methods in field studies was enormously successful, but they also pointed up some puzzling phenomena. For example, quantitative geochemical modeling techniques developed by N. L. Plummer and his associates clearly documented that some deep confined aquifers were partially open systems with respect to carbon dioxide (Plummer, 1977). What could be the source of this carbon dioxide? Many investigators invoked the "dynamochemical," inorganic explanation given by Foster (1950) as the source of carbon dioxide. However, studies of deep injection of liquid wastes (Leenheer, Malcolm, and White, 1976;

Godsy and Ehrlich, 1978), studies of shallow waste-disposal practices (Ehrlich et al., 1982), and studies of ground water produced from deep wells (Godsy, 1980; Dockins et al., 1980) began to show that microorganisms were indeed present in subsurface environments.

The debate as to whether the observed source of carbon dioxide in aquifers was biotic or abiotic in many ways resembles the nineteenth-century debate as to the origin of fermentation. In the nineteenth century it was not at all obvious that fermentations were caused by microorganisms. Similarly, in the early 1970s, it was not obvious that carbon dioxide production in ground-water systems was microbially mediated. The idea that microbial metabolism was the principal source of carbon dioxide in deep aquifer systems, originally proposed by Cedarstrom (1946) and later rejected by Foster (1950), was revived by Chapelle et al. (1987). As was the case in the nineteenth-century biotic-abiotic fermentation debate, however, observational evidence was insufficient to settle the issue. It took several experimental studies, conducted by different laboratories and using different methods (Chapelle et al., 1988; Phelps et al., 1989a), before the biologic origin of the carbon dioxide in deep aquifer systems was firmly established.

From a historical perspective, the resolution of the deep aquifer–carbon dioxide debate was pivotal to the geochemical community. First of all, here was evidence that microorganisms were involved in a geochemical process that impacted many aquifer systems. Second, it gave an example of how experimental microbiologic techniques could be applied in a practical way to the study of hydrologic systems. This realization, together with evidence from studies of shallow aquifer systems being performed by microbiologists, catalyzed the acceptance of these concepts in the geochemical community.

1.3.2 Subsurface Microbiology and Microbial Ecology

Soil microbiology has been an important subdiscipline of microbiology since the pioneering studies of Winogradsky and Beijerinck in the early twentieth century. However, soil microbiology traditionally has been preoccupied with soil fertility as it relates to agricultural practice. Consequently, prior to 1970, there was virtually no microbiologic investigations that extended below the root zone of plants.

In the 1970s, however, it became increasingly evident that certain waste-disposal practices were contaminating subsurface environments below the root zone. There was considerable concern about the effects of these waste-disposal practices on ground-water quality, and this concern began to draw microbiologists toward studying subsurface environments. In 1973 two Environmental Protection Agency microbiologists, William Dunlap and James McNabb, driven by concern about contaminated aquifers, prepared a literature search that turned up many of the early references from the geologic and petroleum literature. At this point, the historical differences between microbiology and geology began to make an impact. The problem was that the early studies by Rogers (1917) and Cedarstrom (1946) were entirely observational and thus, by microbiologic standards, unreliable. Even early studies that included normal microbiological methods seemed to be of dubious reliability. Normal microbiologic standards of aseptic technique did not appear to have been followed in most of these studies. Furthermore, the possibility that microorganisms could have been introduced to the deep formations through the drill-

ing process or through the finished wells made the microbiologists deeply suspicious of the early work from the petroleum industry.

Microbiologists, who were trained to rely on rigorous experimental procedures, began working on ways to evaluate the possibility that drilling procedures or wells introduced microorganisms into previously sterile subsurface environments. One particularly clever study was described by Olson et al. (1981). These investigators were studying ground water from the deeply buried Madison aquifer of Montana, which produced fairly hot (47–54°C) ground water. It was found that sulfate-reducing bacteria were present that would grow at 50°C but not at 22°C. Accordingly, this suggested that the microorganisms were adapted to the temperature of the aquifer and therefore were certainly indigenous to it. Water from wells was used by a number of microbiologists to isolate microorganisms from pristine (Godsy, 1980) and contaminated aquifers (Godsy and Ehrlich, 1978; Ehrlich et al., 1982).

The development of aseptic procedures for sampling deeply buried sediments was of particular importance in studying subsurface microbiology. Dunlap et al. (1977) described a method by which a bore-hole was drilled with augers, sediment samples were collected with a split-spoon sampler, and the outer (i.e., contaminated) portion of the core removed with a paring device. With some modifications, these sampling procedures were used to collect aseptic samples for a variety of experiments and procedures by Wilson et al. (1983), Ghiorse and Balkwill (1983), and White et al. (1983). These aseptic coring procedures have been widely accepted and are standard techniques for sampling shallow water table aquifers.

Auger drilling is not suitable for coring sediments more than about 30 meters deep. For such deeper sediments, mud-rotary, or air-drilling methods are necessary. This greatly complicates the process of obtaining uncompromised sediment samples. Chapelle et al. (1987, 1988) used a combination of mud-rotary drilling and split-spoon coring to obtain sediments as deep as 200 meters. Phelps et al. (1989b) described sampling procedures using mud-rotary coring. Development of these sampling procedures made it possible to investigate microbial processes in deeply buried sediments without being restricted to water samples from wells.

Many of the microbiologists who were involved in early investigations of subsurface environments were microbial ecologists by training. It's not surprising, therefore, that many of these studies focused on ecological issues, such as microbial diversity (White et al., 1983; Hirsch and Rades-Rohkohl, 1983) and nutritional status (White et al., 1983; Balkwill and Ghiorse, 1985). The most important focus, however, was on the ability of subsurface microorganisms to degrade potential pollutants (Wilson et al., 1983; Ehrlich et al., 1982).

Consideration of microbial ecology issues, however, pointed out some limitations of relying solely on asceptically cored sediments for investigating subsurface microbiology. Specifically, coring deep holes using mud rotary methods was extremely expensive. Obtaining enough core holes to cover adequately the large areas of most deep aquifer systems was an economic and practical impossibility. This led microbiologists to consider the use of ground-water chemistry in ecological studies of the deep subsurface.

Again, however, microbiologists brought a different perspective to the problem. It was well known from studies of surface aquatic sediments that certain unstable intermediate products of microbial metabolism, such as hydrogen gas and organic

acids, provided information as to the distribution of terminal electron-accepting processes. This raised the possibility that measuring certain metabolites in ground water could provide information as to the distribution of microbial processes in the subsurface. The use of hydrogen concentrations as a tool for delineating iron-reducing, sulfate-reducing, and methanogenic zones of aquifers was first proposed by Lovley and Goodwin (1988). This technique, which has been used in subsequent hydrologic studies (Chapelle and McMahon, 1991; Chapelle and Lovley, 1992), is an effective combination of both microbiologic and hydrologic methods.

In addition to helping delineate the distribution of metabolic processes in subsurface environments, ground-water chemistry has helped in studying the microbial ecology of subsurface environments. Because of the nutrient-limited nature of most aquifer systems, it was expected that rates of microbial metabolism would be very low in the deep subsurface. This was confirmed by experiments with cores of deep sediments that showed the turnover of radiolabeled carbon compounds (Phelps et al., 1989a) was relatively low. However, because the sediments had been disturbed by coring and because artificial nutrients had been used in the assays, it was difficult to extrapolate these experimental results to in situ rates of metabolism. Chapelle and Lovley (1990) used a combination of radiotracer experiments and geochemical modeling to delineate actual in situ rates of microbial metabolism in a deep aquifer system. This approach again showed that a combination of microbiologic and geochemical techniques could be effective in addressing issues of microbial ecology.

1.3.3 Subsurface Microbiology and Contaminant Biodegradation

Throughout most of the nineteenth and twentieth centuries, the principal method of disposing of municipal, industrial, and military wastes was to simply dump them into holes in the ground and forget about them. However, by the 1960s, it was becoming increasingly apparent that these waste-disposal strategies were causing serious environmental damage. Concern over environmental pollution was also raised by incidents like the 1967 sinking of the supertanker *Torrey Canyon,* which spilled millions of barrels of crude oil into the English Channel. At that time, it was widely feared that such spills could result in permanent and irreversible damage to the environment (Atlas, 1981). Public awareness of industrial pollution due to sloppy waste-disposal practices crystallized in the early 1970s with the discovery that an elementary school in Love Canal, New York, had been built on top of an industrial dump. Furthermore, toxic chemicals had seeped through the foundations of the school and had exposed hundreds of children and teachers to potentially poisonous vapors.

The public outcry over the Love Canal incident led to congressional action in the form of the Resource Conservation and Recovery Act (RCRA) of 1976. This act mandated the end to unregulated disposal of hazardous wastes. Later, Congress enacted the Comprehensive Environmental Response Compensation and Liability Act (CERCLA) in 1980 to deal with the problem of industrial wastes that had accumulated over the last hundred years. This act established a billion-dollar fund to help clean up heavily polluted sites. Because of the huge amounts of money involved, CERCLA quickly acquired the nickname "Superfund," which it has been called ever since.

The establishment of Superfund created a new industry for cleaning up environmental pollution. At first, it was widely assumed that physical methods, such as

simply digging up old waste drums and transporting them to more secure repositories, would be sufficient to clean up most sites. Soon, however, it became apparent that much contamination had accumulated in soils or in underlying ground-water systems. Cleaning up this kind of contamination proved to be much more difficult then had originally been anticipated.

Underground storage tanks, which were commonly used to store and distribute petroleum products and which often leaked, were found to be one of the most common sources of ground-water pollution (Council on Environmental Quality, 1981). However, methods for remediating such contamination were problematic. By far the most common remediation strategy was to pump contaminated ground water out of the ground and treat it to remove contaminants. Many of these pump-and-treat systems, as they came to be called, did not consider the possibility that the contaminants were being degraded by microbial processes (Yazicigil and Sendleing, 1981). The reason for this was that, in the early 1980s, little was known about the microbiology of shallow aquifers (Wilson et al., 1983). Early studies of soil microbiology had indicated that numbers of bacteria dropped off sharply with depth (Waksman, 1916), and it was widely assumed that shallow aquifers were sterile. With the increasing concern about ground-water contamination, however, the microbiology of shallow aquifers became an active topic of investigation (White et al., 1983; Hirsch and Rades-Rohkohl, 1983). Several of these studies focused on the ability of subsurface microorganisms to degrade petroleum hydrocarbons. In particular, Wilson et al. (1983) showed that toluene, a common component of gasoline, was easily degraded by microorganisms in shallow aquifers.

Throughout the 1980s, laboratory studies of microbial activity in shallow aquifer systems, and the ability of those microorganisms to degrade petroleum hydrocarbons, was a topic of intense inquiry (Wilson et al., 1986; Heitkamp and Cerniglia, 1988; Fredrickson et al., 1991). In addition, numerous field studies showed that microbial oxidation of petroleum hydrocarbons was clearly reflected in ground-water chemistry (Cozzarelli et al., 1989; Chiang et al., 1989). These field studies were important because they suggested that petroleum hydrocarbons were being oxidized under anaerobic conditions. Throughout most of the 1970s, it was widely believed that molecular oxygen was necessary to biodegrade petroleum hydrocarbons (Bailey et al., 1973). In fact, the first microorganism capable of completely mineralizing an alkyl benzene under strictly anaerobic conditions was only first isolated in 1988 (Lovley and Phillips, 1988). Laboratory studies have now shown that petroleum hydrocarbons are degraded under nitrate-reducing (Hutchins et al., 1991a), Fe(III)-reducing (Lovley et al., 1989), sulfate-reducing (Edwards et al., 1992), and methanogenic (Grbi-Gali and Vogel, 1987) conditions. For many years, the anaerobic oxidation of petroleum hydrocarbons was controversial, largely because aerobic microbial processes had been the focus of the early studies of the marine environment. Nevertheless, the importance of anaerobic oxidation processes in the biodegradation of petroleum hydrocarbons became firmly established (Lovley, 1997).

But petroleum hydrocarbons were only part of the contamination problem. Industrial solvents like trichloroethene (TCE) and perchloroethene (PCE) had been used since the early 1900s and huge amounts of these chemicals had been simply dumped into seepage basins and unlined landfills. Because solvents like TCE and PCE are entirely synthetic compounds, with no natural analogues, it was widely believed in the 1970s that microorganisms could not degrade them. But in 1985,

J. T. and B. H. Wilson showed that aerobic methane-oxidizing bacteria were capable of co-oxidizing TCE to carbon dioxide. Later, it became evident that chlorinated ethenes were also subject to reductive biodegradation, with chlorine atoms being sequentially stripped off of the ethene molecule to form dichloroethene (DCE) and chloroethene (which is usually called vinyl chloride, or VC) (Vogel and McCarty, 1985; Barrio-Lage et al., 1987; Barrio-Lage et al., 1990; McCarty and Semprini, 1994). Later still, it was discovered that VC and DCE were also subject to oxidation by microorganisms (Davis and Carpenter, 1990; Bradley and Chapelle, 1998).

Starting from the initial assumption that chlorinated ethenes were not biodegradable at all, it is now evident that there are a number of microbial processes that biodegrade these compounds in ground-water systems, and that they are very important in restricting contaminant transport. This experience has been repeated with a number of so-called "recalcitrant" contaminants. Polychlorinated biphenols, explosives such as TNT, and gasoline additives such as methyl-*tert* butyl ether (MTBE) are other examples of compounds that were initially thought to be completely inert, but which now are known to undergo microbial degradation in ground-water systems. This, of course, is the normal progression of scientific inquiry. Understanding the behavior of human-produced contaminants in ground-water systems requires both an observational approach (looking for evidence that degradation is occurring in situ) and an experimental approach (showing that a particular microbial process occurs under controlled laboratory conditions). This takes time. From the time that TCE was first identified as an important contaminant of ground-water systems (~1970) it took 15 years to identify a cometabolic microbial degradation process, and another 10 to fully appreciate the importance of reductive and oxidative degradation processes. As new human-produced contaminants are introduced in the future, it is safe to assume that an understanding of their microbial degradation processes will lag in a similar fashion.

1.4 CHAPTER SUMMARY

The preceding sections show that the history of subsurface microbiology has been one of convergence between the different disciplines of geology and microbiology. Observations of ground-water chemistry had suggested, from at least the 1920s, the possible importance of microbial processes. However, an observational approach was not sufficient to demonstrate that this was, in fact, the case. Rather, rigorous sampling methods and experimental techniques were required to demonstrate adequately the role of microbial processes.

On the other hand, experimental methods themselves had significant disadvantages, such as extreme cost, disturbance of sediments, and an inherent small scale that was inappropriate for large hydrologic systems. In the end, an integration of microbiology and the geosciences has proved to be the most effective means of studying subsurface microbiology. This integrated approach, which requires that microbiologists and geologists understand each other's unique and important perspectives, holds much promise for unraveling important environmental problems in terrestrial subsurface environments.

QUESTIONS TO CONSIDER

1. What kinds of observations did early geoscientists make that indicated that the earth was much older than indicated by the Bible?
2. Give three examples of how Louis Pasteur established rigorous experimentation as the basis for modern microbiology. How did Robert Koch's work contribute to establishing experimental microbiology?
3. What observational evidence initially suggested that viable microorganisms were present in oil field brines? Could this evidence, by itself, conclusively demonstrate the presence of microorganisms in deep subsurface environments?
4. In the 1930s and '40s, geoscientists widely acknowledged that microorganisms inhabited deep subsurface environments. What events led this understanding to fall into disrepute in the 1950s and 1960s?
5. What upheaval in American society led to renewed interest in subsurface microbiology in the 1970s and 1980s?
6. Why was the biodegradation of petroleum hydrocarbons of such interest to microbiologists in the 1970s?
7. Why were compounds like chlorinated ethenes, polychlorinated biphenols, and TNT initially thought to be nonbiodegradable?
8. How did an observational approach advance the understanding of chlorinated ethene biodegradation processes in ground-water systems?
9. How did an experimental approach advance the understanding of chlorinated ethene biodegradation processes?

CHAPTER 2

MICROORGANISMS PRESENT IN THE GROUND-WATER ENVIRONMENT

The kinds of microorganisms encountered in the biosphere are as varied and diverse as the kinds of environments present on earth. If one considers the diversity of potential habitats in the biosphere (surface sediments in freshwater or saltwater bodies, subsurface sediments in either aerobic or anaerobic environments, the water column of deep or shallow water bodies, hot hydrothermal waters, frozen sediments in the Arctic and Antarctic plains, extreme pressure in deep ocean waters, and the bodies of higher plants and animals), it's little wonder that microorganisms display such astonishing diversity.

In spite of this diversity, there are only three primary lineages, referred to as domains, to which all life on earth (not just microorganisms) belong. These domains are the Archaea, the Bacteria, and the Eucarya (Woese et al., 1990). If we include viruses, which many microbiologists consider to be noncellular life-forms, there are just four basic kinds of microorganisms to consider when studying ground-water systems.

The bacteria are easily distinguished from eucarya on the basis of cellular architecture. The bacteria, also called procaryotes, are characterized by the lack of a true nucleus (*pro* meaning "early" or "primitive," and *karyo* meaning "nucleus") and includes the bacteria and the cyanobacteria. Cyanobacteria were formerly called "blue-green algae," but are now recognized as members of the Domain Bacteria. The eucaryotic cell has a true nucleus (*eu* meaning "true") and includes algae, fungi, and protozoa.

The archaea, which include the methane-producing "bacteria," are morphologically indistinguishable from bacteria and were once considered to be procaryotic microorganisms. Studies in the last 30 years, however, have shown that archaea are not bacteria and that they represent a previously unrecognized domain. Archaea are restricted to anaerobic environments, such as organic-rich sediments and the intestines of higher animals, or hypersaline environments. Geologically, the archaea are important because they inhabit virtually all subsurface environments, are an important source of commercial methane, and greatly impact the chemistry of ground-water systems.

Viruses are distinct from other types of microorganisms in that they are obligate parasites. That is, they do not have the capability to live and reproduce without having a host cell to provide energy. Viruses are important in subsurface microbiology primarily because ground water may transport viruses to wells or other drinking water supplies and thus spread infectious diseases. It is almost certainly true that viruses use subsurface bacteria as hosts and therefore are probably present wherever bacteria are present. That topic, however, has yet to be explored systematically.

2.1 THE BACTERIA

Bacterial microorganisms are characterized by their distinctive and relatively simple cellular structure. Figure 2.1 shows the kinds of structures that are typically observed in bacteria.

The bacterial chromosome consists of a single molecule of DNA that, in spite of many loops and twists, is arranged into a closed circle. This closed circle arrangement is found only in procaryotes and the archaebacteria. The DNA is otherwise identical to that of other organisms and is characterized by its double helix structure. The DNA carries genetic information needed for the cell to carry out metabolism and growth, as well as carrying the information needed for replication. Each bacterial chromosome consists of anywhere between 2,000 and 10,000 units of heredity called genes. Genes are segments of the DNA strand that code for a particular protein or polypeptide. Most of the time, procaryotic cells have just one copy of their chromosomal DNA and are therefore referred to as being haploid. Just before cell division, however, two or more copies of the bacterial chromosome may be present. These masses of DNA are sometimes visible under the microscope and are referred to as nucleoids.

Bacteria may also contain smaller circles of DNA, distinct from chromosomal DNA, that are termed plasmids. Plasmids are not involved in cell replication but

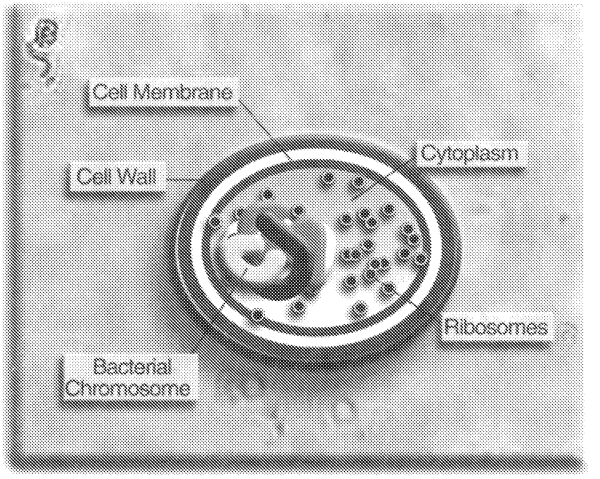


Figure 2.1. Cellular structure of bacteria.

are nevertheless very important. Plasmids code for enzymes or other proteins that have specific functions in helping the microorganism deal with its environment. For example, plasmids often code for proteins that detoxify or otherwise neutralize antibiotics. Plasmids may also code for proteins that aid in the decomposition of particular organic compounds, enabling the bacteria to use those compounds as an energy source. Much research has gone into identifying plasmids that code for the decomposition of toxic chemicals, because such capability could increase the effectiveness of bioremediation strategies. This topic is discussed in greater detail in Chapter 9.

Ribosomes are small, dark structures that are embedded in the cytoplasm of the cell. Ribosomes are protein assembly structures and provide a surface upon which amino acids can be brought together and assembled in the proper sequence. Ribosomes in procaryotes are made from two subunits. The smaller subunit is called the 30S subunit and the larger one is called the 50S subunit. The *S* stands for "Svedberg" units, a measure of the rate of sedimentation in an ultracentrifuge and hence a measure of molecular size. Because the 50S subunit settles faster than the smaller 30S subunit, it is proportionally more massive. Each subunit consists of an RNA molecule, which on its own may be either 23S, 16S, or 5S, and associated proteins that aid in assembling amino acids.

The cytoplasm of procaryotes always contains individual ribosomes. Often, however, ribosomes are arranged into complexes that are called polysomes. These polysomes work simultaneously to assemble different polypeptides which may constitute parts of one protein. Sometimes polysomes are embedded into the cell membrane.

The cell membrane, also termed the cytoplasmic membrane, acts as the boundary between the interior of the cell and the outside environment. As such, the cell membrane has numerous functions that regulate the chemical environment inside and outside the cell. Figure 2.2 shows a schematic diagram of the cell membrane. It consists of approximately 60% protein and 40% phospholipids. The phospholipids are arranged into a bilayer, in which the hydrophobic (non-water-soluble) portions point outward. This arrangement helps the cell to regulate its water balance. Embedded in the phospholipids are proteins that exhibit a variety of arrangements. The purpose of cell membrane proteins is to regulate the transport of chemicals into and out of the cell. Some proteins, for example, act as "ports" for bringing simple sugars into the cell, where they can be utilized for energy. Other proteins act as "switches," transferring electrons in the cell's electron transport system.

The cell membrane is semipermeable and allows some substances, such as water, to cross in response to concentration gradients. This type of transport occurs spontaneously and does not require the cell to expend energy. Other substances are actively carried across the cell membrane by specialized proteins. This type of transport is termed facilitated transport and requires the cell to expend energy.

The cell membrane is enclosed within the cell wall (Fig. 2.1), which gives the cell rigidity and helps to protect it against osmotic stress. A substance called peptidoglycan, which is unique to procaryotic microorganisms, provides much of the cell wall's structural strength. Peptidoglycan is a three-dimensional polymer of sugars and amino acids that are cross-linked with short peptide bridges.

There are two major types of cell walls (Fig. 2.3). The gram-positive cell wall consists of an inner membrane with a relatively thick layer of peptidoglycan cov-

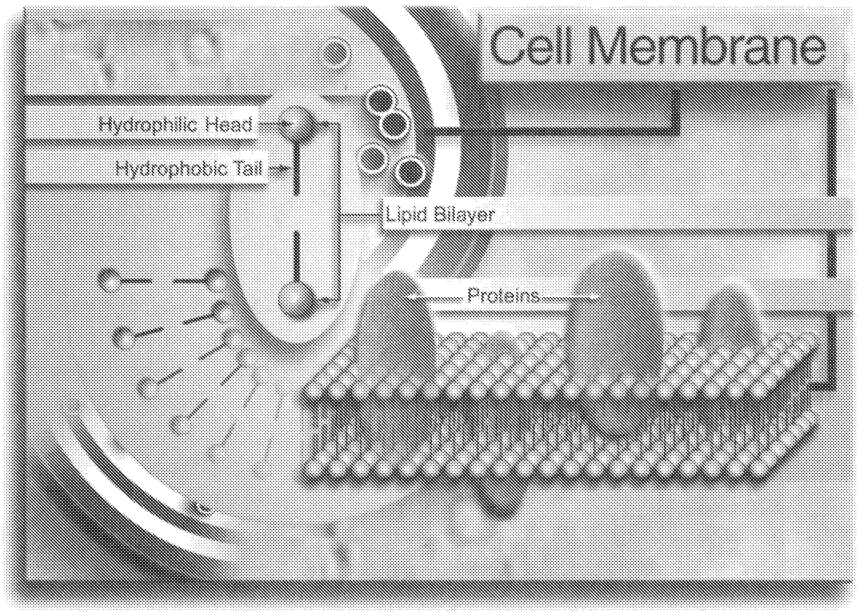


Figure 2.2. Schematic diagram of the cell membrane, sowing the arrangement of proteins and phospolipids.

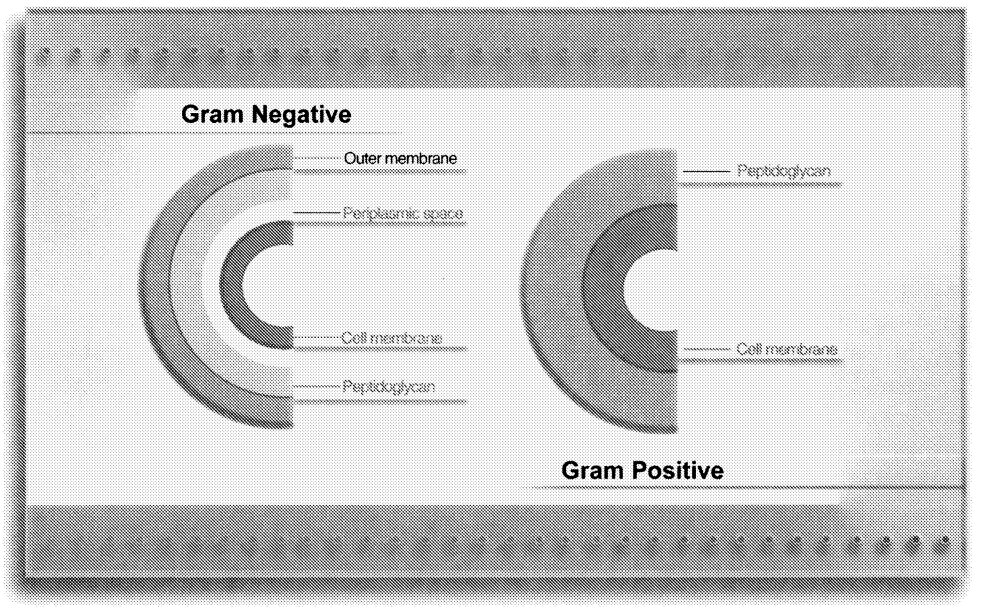


Figure 2.3. Diagram showing the configuration of (a) the gram-negative and (b) the gram-positive cell wall.

ering it. There are also varying amounts of teichoic acids, polymers of sugar alcohols and phosphates, present in gram-positive cell walls. This thick peptidoglycan layer has the characteristic that it retains the crystal violet pigment in Gram's stain, even when washed with ethyl alcohol—hence the term "gram positive."

The gram-negative cell wall (Fig. 2.3) has a layer of phospholipids and lipoproteins outside a thinner peptidoglycan layer. The gram-negative cell wall does not retain Gram's stain when washed with ethyl alcohol. In the gram-negative cell wall, there is a space between the cell membrane and the peptidoglycan layer, termed the periplasmic space. The periplasmic space is absent from gram-positive organisms and reflects a basic difference in how substrate-degrading enzymes are utilized by the two types of microorganisms.

Bacteria often have external coatings on their cell walls. These coatings are called the glycocalyx and consist of either polysaccharides or proteins. If the coating is hard and dense, it is often referred to as a capsule. If the coating is soft and pliable, it is often termed a slime layer. Cells growing on culture media often produce a thick glycocalyx, and this is what gives individual bacterial colonies a smooth appearance. Cells that do not produce abundant glycocalyx often produce colonies that appear rough.

One important function of the glycocalyx is that it facilitates the attachment of bacteria to surfaces in the environment. For example, *Streptococcus mutans,* a bacterium that lives in the mouths of human beings, produces a thick glycocalyx slime designed to allow it to stick to its host's teeth (thus avoiding being swallowed and killed). *S. mutans* produces this polysaccaride glycocalyx from sucrose (refined sugar), so that eating a candy bar is a boon for slime production. The "cottony" feeling in your mouth that becomes noticeable 10 or 15 minutes after eating candy comes from the slime produced from sucrose and used to coat your teeth. This slime, also called plaque, contributes greatly to tooth decay.

In the same way that *S. mutans* uses a glycocalyx to cling to teeth, bacteria in sediments use these coatings to cling to mineral surfaces. This ability helps bacteria gain access to nutrients associated with sediment particles and provides a stable environment for subsequent reproduction.

2.2 THE EUCARYA

Algae, fungi, and protozoa have cells that are much different from bacterial cells and are classified as eucaryotes. In contrast to the relatively simple structures present in bacterial cells, the eucaryotic cell is extremely complex. The eucaryote is characterized by the presence of a distinct nucleus (Fig. 2.4). Many, but not all, eucaryotes have a cell wall, and all eucaryotes have a cell membrane. Also present are large folded structures, called the endoplasmic reticulum, oval membrane disks called Golgi bodies, and organelles called mitochondria, lysosomes, and chloroplasts (Fig. 2.4).

The cell wall in eucaryotes performs many of the same functions as that in bacteria, such as protecting against osmotic shock and giving tensile strength to the cell. The cell walls of eucaryotes are formed from polysaccharides, but peptidoglycan is absent. The cell membrane in eucaryotes is similar to that of procaryotes, the main differences being in the composition of the lipids. The eucaryotic

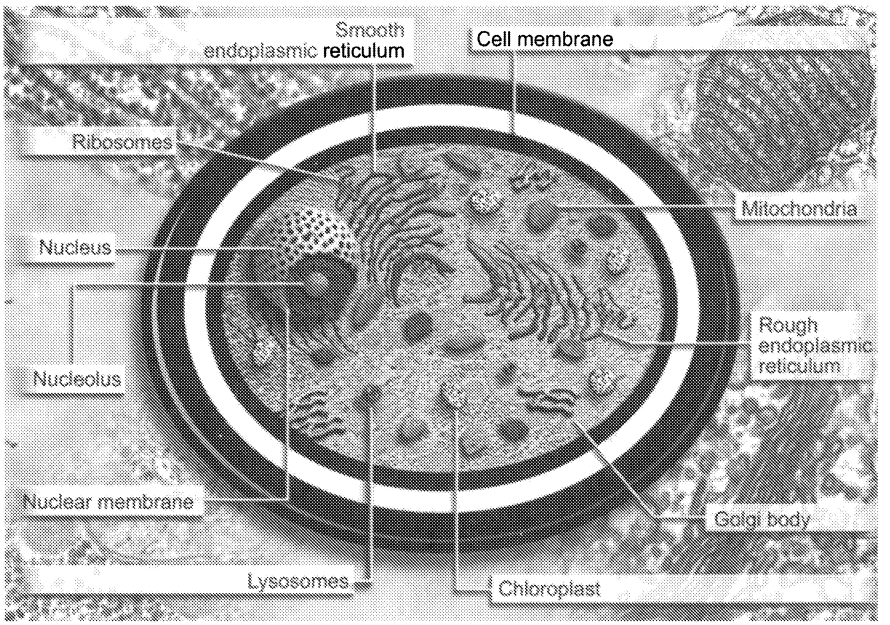


Figure 2.4. Structures present in eucaryotic cells.

cell membrane carries out facilitated and active transport, as in procaryotic cells. Many of the metabolic functions carried out in procaryotic cell membranes, however, have been taken over by specialized organelles in the eucaryote.

In the eucaryotic cell, energy production occurs in the mitochondria, photosynthetic activity is carried out by chloroplasts, and digestive enzymes are stored in lysosomes. All of these functions are carried out in the cell membranes of bacteria. Protein assembly in eucaryotes is carried out by ribosomes, as in procaryotes. However, eucaryotic subunits are larger (40S and 60S) than in procaryotes. Furthermore, eucaryotic ribosomes are often arranged along the surface of a folded membrane, the endoplasmic reticulum. This structure aids in the synthesis of very complex proteins that require numerous steps during assembly. Further processing of enzymes is performed by Golgi bodies separate from the endoplasmic reticulum.

Algae are photosynthetic eucaryotes and have the distinction of producing most of the world's oxygen. As such, algae contain photosynthetic chloroplasts. Algae have cell walls composed of cellulose, pectin, or silica, and may live singly or in colonies. There are six groups of algae recognized: green algae, euglenids, diatoms, dinoflagellates, brown algae, and red algae.

Unlike the photosynthetic algae, the fungi are heterotrophic; that is, they are decomposers and obtain energy and nutrients from preexisting organic carbon sources. The fungi have developed mechanisms for degrading almost any kind of organic carbon compound found in nature. Most fungi are obligate aerobes, meaning that they must have oxygen in order to respire. However, some fungi, notably the yeasts, are capable of fermentation.

The special function of fungi in the environment is to recycle the remains of plant and animal debris. In this way, fungi often compete directly with heterotrophic

bacteria for available resources. The greater complexity of eucaryotic structure, which allows the synthesis of many degrading enzymes lacking in the procaryotes, often gives fungi a competitive advantage over bacteria. Fungi, for example, are particularly adept at degrading lignins in decaying plants, a class of compounds that bacteria attack, but less efficiently. Also, the metabolic flexibility of fungi allow them to live in particularly stressful environments, such as hypersaline lakes. This resistance to osmotic stress is the main reason that molds, which are a type of fungi, are able to grow on preserved foods, such as jams, that are resistant to attack by bacteria.

The protozoa are single-celled heterotrophic eucaryotes that are characterized by extremely complex cell structure. The most familiar protozoa to geoscientists are foraminifera, radiolaria, and dinoflagellates, all of which are prominently represented in the fossil record. Some protozoans are predatory and are able to attack and ingest other microorganisms. Many predatory protozoans are specifically adapted to feeding on bacteria, and this grazing is often a limiting factor in bacterial populations in both surface and ground-water systems. Most protozoa, however, belong to floating communities called zooplankton.

2.2.1 Eucaryotes in Ground-Water Systems

The presence and distribution of eucaryotic microorganisms in ground-water systems has been studied seriously only recently. The first microbiologic investigations of shallow water table aquifers indicated that procaryotes were the dominant microorganisms present and that eucaryotes might be absent altogether (White et al., 1983; Balkwill and Ghiorse, 1985). Subsequent studies, using more sensitive techniques, were able to show the presence of limited populations of eucaryotes in ground-water systems.

Sinclair and Ghiorse (1987) used a most-probable-number counting procedure to document the presence of protozoa in the shallow pristine water-table aquifer at Lula, Oklahoma. Many of the horizons sampled, to a depth of 8 meters, had fewer than 0.2 protozoa/gram of dry sediment. A gravelly bed at a depth of 7.5 meters, however, exhibited between 2 and 5 protozoa/gram of dry sediment. Comparable numbers were found in other sandy horizons. Surface soils from this site, in contrast, were characterized by counts of protozoa in the 102–106 cells per gram dry sediment. So, while protozoa were demonstrably present, they were present in very low numbers. A latter study of buried-valley aquifer sediments in Kansas (Sinclair et al., 1990) showed a similar pattern of protozoa abundance.

While the presence of eucaryotic cells is low in pristine aquifer sediments, there is evidence that, in aquifers contaminated by organic chemicals, the abundance of eucaryotes may be much higher. For example, Madsen et al. (1991), in a study of an aquifer that was contaminated by polyaromatic hydrocarbons, showed that numbers of protozoa were much higher in the plume of contamination than outside the plume. This was interpreted as reflecting much greater growth rates of bacteria in the contaminated zone, which in turn supported a significantly higher population of protozoan grazing on the bacteria. In contrast, little relationship was found between the presence of fungi and contamination. On the basis of this data, Madsen et al. (1991) suggested that the population size of protozoans could be used as evidence for chemical contamination of aquifers.

A similar pattern of higher numbers of protozoa being present in contaminated versus uncontaminated aquifer sediments was demonstrated at a site contaminated with jet fuel (Sinclair et al., 1993). In this case, an engineered bioremediation system designed to add hydrogen peroxide to ground water, and thus to increase rates of aerobic degradation of petroleum hydrocarbons, was in place. A major limitation to this kind of engineered bioremediation system is clogging of sediments due to growth of bacterial biomass. However, at this site, no such clogging was observed. Rather, it appeared that protozoa were grazing on bacteria in the zone of treatment, and that this grazing maintained bacterial numbers below a level that would cause clogging. This grazing did not, however, appear to decrease the efficiency of petroleum hydrocarbon degradation (Sinclair et al., 1993).

Giardia *and* Cryptosporidium In 1993, over 400,000 people in Milwaukee, Wisconsin, became ill with a disease that caused diarrhea and vomiting. Some of the victims, especially those with compromised immune systems, died. Later study showed that the disease was caused by the protozoa *Cryptosporidium parvum,* which had contaminated the city's drinking water supply. Another water-borne protozoa, *Giardia lamblia,* is also known to cause intestinal disease in humans. These cases have led to widespread concern that these protozoa may contaminate ground water as well as surface water supplies.

Both *G. lamblia* and *C. parvum* are protozoan parasites that infect the digestive tract of humans and other mammals. It has been demonstrated that aquatic mammals, such as beavers and muskrats, can serve as zoonotic hosts, maintaining populations of *Giardia* in otherwise pristine surface waters. Domestic mammals, particularly ruminants such as cows, can serve as an infective host for *Cryptosporidium.*

Because both *Giardia* and *Cryptosporidium* inhabit surface water, it is possible that induced recharge of surface water to ground-water systems, either due to pumpage or to bank storage during flood events, can cause contamination of ground water. The vulnerability of ground-water supplies to contamination, therefore, depends on the transport of the protozoa in ground-water systems.

The transport of *Giardia* and *Cryptosporidium* in ground-water systems has not been studied directly. However, the transport of protozoa in a sandy aquifer underlying Cape Cod, Massachusetts, was studied by Harvey et al. (1995). In this study, an indigenous strain of a flagellated protozoa was recovered from aquifer sediments, grown in culture and stained, and then injected into the aquifer with a bromide tracer. A tenfold reduction in protozoa numbers was observed in the first meter of flowpath, and numbers of protozoa became undetectable 3.6 meters downgradient. These data suggest that protozoa can be transported in ground-water systems, but that they are probably filtered out relatively rapidly. While contamination of ground water adjacent to surface-water bodies by *Giardia* and *Cryptosporidium* is possible, such contamination is unlikely to extend significant distances (>10 meters) from the point of recharge.

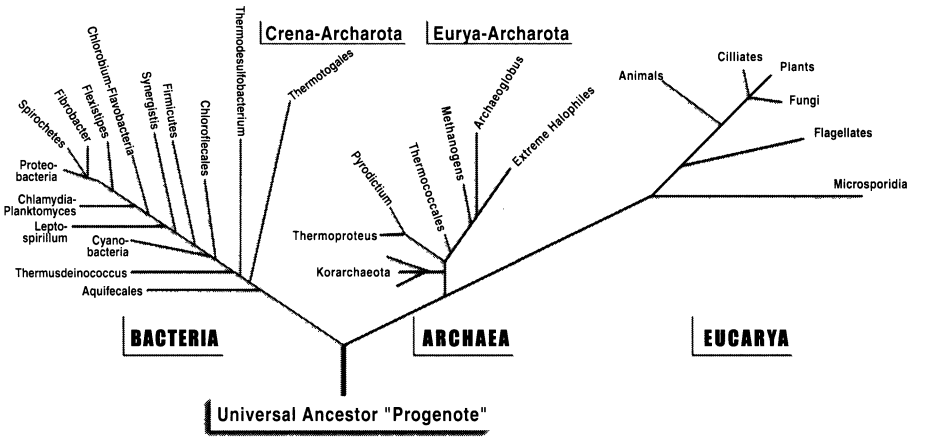
2.3 THE ARCHAEA

The distinction between bacterial and eucaryotic microorganisms was first defined in terms of the internal structures that were visible with a microscope (Figs. 2.1

and 2.4). On this basis, as well as on the basis of differences between the biochemistry of their enzyme systems, biologists constructed a tree in which all cells could be classified under either the procaryotic or eucaryotic "stems."

This dichotomous classification was questioned in the 1970s by Carl R. Woese and his associates, who were working at the University of Illinois. Woese's group was interested in being able to quantify genetic similarities between microorganisms. In order to do this, they decided to look at the nuceotide sequences of ribosomal RNA (rRNA). The choice of rRNA was based on the fact that it was relatively easy to isolate from bacterial cells and that sequencing discrete portions of it was technically feasible. There are three kinds of rRNA in bacteria. The "large" ribosomal unit has a Svedberg unit value of 23S and is approximately 2,900 nucleotide units long. There is also a very "small" one that is 5S and is only 120 nucleotides long. Intermediate between these two is the 16S rRNA that is about 1,540 nucleodides long. The 23S was too long to be conveniently sequenced in the early 1970s whereas the 5S did not contain enough nucleotides to give much information. Thus, Woese's group settled on the 16S as the rRNA unit of choice and began sequencing the 16S rRNA of many different types of procaryotes and eucaryotes.

As the sequencing proceeded, a surprise emerged. As expected, eucaryotes were most closely related to eucaryotes. Among the bacteria, however, it appeared as if the methane-producing bacteria were distinct from other types of bacteria. In fact, the methanogens were no more closely related to the other bacteria than they were to eucaryotes. The implications of this finding were straightforward. There were not just two but three stems in the tree (Fig. 2.5). Woese named these three stems the eubacteria (true bacteria), the eucaryotes (true nucleus), and the archaebacteria (ancient bacteria). This nomenclature reflected Woese's belief that archaebacteria evolved very early in earth's history. More recently, however, Woese has suggested that using the word *bacteria* in archaebacteria implies is misleading, and proposed the term "Archaea" instead (Woese et al., 1990).



THE UNIVERSAL PHYLENOGENETIC TREE OF LIFE

Figure 2.5. Diagram showing the relatedness of the three domains.

In addition to their distinctive 16S rRNA sequences, archaea exhibit other differences from the procaryotes. The lipids of both bacteria and eucaryotes consist mainly of two straight-chain fatty acids bound at one end to a glycerol molecule by an ester linkage (-CO-O-). In archaea, on the other hand, the glycerol and the acid chains have an ether (-O-) link. In addition, the cell walls of bacteria contain peptidoglycan, whereas the cell walls of archaea contain pseudopeptidoglycan or just protein.

The archaea include three distinct kingdoms. Kingdom Crenarchaeota consists mainly of thermophilic (heat-loving) organisms. Kindom Euryarchaeota includes the methanogens (microbes that produce methane from carbon dioxide and hydrogen), extreme halophiles (microbes that live in concentrated salt brines), and thermoacidophiles (microbes that live under extreme conditions of heat and low pH). Interestingly, a third kingdom, Kingdom Korarchaeota, has been proposed based solely on the 16S rRNA sequences of uncultured microorganisms found in terrestrial hot springs. Of these types, the methanogens most commonly affect the geochemistry of ground water. Early in the earth's history, methanogens could have existed almost anywhere. Today they are restricted to environments where oxygen has been excluded and where hydrogen and carbon dioxide are available. Because subsurface environments are often anoxic, they are extensively colonized by methanogens.

2.4 THE VIRUSES

Viruses are very small infectious agents. They are too small to be seen with a light microscope, and their presence was discovered indirectly. In 1886, an American bacteriologist, A. E. Mayer, described a mottling disease of the tobacco plant, called "tobacco mosaic disease." He noted that the disease could be transmitted by injecting the sap of infected plants into healthy plants. A few years later, O. Iwanowski discovered that the causative agent of tobacco mosaic disease was filterable; that is, the ground-up extract of diseased plants could infect healthy plants even when the extract was passed through a very fine filter. Iwanowski's experiments were reproduced by Martinus Beijerinck in 1898. Clearly, the infectious agent was not a bacterium. These experiments had the effect of extending microbiology beyond the study of bacteria and eucaryotes to the study of infectious agents, viruses, which were too small to be seen.

Exactly what kind of infectious agent viruses were was largely a mystery in the early part of the twentieth century. A major contribution to virology was made in 1935 when the American Wendell Stanley showed that the tobacco mosaic virus could be crystallized. This discovery led to studies of viral size and shape using X-ray defraction techniques. It was not until the advent of the electron microscope, however, that viral particles could be directly observed.

What is a virus? The best way to answer this question is to say what it is not. Viruses are not cells, for example, because they cannot reproduce independently and because they have no independent metabolism. One of the classic (and unresolved) debates in microbiology is whether or not viruses are living organisms. When it was first shown that "inanimate" crystals of the tobacco mosaic virus could cause the disease, many people argued that viruses were merely toxic chem-

icals. On the other hand, these particular toxic chemicals appeared to have the ability to reproduce themselves; was this not a clear characteristic of life? Not necessarily, came the rejoinder. Many crystalline substances "grow" from supersaturated solutions. Was this not "reproduction"? This debate has never been settled to everyone's satisfaction, and most people have come to the conclusion that there is not much to be gained by arguing about it. It does point out, however, some of the unique features of viruses.

At the simplest level, a virus is simply a genome, genetic information stored on DNA or RNA, wrapped in an exterior coat of protein. In the tobacco mosaic virus (Fig. 2.6a), a single strand of RNA is surrounded by protein subunits (called capsomeres) that link together to form the protein coat (called a capsid). Viral particles are called virions and are characterized by a regular geometrical arrangement of the capsomeres. Common shapes of virions are spheres (Fig. 2.7), cylinders, wedges, or prisms. Some virions are covered by an envelope and are termed enveloped; others have no envelope and are termed naked.

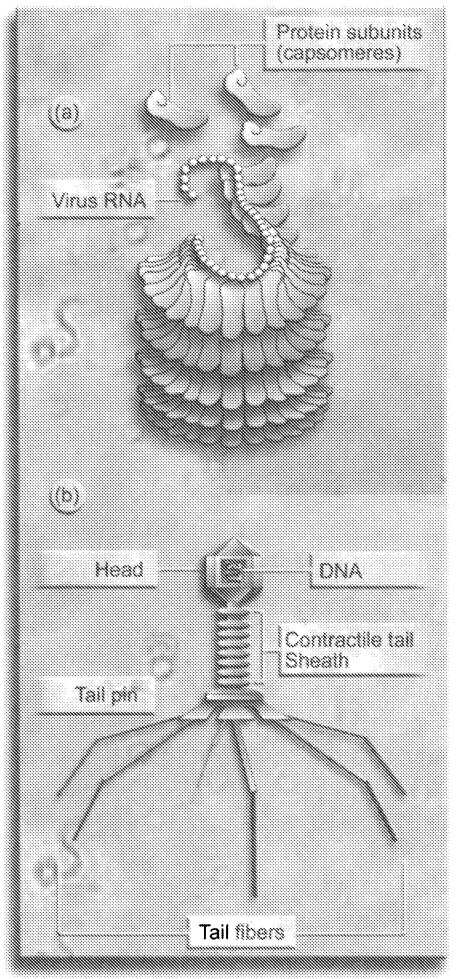


Figure 2.6. Structures present in (a) tobacco mosaic virus, and (b) a typical bacteriophage.

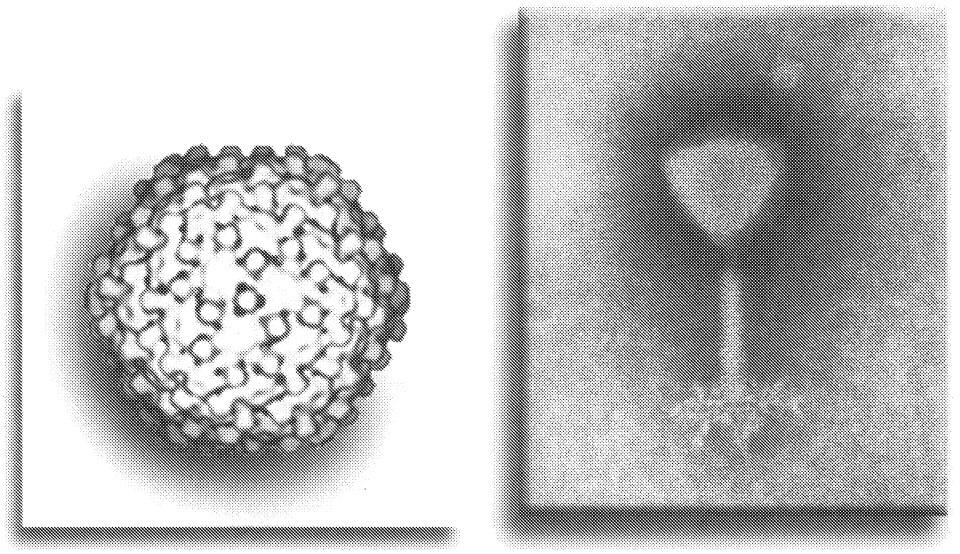


Figure 2.7 Electron micrographs of (a) a circular virus and (b) bacteriophage T-14.

Viruses that are parasitic to bacteria are called bacteriophages and have a somewhat more complicated stucture (Fig. 2.6b). The head of the virus contains the nucleic acid genome (in this case, DNA) that is set upon the contractile tail sheath. At the base of the tail sheath is the tail pin and the tail fibers. These stuctures enable bacteriophages, such as the T4 bacteriophage, to attack a bacterium. First, the tail fibers find and attach to a target protein on the cell wall. Second, the cell wall is pierced by the tail pin. Finally, the tail sheath contracts and injects DNA into the bacterium. The DNA promptly takes over the cell's metabolism and redirects it to manufacture more virus particles.

It is convenient to classify viruses according to the nucleic acid that makes up its genome. The DNA viruses include the herpesvirus, which causes herpes simplex types I and II, and adenoviruses, which cause symptoms of the common cold. The RNA viruses include enteroviruses, which cause polio and gastrointestinal illness; rhinoviruses, which also cause symptoms of the common cold; and the HIV virus, which causes the deadly disease AIDS in humans.

2.4.1 Viral Ecology

Given the history of how viruses came to be studied by microbiologists, it is entirely understandable that most of what is known about viruses deals with how they infect humans, animals, and plants. Very little attention has been given to the role that viruses play in the ecology of natural systems.

In the late 1980s, however, microbiologists made the surprising discovery that a milliliter of ordinary seawater often contained as many as 10^8 viral particles (Proctor and Furhman, 1990; Suttle, 1993). Further studies showed that viruses were intimately involved in the ecology of photosynthetic cyanobacteria that form the base of marine ecosystems. Specifically, viruses infect cyanobacteria, hijack

their cellular metabolism into reproducing more viral particles, and kill them (Proctor and Furhman, 1990). For cyanobacteria, life is a race to reproduce before they are infected and killed by the viruses. For viruses, on the other hand, "life" consists of trying to locate a cyanobacteria, attach to specific proteins on the bacterial cell wall, and injecting their DNA. If, after a few hours, a viron cannot locate and infect a host, it loses its ability to attach to bacterial cells and, in effect, "dies."

The ecological advantage for a viron infecting a cyanobacteria is obvious—it allows the viron to reproduce. However, the ecological advantage for the cyanobacteria is less obvious. Studies have shown that cyanobacteria introduced into virus-free seawater do not thrive, as one might expect. Rather, their rate of growth slows down and stops. The reason for this puzzling behavior, it turns out, is due to the cycling of nutrients. In natural seawater, the death of individual cyanobacteria due to viral infection is a principal source of essential nutrients such as nitrogen and phosphorus for growing cells. Without that source of nutrients, the cyanobacteria can't grow (Suttle, 1999). Thus, while viral infection is a disaster for individual cyanobacteria, it actually benefits the health of the overall cyanobacterial population. This is a classic example of a host–parasite relationship that has benefits for both species.

Other than the studies of seawater cited above, very little is known about the ecology of virus–host interactions in terrestrial environments or in ground-water systems. Nevertheless, by analogy to the ecology of seawater, it may be anticipated that such relationships exist, and that they may be integral parts of the ecology of these systems.

2.4.2 Viruses in Ground-Water Systems

The study of viruses in water science has focused largely on the mobility of pathogenic viruses from sewage effluents. Infectious hepatitis is the most closely studied disease that has been documented as being transmitted by a water-borne virus. Many epidemics of hepatitis have been traced to fecal contamination of drinking water or fecal contamination of shellfish that are subsequently consumed by humans. Although the disease hepatitis has been closely studied, not much is known about the virus itself. This is largely because of the extreme difficulty in studying the hepatitis virus in the laboratory.

There is considerable debate as to the possibility of pathogenic viruses being transported in ground water. Fecal contamination of shallow water table aquifers could result in the transmission of infectious hepatitis. However, solid evidence that such transmission has occurred is generally lacking. Studies have shown, however, that viruses can persist in ground-water environments. For example, Keswick et al. (1982) showed that polioviruses, coxsackieviruses, and rotaviruses survive much longer in subsurface environments—on the order of weeks or months—than had generally been assumed. A field study by Wellings et al. (1975) gave direct evidence that viruses could survive up to 28 days in ground-water systems. These survival times are sufficient for viruses to move through shallow aquifers to wells from, for example, septic system effluents.

In one such study, a septic tank was seeded with a model enterovirus, and the survival and transport of virons in the tank itself and in the septic drainfield was monitored for three months (Scandura and Sobsey, 1997). The results showed that

the numbers of the model viron decreased rapidly in the septic tank itself as effluent washed out into the drainfield. Furthermore, the model viron was detected just one day later in a monitoring well placed near the drainfield outfall. Monitoring data showed that virons were transported at least 15 meters downgradient of the outfall.

The development of polymerase chain reaction (PCR) technology has greatly aided the study of human viruses in wastewater and ground water. These methods, which are described in greater detail in Chapters 5 and 6, increase the detection sensitivity for specific viruses and decrease the time required for detection. Standard cell culture methods for the detection of human pathogenic viruses in water samples is expensive and time-consuming, requiring up to a month for confirmed positive results (APHA, 1989). With PCR technology, target nucleic acids can be amplified using specific oligonucleotide primers. Using repeated cycles of PCR, a 10^6-fold amplification of a single copy of target DNA can be completed in a few hours. The decreased time and increased sensitivity of PCR allow the detection of small amouts of DNA and RNA in water samples (Reynolds et al., 1997). Studies using PCR technology have shown that enteroviruses can be detected in well water that has been exposed to sewage effluent (Abbaszadegan et al., 1993).

Because land application is widely used to dispose of sewage effluents, the delivery and transport of viruses to ground water is a matter of environmental concern (Berg, 1983). These studies indicate that viron survival in water is affected by physical (light, temperature, adsorption, aggregation) processes, chemical (pH, ionic strength, redox) processes, and biologic (types of virons, bacterial activity, protozoan activity) processes. Solar radiation, for example, was shown to greatly increase the rate of inactivation of poliovirus and coliphage T4 viruses—a fact of importance for land application of sewage effluents. Similarly, it was shown that increased ionic strength increased the inactivation rate of poliovirus, and that the adsorption of viruses onto soil particles was pH dependent.

Because the kinds of cells needed for the replication of human pathogenic viruses are either absent or present in low concentrations in ground-water systems, it is unlikely that human pathogens such as poliovirus are active in ground-water systems for long periods of time (>3 months). While this seems perfectly logical, firm data demonstrating this to be the case is not available. Additional studies on the persistence of viruses in subsurface environments are needed to verify this. In addition, studies on the role of viruses in the ecology of subsurface environments are warranted as well.

2.5 BACTERIA IN GROUND-WATER SYSTEMS

This book is concerned largely with bacteria—that is, procaryotic microorganisms—and how they influence the chemistry of ground water. In this context, the archaea, even though they are not procaryotic microorganisms, will be considered together with bacteria. While eucaryotic microbes are present in ground-water systems, their abundance is typically three or four orders of magnitude less than that of bacteria. Also, because most eucaryotes are restricted to oxygenated environments, they are excluded from many deep subsurface environments. Similarly, viruses are present in some subsurface environments. Because they have no independent metabolism, however, their impact on ground-water chemistry is probably

limited to the effects that they have on indigenous bacteria. Thus, the emphasis on bacteria and bacterial processes in this book reflects the relative importance of the different types of microorganisms on the chemistry of ground water.

2.5.1 Classifying Bacteria

The classification of living organisms is called taxonomy. The purpose of bacterial taxonomy is really twofold. First, its purpose is to be able to distinguish between different bacteria. Second, and equally important, its purpose is to identify similarities between different bacteria so that phylogenic relationships can be determined. Because of these two aims, classifications of bacteria are hierarchical; that is, groups of closely related species are arranged into genera, similar genera are arranged into families, families into orders, and so on. The names given to the microorganisms reflect this hierarchical classification scheme.

Bacteria are named according to a binomial nomenclature, with each distinct species given a name consisting of two words. Take, for example, the bacterium *Escherichia coli*. The first part of the name, *Escherichia* is the genus to which the microorganism belongs and is always capitalized. The second part of the name, *coli,* is the name of the species and is not capitalized. It is proper usage to italicize or underline the proper names of microorganisms. The root of the genus name is either a Latin or a Greek word that may in some way be descriptive of the organism or be in honor of a particular person. It is common practice to abbreviate the names of microbes to the first letter of the genus, followed by the species name. *Escherichia coli,* therefore, is commonly referred to as *E. coli. E. coli* belongs to a family of related genera that inhabit the intestines of higher animals and are therefore called the Enterobacteriaceae, or "intestine bacteria." The Enterobacteriaceae belong to the order Eubacteria, or "true bacteria." This order includes the greatest number of bacterial species that inhabit ground-water systems.

Criteria Used to Classify Bacteria Bacteria are classified on the basis of cell morphology, type of cell wall, cell growth, biochemical transformations carried out by the cell, nutrition, and sequences of rRNA and DNA. All of these characteristics, of course, are determined by the genetic information stored in the cell's chromosomal DNA. Because of this, similarities and differences of base sequences in chromosomal DNA between different strains of bacteria is the basis of modern bacterial taxonomy. However, it is still useful to characterize bacteria based on morphological characteristics that can be determined under the light microscope and physiological characteristics that can be determined by growth on different substrates.

The gram stain is used to determine cell shape and type of cell wall. In this process, the bacteria are obtained in pure culture, smeared onto a glass slide, and fixed (heated) so that the smear will adhere to the slide. The fixed smear is subjected to the following solutions in this order: crystal violet, iodine solution, ethyl alcohol (a decolorizing agent), and safranin. Bacteria that have peptidoglycan as their outer cell wall retain the crystal violet, are stained a deep purple color by this procedure, and are termed gram positive. Bacteria that have an outer cell wall consisting of lipopolysaccharides and proteins do not retain the crystal violet stain, are colored red by the counter stain safranin, and are termed gram negative.

The shape and arrangement of the cells (Fig. 2.8) are also observed using the Gram stain technique. A coccus (plural, cocci) is a spherical cell (Fig. 2.8a), a bacillus (plural, bacilli) is a rod-shaped cell (Fig. 2.8b), a spirillum (plural, spirilla) is a helical rod (Fig. 2.8c), and a vibrio is a V-shaped cell (Fig. 2.8d). It is common to refine these basic shapes by terming different cells long rods, short rods, or whatever term the individual observer deems important. The arrangement of cells is also important. Some cells, notably those of the genus *Streptococcus,* are characterized by cells arranged in chains. Others, such as *Micrococcus,* are typically found in grape-like clusters. Some bacteria tend to occur in pairs or in clusters of four, called tetrads. All of these characteristic cell arrangements are useful in taxonomy.

Motility in bacteria is conferred by the presence of extremely thin, hair-like appendages, called flagella, that protrude from the cell wall (Fig. 2.9). Because flagella confer motility and because not all species of bacteria are flagellated, it follows that there are motile and nonmotile species. As such, the presence or absence of motility is a convenient characteristic for the classification of bacteria.

Flagella are hooked structures that are embedded in granular bodies just beneath the cell wall in the cytoplasm. As the flagellum is rotated, much like a motorboat's propeller, the bacterium is propelled forward, allowing it to "swim." There are many different arrangements of flagella found in nature. If a single flagellum is found on one or both ends of a rod-shaped bacterium, it is termed a polar flagellum. All bacteria with a polar flagellum are classified into the order Pseudomonadales.

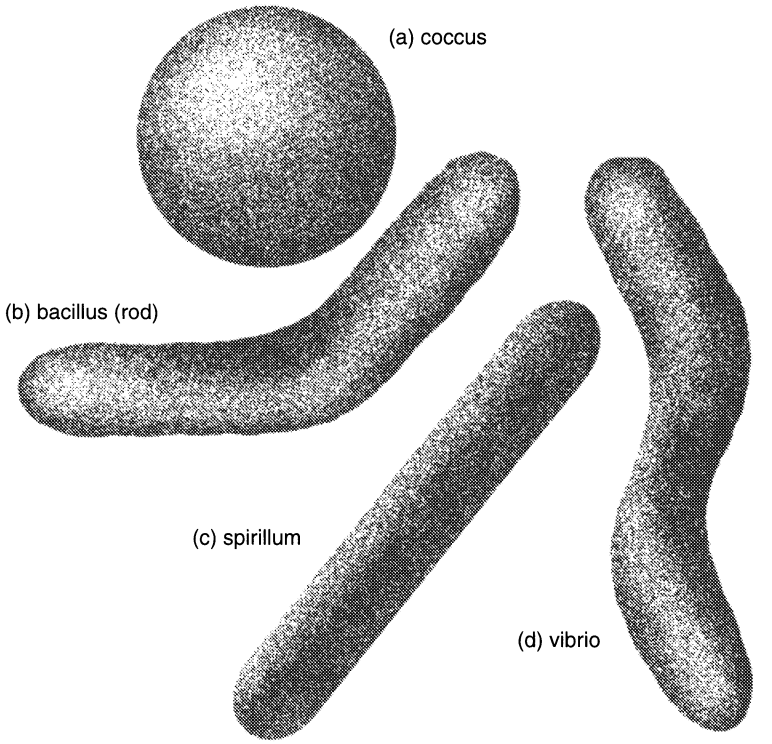


Figure 2.8. Common shapes exhibited by bacterial cells.

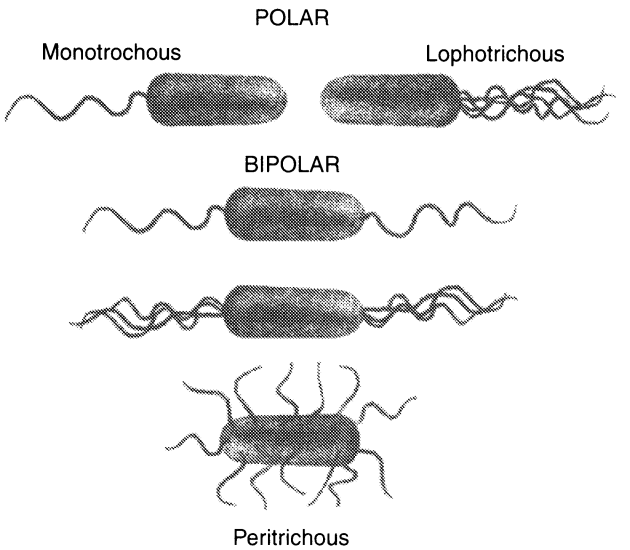


Figure 2.9. Different arrangements of flagella in bacterial cells.

Other types of rod-shaped bacteria have many flagella arranged over much of the cell. These are termed peritrichous flagella, and all bacteria showing this arrangement are classified in the order Eubacteriales.

While morphology can vary significantly in bacteria (Fig. 2.10 and Fig. 2.11) other classification criteria are useful. Nutrition, or the types of substances used for carbon and energy sources, is another logical basis upon which to classify bacteria. Heterotrophs are organisms that use organic carbon as energy and carbon sources.

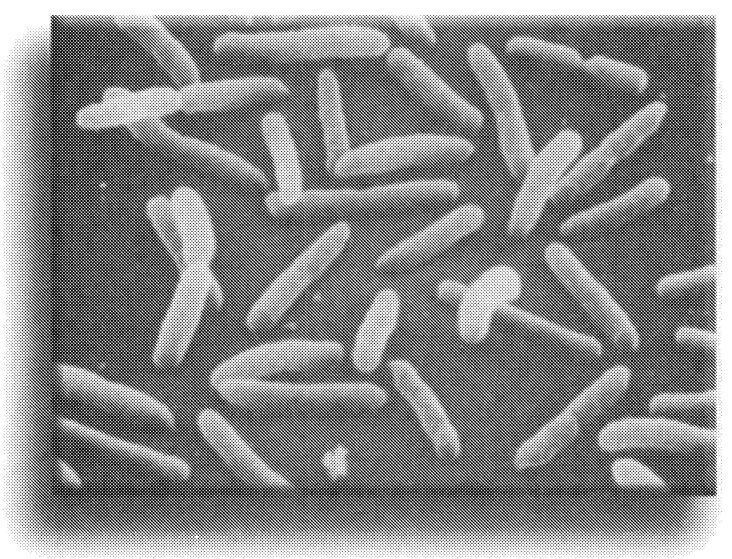


Figure 2.10. Micrographs of rod-shaped bacteria.

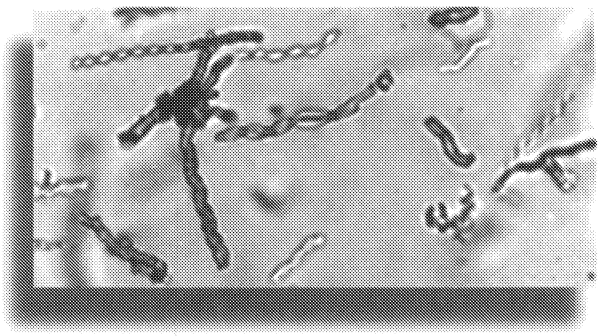


Figure 2.11. Micrograph of the iron-oxidizing bacteria *Gallionella.*

Lithotrophs use inorganic carbon, such as carbon dioxide or bicarbonate, as carbon sources and an external source of energy. Chemolithotrophs are a type of lithotroph that obtain energy by oxidizing reduced inorganic chemicals, such as ferrous iron or sulfides. Photolithotrophs are lithotrophs that are able to obtain energy from light.

Metabolism, the ways in which bacteria utilize their nutrients, is also used in classification. In order to obtain energy from a substrate, microorganisms remove electrons and transfer them to other chemicals that serve as electron acceptors. The use of inorganic chemicals, such as oxygen, ferric iron, or sulfate as electron acceptors is called respiration. The use of organic chemicals as an electron acceptor is called fermentation. Bacteria that use oxygen as an electron acceptor are called aerobes. Bacteria that can use only oxygen as an electron acceptor are called obligate aerobes. Bacteria that use oxygen when it is available but are also capable of fermentation are termed facultative anaerobes. Microorganisms that grow only in the absence of oxygen are called obligate anaerobes.

The most direct means for taxonomy is the composition and structure of chromosomal DNA that codes for all of the characteristics—size, shape, nutrition, and metabolism—exhibited by microorganisms. One common method of comparing the DNA of different microorganisms is to compare the relative abundance of the nucleotides guanine plus cytosine (G + C) to total nucleotide abundance. For example, based just on G + C, the enteric bacteria *Escherichia, Shigella,* and *Salmonella* (48–52% G + C) can be easily distinguished from most species of *Pseudomonas* (66–68% G + C).

Measuring G + C has the advantage that it is relatively easy to do. However, because most of the information in DNA is coded by the sequence of nucleotides, not just by their relative abundance, this method is not ideal. The order that nucleotides occur in a genome contains much more information and is a more direct way to classify bacteria. The attempts to use DNA to classify bacteria date to the 1960s when investigators began experimenting with DNA homology (Schildkraut et al., 1961). DNA homology is the degree to which DNA of one bacterial strain matches another, and in theory, at least, is an ideal way to classify bacteria. This method has been used with some success for microbial taxonomy (Grimont, 1988). In practice, however, DNA homology has some serious drawbacks. Whereas this method is very good at distinguishing differences between closely related species,

it gives very little information about the relationship of more distantly related species, and does not help place strains within higher taxa, such as genus, family, order, division, and kingdom.

For these reasons, RNA present in bacteria also have been extensively used for classification purposes. Originally, the approach was to separate and sequence the 16S ribosomal RNA (rRNA) from bacterial cells (Woese, 1981). Because there are many fewer nucleotides in the 16S rRNA (about 1,500 base pairs) than in chromosomal DNA itself (about 4.5 million base pairs), this was a much more manageable task. This tool has been especially useful for determining the "relatedness" of different eucaryotic, bacterial, and archaeal species. With the advent of polymerase chain reaction (PCR), cloning, and sequencing technology (Chapter 5), it has become possible to analyze bacterial DNA for specific 16S rRNA genes and use these as a basis for taxonomic classification. This has been greatly facilitated by the development of large databases that include phylogenetically ordered alignments of rRNA for large numbers of microorganisms (Maidak et al., 1994). It is now possible to take an unknown bacterial isolate, amplify a fragment of its 16S rRNA gene, sequence a portion of the fragment, and compare the resulting sequence to a database. It is usually possible to identify a microorganism to the genus level by sequencing between 300 and 500 base pairs. These PCR-based methods are now the most reliable ways for identifying and classifying bacteria found in natural and human-created environments (Amann et al., 1995).

2.5.2 Gram-Negative Bacteria Found in Ground-Water Systems

Gram-negative bacteria are found extensively in ground-water systems. For example, in a shallow water table aquifer in Oklahoma (Balkwill and Ghiorse, 1985), about two-thirds of the bacteria isolated were gram negative. In a carbonate aquifer in the Atlantic Coastal Plain (Chapelle et al., 1988), about 70% of the isolates recovered were gram negative. Finally, in clastic aquifers of the Atlantic Coastal Plain (Balkwill, 1989), anywhere from 60% to 90% of the isolates recovered were gram negative. This group of microorganisms, therefore, are important components of the terrestrial subsurface flora.

Aerobic Gram-Negative Rods Aerobic gram-negative rods are widely distributed in soils and shallow ground-water systems. Most of these bacteria have an enzyme called cytochrome oxidase that enables the organism to transfer electrons onto molecular oxygen, thus using oxygen as a terminal electron acceptor. Such microorganisms are termed oxidase positive. In the shallow aquifer near Lula, Oklahoma (Balkwill and Ghiorse, 1985), all of the bacteria isolated and characterized were oxidase positive, meaning that they carried this particular enzyme. In deeper anaerobic aquifers, as one might expect, the presence of gram-negative, aerobic rods becomes less common.

Members of the genera *Pseudomonas, Azotobacter, Rhizobium, Alcaligenes, Flavobacterium,* and *Bordetella* are representative of aerobic gram-negative rods. Pseudomonas appears to be particularly common in ground-water systems. Of 29 strains of bacteria identified to the genus level in the carbonate Floridan aquifer system of South Carolina, 11 could be assigned to the genus *Pseudomonas* (Chapelle et al., 1988). At the Lula, Oklahoma, site (Balkwill and Ghiorse, 1985), 3 of the 6

isolates characterized appeared to be *Pseudomonas*. Out of 138 strains of aerobic bacteria isolated from deep coastal plain sediments in South Carolina, 10 were identified as *Pseudomonas* based on 16S-rRNA sequences (Balkwill and Boone, 1997). Pseudomonads have also been identified in a volcanic tuff aquifer at the Nevada Test Site (Haldeman and Amy, 1993), and from fluvial and lacustrine sediments in Hanford, Washington (Balkwill and Boone, 1997).

One reason pseudomonads are common in shallow water table aquifers is that they are extraordinarily versatile in the kinds of organic substrates on which they can grow. In addition, many species do not require specific vitamins, growth factors, or amino acids and readily live on a number of different carbon sources. In one study, for example, a species of *Pseudomonas* was shown to grow on 127 different organic carbon compounds. In fact, the only reason that study stopped at 127 was that the investigators ran out of ideas as to what to feed it! This adaptability makes it an ideal candidate for inhabiting aerobic aquifers, where organic carbon substrates are largely limited to dissolved organic carbon leaching out of the soil zone above.

Pseudomonads are always respirative and do not have the ability to ferment if conditions become anaerobic. Some species of *Pseudomonas*, however, do have the ability to use alternative pathways of respiration. For example, some strains are able to use nitrate as an alternative electron acceptor if oxygen concentrations become limiting for growth. In addition, some strains of Pseudomonas are capable of dissimilatory Fe(III) reduction. The flexibility of these microorganisms, both in terms of the organic substrates utilized and the electron acceptors used, makes them well adapted for the variable conditions encountered in the terrestrial subsurface.

Azotobacter and *Rhizobium* are notable in that some members of these genera are capable of nitrogen fixation; that is, these bacteria take inorganic nitrogen gas, N_2, and transform it into organic nitrogen compounds. Because all of the higher plants and animals require organic nitrogen for protein synthesis, nitrogen fixation is extremely important in agricultural microbiology. Bacteria are the only organisms on earth capable of this critical function.

Members of the genus *Rhizobium* form symbiotic associations with the roots of legume plants, such as soybeans. Because of this symbiotic strategy, members of this genus are probably not widespread in subsurface environments below the root zone. *Azotobacter,* unlike *Rhyzobium,* are free-living and are known to inhabit soils. As nitrogen-fixation activity has been observed in samples from shallow water table aquifers and from deeper aerobic aquifers (Fredrickson et al., 1989), it seems probable that representatives of the genus *Azotobacter* are present in such systems. The only identified member of *Azotobacter* that has been identified in ground-water systems, however, was associated with drilling fluid and not uncontaminated sediments (Chapelle et al., 1988). It has yet to be shown, therefore, that members of the genus *Azotobacter* inhabit subsurface environments below the soil zone.

Flavobacterium is widely distributed in soils and water. They have not been reported as being isolated from cored aquifer sediments, but they have been reported in ground water produced from aerobic aquifers. Of 500 isolates recovered from ground water produced from an alluvial aquifer in Arizona, between 3% and 10% were identified to be *Flavobacterium* (Stetzenbach et al., 1986). It is not possible to determine unequivocally if these bacteria occur naturally in the aquifer or if they colonized the well subsequent to drilling. However, because these bacteria

are so common in soils, it's likely that they are present in some aerobic ground-water systems. An interesting attribute of *Flavobacterium* is that they are nutritionally diverse and are able to utilize many kinds of organic compounds as substrates. Some isolates of *Flavobacterium* are even reported to hydrolize ligin, a metabolic strategy most often associated with the fungi.

Other aerobic gram-negative rods include the genera *Agrobacterium, Mycrocyclus, Gallionella,* and *Caulobacter. Agrobacterium* is notable mostly in that it contains a tumor-inducing characteristic that produces abnormal growths on some plants. *Agrobacterium* has not been reported from cored sediments but has been isolated from ground-water samples (Hirsch and Rades-Rohkohl, 1983). *Mycrocyclus* has been recovered from cored sediments of the Atlantic Coastal Plain (Chapelle, unpublished data) and from samples of ground water (Hirsch and Rades-Rohkohl, 1983).

Gallionella is a chemolithotrophic bacterium that obtains energy by oxidizing dissolved ferrous iron to ferric oxyhydroxides. Since it is an obligate aerobe and because ferrous iron is largly absent from aerobic water, *Gallionella* generally lives where aerobic and anerobic waters mix. This commonly occurs at the bottom of streambeds receiving discharge from anaerobic aquifers. Unfortunately, wells that tap anaerobic aquifers also provide such an aerobic-anaerobic interface. The growth of *Gallionella* in wellbores is a significant problem because the ferric oxyhydroxides that it produces will eventually clog the well.

Caulobacter is interesting morphologically because it is characterized by a stalk that is a continuation of the long axis of the cell. *Caulobacter* is an *oligotroph,* or a bacterium adapted to conditions of extreme nutrient deprivation. These adaptations include the ability to enter a vegetative state, which reduces energy requirements, and the ability to store energy in the form of poly-beta-hydroxybutyric acids. Given the oligotrophic (nutrient-poor) conditions of most aquifer systems, *Caulobacter* is probably widely distributed in shallow aquifer systems. Members of this genus have been isolated from ground-water samples by Hirsch and Rades-Rohkohl (1983).

Brucella, Bordetella, and *Francisella* are other genera of aerobic gram-negative bacteria. These genera contain species that are important human pathogens but have not yet been observed in ground-water systems.

Aerobic Gram-Negative Cocci There are relatively few genera of aerobic gram-negative cocci among the bacteria. Most of these genera, which include *Neisseria, Moraxella,* and *Acinetobacter,* are notable in that they are important human pathogens. *Neisseria gonorrhoeae,* for example, causes the human venereal disease gonorrhea, and *Neisseria meningitidis* causes a particularly severe form of meningitis. *Neisseria*-like microorganisms have been recovered from sediments of aerobic aquifers (Jimenez, 1990), and several *Moraxella* species have been recovered from groundwater produced from aerobic aquifers (Stetzenbach et al., 1986). However, there is no evidence that the species observed are pathogenic.

Members of the genus *Acinetobacter* are widely distributed in soils and water, and so it is reasonable to expect that they also inhabit aerobic ground-water systems. In an analysis of 138 bacterial isolates from aerobic aquifers of the Atlantic Coastal Plain in South Carolina, 25 were assigned to *Acintobacter* on the basis of 16S rRNA sequences (Balkwill and Boone, 1997). A similarly high percentage of

strains was found to be *Acinetobacter* in volcanic tuff aquifer at DOE's Nevada Test Site (Haldeman and Amy, 1993). Interestingly, however, *Acinetobacter* was much less in evidence in a core hole at DOE's Hanford, Washington, site (Balkwill and Boone, 1997). This probably reflects the fact that ground water at the Hanford site is largely anaerobic, which appears to exclude aerobic bacteria such as *Acinetobacter.* Species of the genus *Acinetobacter* have also been reported in well water produced from deep aerobic aquifers. Of 500 different isolates recovered from well water produced from a deep (450–500 feet deep) aerobic aquifer in Arizona (Stetzenbach et al., 1986), 54% were identified as *Acinetobacter.*

Facultatively Anaerobic Gram-Negative Rods The enteric bacteria, or Enterobacteriaceae, are the most important members of this group of bacteria. They have been carefully studied because of their importance in human and animal health. The single most studied microorganism in microbiology, *Escherichia coli,* belongs to the family Enterobacteriaceae. Other members of this family include *Shigella, Salmonella, Klebsiella,* and *Enterobacter,* all of which include members that are human pathogens. These bacteria all have the capability of respiratory metabolism if molecular oxygen is available. If molecular oxygen is not available, these bacteria are able to switch to fermentation as an alternative metabolic strategy. This enables them to grow under either aerobic or anaerobic conditions.

The presence of enteric bacteria in ground water implies the presence of fecal contamination, either from humans or animals, that has been transported from land surface. As such, *E. coli* is widely used as an indicator of sewage contamination of ground water (Keswick et al., 1982). Some members of the Enterobacteriaceae, however, are indigenous to soils. An example of this type of soil microorganism is *Aerobacter aerogenes.*

Because the Enterobacteriaceae are so closely related and because they are of such medical and commercial importance, a considerable amount of work has gone into distinguishing between species. One key taxonomic characteristic are the kinds of fermentation products produced by the microorganisms. Two broad patterns are recognized: the mixed-acid fermentation and the butylene glycol fermentation. In mixed-acid fermentation, three acids—lactic, acetic, and succinic—are formed, as well as ethanol, CO_2, and H_2. In butylene glycol fermentation, smaller amounts of acid are formed, with butylene glycol, ethanol, CO_2, and H_2 being the main end products. Mixed-acid fermentation yields equal amounts of CO_2 and H_2, whereas that of butylene glycol yields more CO_2 than H_2. This is because mixed-acid fermenters produce CO_2 and H_2 only from decomposition of formate

$$HCOOH \rightarrow H_2 + CO_2$$

by means of the formic hydrogenylase enzyme system. The butylene glycol fermenters also produce CO_2 and H_2 from formic acid, but they produce additional CO_2 during the reactions that lead to butylene glycol. *E. coli* is an example of a mixed-acid fermenter, and *Aerobacter aerogenes* is an example of a butylene glycol fermenter.

The presence of facultatively anaerobic gram-negative rods have not been extensively described in ground-water systems. *Chromabacterium,* a member of this group, has been recovered from a shallow water table aquifer in Lula, Oklahoma

(Balkwill and Ghiorse, 1985), and from a shallow water table aquifer in the Atlantic coastal plain (Chapelle et al., 1988). However, because these kinds of bacteria are relatively common in soils, it is probable that they are also widely distributed in shallow ground-water systems. Data on the presence of this type of bacteria are uniformly lacking from deeper, anaerobic aquifer systems.

Anaerobic Gram-Negative Rods Truly anaerobic bacteria are able to grow without molecular oxygen as an electron acceptor. In fact, many anaerobic bacteria do not tolerate the presence of oxygen. When molecular oxygen is dissolved in water, hydrogen peroxide is formed by the reaction

$$H_2O + 1/2\ O_2 \rightarrow H_2O_2$$

Hydrogen peroxide formed by this reaction is a strong oxidant and is highly toxic to all bacteria. Aerobic bacteria, which must live in oxygenated environments, produce an enzyme called catalase, which breaks down the peroxide to oxygen and water and keeps the peroxide from damaging the cell. Anaerobic bacteria generally lack catalase and therefore have no defense against the toxic effects of peroxide.

Several anaerobic, gram-negative, rod-shaped bacteria belong to the family Bacteroidaceae and include the genera *Bacteroides* and *Fusobacterium.* Species of *Bacteroides* inhabit the oral cavities and gastrointestinal tracts of mammals.

Many species of sulfate-reducing bacteria, notably *Desulfovibrio,* are gram-negative anaerobic rods. These microorganisms can use H_2 or simple organic compounds such as acetate as energy sources and sulfate as a terminal electron acceptor. *Desulfovibrio* is not autotrophic; that is, it cannot obtain all of its carbon from CO_2 but, rather, requires organic compounds.

Sulfate-reducing bacteria are widely distributed in ground-water systems. They have been reported from petroleum reservoirs (Bastin, 1926; Davis, 1967), and anaerobic ground-water systems (Dockins et al., 1980; Chapelle et al., 1987). Interestingly, sulfate-reducing bacteria, which are poorly tolerant of molecular oxygen, have also been reported from aerobic ground-water systems (Jones et al., 1989). It is probable that anaerobic microenvironments in predominantly aerobic aquifers support the growth of these organisms. Thermophilic sulfate-reducing bacteria have been reported from subsurface environments as deep as 2.77 km (Balkwill and Boone, 1997). These microorganisms, while apparently related to the genus *Desulfotomaculum*, exhibit only a 94.6% similarity with known species of that genus.

Because of their widespread occurrence, sulfate-reducing bacteria are one of the most important types of microorganisms in terms of their impact on ground-water geochemistry. An important limitation of sulfate-reducing bacteria is that they are not capable of completely metabolizing carbohydrates to CO_2 and water with the reduction of sulfate. Rather, they are dependent on associated fermentative bacteria to oxidize partially the carbohydrates to simple organic acids and hydrogen (H_2). These organic acids and H_2 are then coupled with sulfate reduction in order to provide energy for growth. Because they consume the products of fermentation, sulfate-reducing bacteria prevent the buildup of fermentation products, which in turn aids the metabolism of fermentative microorganisms. Sulfate-reducing bacteria and fermenters thus have a symbiotic relationship with each other, each contributing to the energy metabolism of the other.

Another important physiologic type of anaerobic gram-negative rods are the Fe(III)-reducing bacteria. This particular type of respiration, using Fe(III) oxyhydroxides as a sole terminal electron acceptor, had been long recognized by microbiologists. Curiously, however, it wasn't until 1987 that the first microorganism capable of growth on organic compounds coupled to the reduction of Fe(III) was isolated in pure culture (Lovley and Phillips, 1988). This microorganism was later assigned to a new genus and named *Geobacter metallireducens* (Lovley et al., 1993). *G. metallireducens* is a nonmotile, nonspore-forming, gram-negative rod. In addition to reducing Fe(III), this microorganism is capable of reducing Mn(IV) and U(VI). Several *Geobacter* species have been recovered from deep subsurface environments (Lovley et al., 1990), and *Geobacter* appears to be widely distributed in ground-water systems (Lovley, 1997).

2.5.3 Gram-Positive Bacteria in Ground-Water Systems

There are not as many kinds of gram-positive bacteria as there are gram-negative bacteria. The gram-positive bacteria include, however, many important human pathogens and have therefore been closely studied. Gram-positive bacteria also have been widely reported from many ground-water systems and may play important roles in modifying ground-water chemistry.

Gram-Positive Cocci The most common gram-positive cocci include the genera *Micrococcus, Staphylococcus,* and *Streptococcus.* Several species of *Staphylococcus* and *Streptococcus* are important human pathogens but are not known to live naturally in ground-water systems. Streptococci are fermentative, do not respire, and do not produce the H_2O_2-neutralizing enzyme catalase. One particular streptococci, *Streptococcus faecalis,* is a normal inhabitant of the large intestines of humans and animals. As such, fecal strep is widely used as an indicator organism for fecal contamination of water supplies.

Staphylococcus and *Micrococcus* are indistinguishable based just on morphology, because both tend to form grape-like clusters when gram-stained. *Micrococcus,* unlike *Staphylococcus*, is widely distributed in soils and surface water. Not surprisingly, *Micrococcus* has been isolated from a variety of subsurface environments, including carbonate aquifers (Chapelle et al., 1988), clastic coastal plain aquifers (Balkwill and Boone, 1997), volcanic tuff aquifers (Haldeman and Amy, 1993), and fluvial-lacustrine aquifers (Balkwill and Boone, 1997).

Coryneform Bacteria The coryneform bacteria have variable shapes that grade from cocci to club-shaped irregular rods. They are aerobic, nonmotile, and often show pronounced intracellular granules. Such bacteria have been isolated from the shallow water-table aquifer at Lula, Oklahoma (Balkwill and Ghiorse, 1985), and from a shallow carbonate aquifer in South Carolina (Chapelle et al., 1988).

The genus *Arthrobacter* contains many species of bacteria that are widely distributed in soils and water. *Arthrobacter* also appears to be widely distributed in ground-water systems. Twenty-five of 138 strains of aerobic bacteria isolated from aerobic aquifers of the Atlantic Coastal Plain underlying DOE's Savannah River Site in South Carolina were assigned to the genus *Arthrobacter* on the basis of 16S rRNA sequences (Balkwill and Boone, 1997). In addition, a high percentage of isolates from a volcanic tuff aquifer at the Nevada Test Site (Haldeman and Amy,

1993) and from a fluvial-lacustrine aquifer at the Hanford site in Washington (Balkwill and Boone, 1997) were assigned to the genus *Arthrobacter*. Clearly, *Arthrobacter* species are widespread in ground-water systems.

Spore-Forming Rods The ability to form endospores, or specialized structures that develop when environmental conditions become adverse, is characteristic of some gram-positive bacteria. Sporulation (spore formation) is a complex process that is initiated when the cell senses the presence of adverse conditions such as a lack of substrates or excessive heat or dryness. First, the bacterium makes a copy of its chromosomal DNA and forms a membrane called the spore septum around the DNA. Thick coats of peptidoglycan are deposited around the septum, together with a substance called dipicolinic acid, to form the cortex. These materials shield the genome of the bacteria from drying and heat stress. In this form, the genome of the bacterium can maintain itself virtually indefinitely.

Two important genera of spore formers are *Bacillus* and *Clostridia. Bacillus* is widely distributed in soils and is capable of both fermentative and respirative (aerobic) metabolism. *Clostridia* is equally widely distributed in soils but is always fermentative. *Clostridia* is very important in the decomposition of organic material under anaerobic conditions.

Members of the genus *Bacillus* have been isolated from a shallow aerobic ground-water system by Chapelle et al. (1988). In addition, an anaerobic species *Bacillus infernus* has been isolated from a 2.7-km-deep core hole in Virginia. Members of the genus Clostridia are probably widely distributed in anaerobic ground-water systems. They have been isolated from ground-water samples by Hirsch and Rades-Rohkohl (1983). In addition, gram-positive rods capable of autotrophic acetogenesis isolated from clayey sediments of the Atlantic Coastal Plain (Chapelle and Bradley, 1996) are possibly *Clostridia.*

Spore-forming, thermophilic, sulfate-reducing bacteria have been isolated from oil field waters produced from the North Sea (Rosnes et al., 1991). These bacteria grew autotrophically on H_2-CO_2 and heterotrophically on a number of fatty acids and alcohols. The spores were extremely heat-resistant and could survive at temperatures of up to 131°C for limited periods of time. This suggests that spore-forming bacteria may be common in deep subsurface environments, particularly those in which heat tolerance is an asset important to survival.

2.6 CHAPTER SUMMARY

This brief overview serves primarily to emphasize the diversity of microbial life in ground-water systems. While extremely diverse, however, this life is not randomly distributed. Aerobic heterotrophs dominate aerobic ground-water systems whether they are deeply buried (as in the Tucson Basin of Arizona) or whether they are relatively shallow. Many of these aerobic ground-water systems are characterized by the presence of protozoa (which may prey on bacteria), algae, and fungi (which compete for carbon substrates with bacteria). Viruses have been extensively studied in ground-water systems, but these studies deal exclusively with human-related contamination. These studies consistently show that virons can remain viable and infectious for several months in ground-water systems. However, the role that bac-

teriophages play in the ecology of aquifers remains unexplored. Anaerobic bacteria such as fermenters, Fe(III) reducers, sulfate reducers, and methanogens predominate in anoxic ground-water systems. As in anoxic aquatic sediments, these microorganisms form symbiotic food chains in which the waste products of fermenters (H_2, formate, acetate) serve as primary substrates for Fe(III) reduction, sulfate reduction, or methanogenesis. Archaea, such as the methanogens, are an importance part of the biota in ground-water systems.

QUESTIONS TO CONSIDER

1. Why did Carl Woese decide to use the 16S subunit of ribosomal RNA to investigate the relatedness of different microorganisms instead of chromosomal DNA?
2. How common do you think protozoan parasites like *Cryptosporidium* and *Giardia* are in ground water? Discuss some ways that ground water can become contaminated by these microorganisms.
3. It has been shown that land application of sewage effluents can contaminant ground water with human enteroviruses. How long do these viruses remain active, and how far do you think they can be transported in ground-water systems?
4. Why do pseudomonads seem to be so ubiquitous in aerobic ground-water systems?
5. Fe(III)-reducing bacteria are common in anaerobic ground-water systems. What kind of aquifer materials favor this kind of metabolism? What kind of aquifer materials do not favor it?

CHAPTER 3

BACTERIAL GROWTH

The chief characteristic that separates living from nonliving entities is the ability of living organisms to grow and reproduce. In this chapter, the growth and reproduction of bacteria will be discussed. The basis of this discussion is to understand the factors and conditions that encourage, or alternatively discourage, the growth of individual cells and populations of cells.

It is useful to begin by differentiating between the growth of individual cells and the growth of populations of cells. The growth of individual cells is defined as an increase in cellular mass, an increase in the number of cellular components, and an increase in size. The growth of populations of cells, however, results in an increase in the total number of cells. Strictly speaking, the term cell growth refers to the growth of an individual cell. However, because the growth of individual cells invariably leads to reproduction and population growth, there is an obvious functional relationship between the two.

This functional relationship between cell growth and population growth is important for practical reasons. Observing and quantifying the growth of individual cells is fairly difficult because of their small size. However, it is relatively easy to follow the growth of populations. Thus, the most common measure of cell growth is in fact to measure the increase in cell populations.

3.1 BACTERIAL REPRODUCTION

Most bacterial cells reproduce by means of binary fission, in which individual cells split to form two daughter cells. The way in which this occurs is shown schematically in Figure 3.1. First, a parent cell undergoes elongation, a process that is more readily apparent in rod-shaped cells than in cocci, and chromosomal DNA is reproduced. Second, there is internal reorganization, in which each half of the dividing cell receives its complement of DNA and a septum forms in between. Third, the septum develops into a transverse cell wall, and the daughter cells separate.

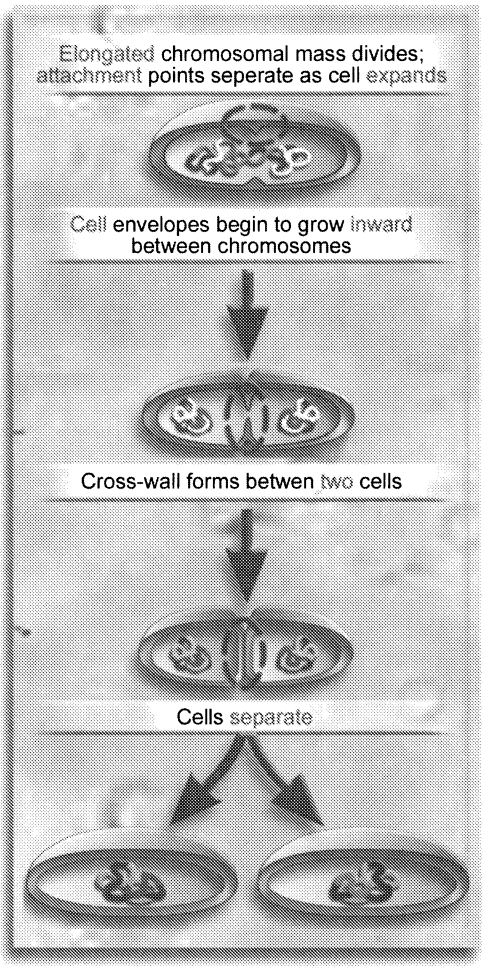


Figure 3.1. Sequence of steps leading to binary fission in bacterial cells.

The time that it takes an individual cell to produce two daughter cells, which is often called its doubling time, is highly species dependent. Some enteric bacteria, notably *E. coli,* are capable of very rapid reproduction and have doubling times on the order of 20 minutes. Other bacteria, such as *Micrococcus,* characteristically grow more slowly and have doubling times on the order of hours. While the fastest doubling time that a bacterium can achieve is largely genetically determined, actual doubling times are dependent on a variety of environmental conditions. Under conditions of nutrient deprivation or in the presence of toxic substances, the division of bacteria can come to a virtual halt.

There has been relatively little work on the doubling times of bacteria that live in ground-water systems. Investigations of soil bacteria show that doubling times are largely dependent on soil temperature, soil moisture, and substrate availability. In ground-water systems, temperature and moisture content are, for all practical purposes, constant. Thus, it might be expected that doubling times would largely reflect the availability of substrates for cell growth.

There is some evidence that this is indeed the case. In studies of a shallow sand aquifer that had been contaminated with sewage effluent, Harvey and George (1987) presented evidence that, based on the incorporation of tritium-labeled thymidine into cellular DNA, doubling times in the contamination plume were on the order of days. Outside the contamination plume, where concentrations of organic carbon were much lower, doubling times were much lower, in some cases on the order of months or years. Thorn and Ventullo (1988) calculated growth rates for bacteria in shallow pristine aquifers of Wisconsin, Michigan, and Oklahoma. Based on this data, doubling times on the order of 1 to 180 days may be calculated with averages on the order of 10 to 20 days. These rates are roughly consistent with the results of Harvey and George (1987). Because of the wider range of aquifer conditions studied, however, Thorn and Ventullo's data give a hint of the variability of growth rates encountered in shallow aquifer systems. This variability is not at all surprising and, as is the case with soils, is certainly a characteristic of these types of systems. While showing the same sort of variability in growth rates as exhibited by soils, rates in shallow aquifers are ubiquitously slower than those in soils.

Studies on doubling times of bacteria that live in deep subsurface environments that are largly cut off from the surface have yet to be performed. It is quite likely, however, that these doubling times will prove to be among the slowest of any terrestrial microbial habitat.

3.2 POPULATION GROWTH KINETICS

A consequence of binary fission being the predominant reproductive strategy of bacteria is that population growth is exponential; that is, if one were to plot the increase of a bacterial population with time, the curve approximates an exponential function of the form:

$$A(x) = A_0 \exp^{kt}$$

where $A(t)$ is the number of cells at any time t, A_0 is the initial number of cells, and k is the first-order growth constant.

Take, for example, the case shown in Figure 3.2, in which a single cell divides with a doubling time of half an hour. After half an hour there are two cells; after 1 hour there are four cells; after 1.5 hours, eight cells, and so on. Initially, the population growth is fairly slow. However, because the rate of growth is dependent upon the number of cells present, after a few hours population growth rapidly accelerates. If growth were to continue unchecked, in few weeks there would be enough bacteria present literally to cover the earth.

Obviously, since the world is not covered with bacteria, the growth of bacterial populations in nature never proceeds unchecked. Environmental factors, such as the lack of substrates, the production of toxic waste products, infection by viruses, or predation by protozoa, always serve to limit bacterial population growth.

In the laboratory, the growth of bacterial populations typically follows a characteristic pattern (Fig. 3.3). Initially, if fresh media is inoculated with a bacterial culture, there is a period in which there is little population growth. This period of

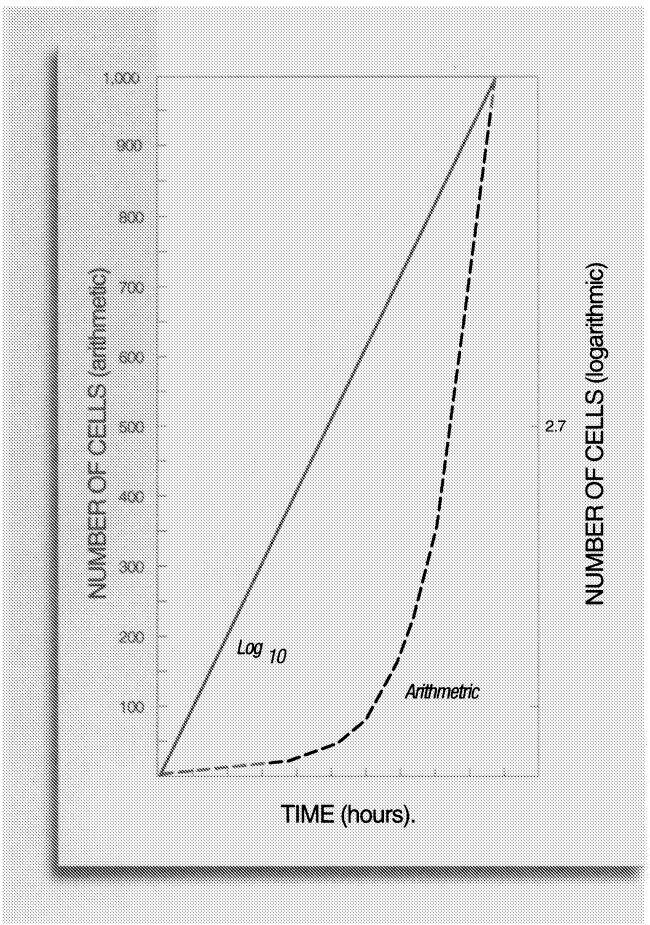


Figure 3.2. Exponential growth of bacteria as a result of binary fission.

slow growth is termed the lag phase and reflects the time needed by the bacteria to adjust to the new conditions. After the lag phase, the population begins a period of rapid growth that is termed the exponential phase, reflecting the characteristic exponential nature of population increase. Eventually, the nutrients present in the media become depleted and/or there is a buildup of toxic waste products, the rate of reproduction equals the death rate, and population growth ceases. The bacterial cells shift utilization of scarce resources away from cellular growth and, instead, concentrate on maintaining themselves in a viable state. This is termed the stationary phase. As time passes and nutrients are completely used up, the population will begin to die off. This is termed the death phase.

In soils where there is a constant flux of nutrients being added from the surface (fertilization) and removed (plant growth), the bacterial population is very dynamic, shifting rapidly between the exponential, stationary, and death phases. It is much less clear how populations change in ground-water systems. In shallow systems that are closely connected with the surface, rapid shifting between exponential growth and death phases may possibly occur. In deeper systems in which changes

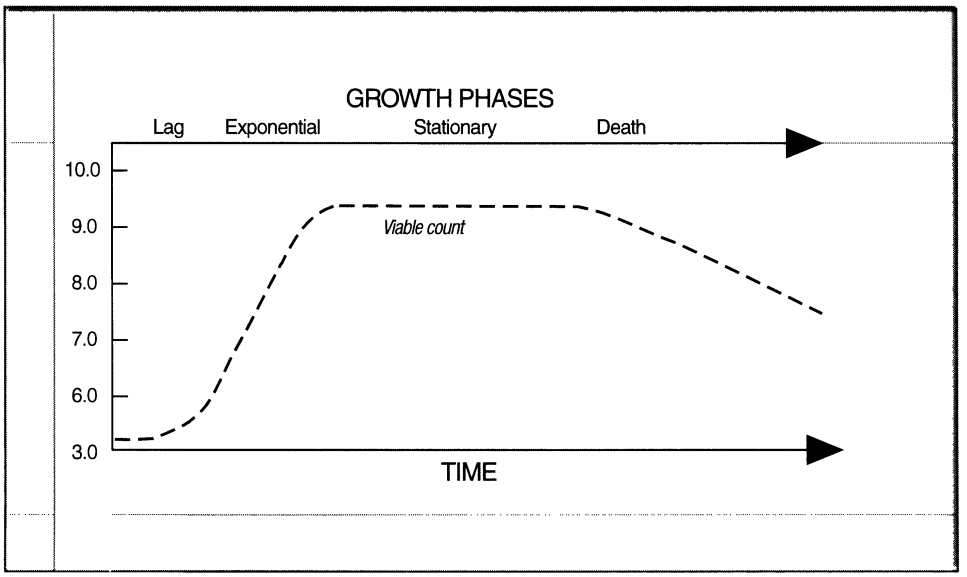


Figure 3.3. The different phases of growth typically observed in bacteria growing in liquid culture media.

in nutrient availability occur very slowly, it is likely that population dynamics are more sluggish.

It is clear that bacteria in most subsurface environments are not in a state of exponential growth. However, it is possible that an unusual substrate-providing event, such as the intrusive migration of hydrocarbons associated with petrogenesis, could result in a "bloom" of bacteria in exponential growth phase.

Most deep subsurface environments are strongly nutrient-deprived and bacterial populations are probably in either stationary or death phase. In either case, the time frame associated with these phases is vastly expanded from what is observed in the laboratory (Fig. 3.3). In the laboratory, it is often observed that the stationary phase predominates for a few days and that the death phase might predominate for a few weeks or even months. In the deep subsurface, however, with sediments and rocks that are typically millions of years old, it is clear that bacterial populations are able to maintain themselves in states approximating stationary phase for long periods of time. It is debatable whether the term "stationary" or "death" phase more properly describes the state of deep subsurface bacterial populations. It may well be that at the time scales considered in these environments, identifying one or the other states is simply not feasible.

3.3 ENVIRONMENTAL CONDITIONS AND BACTERIAL GROWTH

The ability of microorganisms to grow in nature is largely a function of the environmental conditions in which they find themselves. An understanding of how such conditions affect microbial growth is essential to understanding their observed distribution and abundance in nature. It is characteristic of microbial populations that

not all species respond positively to the same set of conditions. In fact, the conditions that are necessary for the growth of one particular strain may be toxic to other strains. More commonly, microbes have a range of tolerance to environmental conditions, and, as long as conditions remain inside that envelope, they can effectively grow. In some cases, microorganisms exhibit remarkable ability to grow under adverse conditions. Bacteria in subsurface environments are particularly notable for their tolerance of difficult growth conditions.

3.3.1 Temperature

The temperature of an environment is an important factor in the growth of microorganisms. Life in microbes, as in all living systems, is supported by a series of enzymatically catalyzed chemical reactions. As temperature increases, the rates of chemical reactions tend to increase. So, to a point, an increase in temperature is favorable for biochemical processes. However, the enzymes and nucleic acids that organize and facilitate these processes are heat-sensitive. If temperatures increase too much, the structures of some proteins may become distorted to the point that they no longer catalyze life-sustaining reactions. Similarly, if the structure of nucleic acids is altered by excessive heat, the information stored in them may become distorted. This, in turn, prevents the production of enzymes needed for growth.

It is universally observed that particular strains of microorganisms exhibit a characteristic envelope of temperature tolerance (Fig. 3.4). First, there is a temperature minimum, below which the microorganism cannot grow. As the temperature increases above this minimum, the growth rate increases until a temperature optimum is reached. This is the temperature at which the organism can grow at its maximum rate. As the temperature increases above the optimum, there is typically a sharp decrease in the growth rate. The temperature maximum is the highest temperature at which a microorganism can grow effectively. The temperature minimum, optimum, and maximum are sometimes referred to as the cardinal temperatures and are characteristic of particular strains. Cardinal temperatures are not, however, completely fixed and may be affected by other environmental factors, such as pH and salinity.

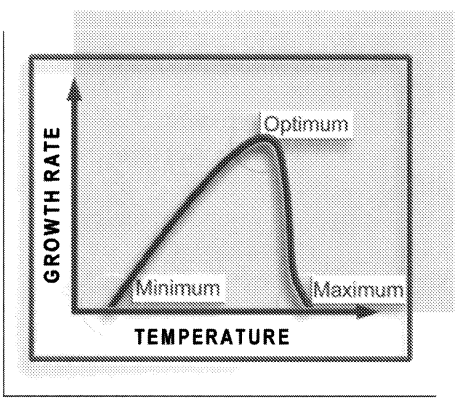


Figure 3.4. A typical envelope of temperature tolerance exhibited by microorganisms.

Microorganisms that have relatively low cardinal temperatures, those between 0° and 20° centigrade, are termed psychrophiles. As one would expect, psychrophilic microorganisms live in environments where cold temperatures typically are found. For example, the bottom waters of the deep ocean basins exhibit temperatures in the 1–3°C range. Bacteria that inhabit these environments are quite capable of growth at these low temperatures, although they may exhibit temperature optima in the 10–20°C range.

Conventional wisdom has long maintained that, on average, psychrophilic microorganisms metabolize more slowly than do mesophiles. However, recent research on petroleum hydrocarbon–degrading microorganisms indicates that this is not necessarily true. In a study comparing the ability of psychrophilic bacteria from a shallow aquifer in Alaska (characterized by an in situ temperature of 5°C) with mesophilic bacteria from a shallow aquifer in South Carolina (with an in situ temperature of 20°C), Bradley and Chapelle (1996) showed that the psychrophilic microorganisms were actually capable of respiring toluene faster at 5°C then the mesophiles were at 20°C. In this particular case, the difference could probably be attributed to the higher nutrient content of the Alaska sediments. This demonstrates that, when microorganisms are adapted to colder temperatures, psychrophilic microorganisms can metabolize at least as fast as, and sometimes faster than, mesophilic microorganisms.

Freezing is generally bacteriostatic—that is, it has the effect of stopping microbial growth but it does not always lead to death. Freezing can be fatal to microorganisms if the formation of ice crystals disrupts essential cellular structures, such as the cell membrane. However, if such cellular damage is avoided, the cell often simply becomes inactive and will remain in that state virtually indefinitely. Because ice crystals are formed less efficiently when freezing occurs rapidly, fast-freezing techniques are commonly used to preserve bacteria in the laboratory.

Microorganisms with temperature ranges in the 20–40°C range are called mesophiles. This is the temperature range of many natural environments, and it is safe to say that the majority of microorganisms that have been characterized are mesophiles. Also, this is the temperature range maintained by warm-blooded animals, and so, many pathogenic bacteria have their optima in this range. It is interesting to note that the temperature maximum is always closer to the temperature optima than the temperature minima (Fig. 3.4). Some researchers think that the mammalian characteristic of producing higher temperatures (i.e., fever) in response to illness is an adaptation designed to take advantage of this characteristic. Increasing body temperature above the temperature optima of the invading microorganisms can be an effective method of fighting some infections.

Most of the bacteria recovered from subsurface environments and characterized are mesophiles and generally grow most efficiently from 20 to 30°C, as this is the most common temperature range of ground-water systems used for water supply. However, many microbiology laboratories habitually maintain the temperatures of their incubators at 37°C, which is body temperature for humans. Some laboratories have had the unfortunate experience of having interesting bacterial strains killed by these "elevated" temperatures.

Microorganisms that grow at temperatures above 45°C are said to be thermophilic. Because of the relatively high temperatures associated with very deep aquifer systems and petroleum reservoirs, thermophilic bacteria are of particular interest in the study of ground-water chemistry.

There is no general agreement as to the highest documented temperature associated with microbial growth. At this writing, investigators culturing bacteria isolated from hot vents on the ocean floor claim to see bacterial growth as hot as 125°C. The optima for most thermophiles that have been characterized, however, is generally in the 55–60°C range.

There is a large body of literature on thermophilic bacteria isolated from the deep subsurface. For example, Zobell (1958) decribed sulfate-reducing bacteria from formation waters that ranged from 60° to 105°C. A number of Russian scientists have made similar observations from oil field brines. Zinger (1966) described sulfate-reducing bacteria in formation waters that were about 70°C; Rozanova and Khudyakova (1974) recovered sulfate-reducing bacteria from water reported to be 84°C; and Rozanova and Nazina (1979) reported an organism designated *Desulfovibrio thermophilus* in formation waters above 80°C, and these same authors reported a strain of *Desulfotomaculum nigrificans* in waters of 60–70°C. Because hot ground water typically cools as it is brought to the surface, it is often difficult to be precise as to the in situ temperature.

One of the most interesting reports of thermophilic bacteria in the deep subsurface is found in the work of Olson et al. (1981). Olson and her coworkers isolated a number of strains of sulfate-reducing and methanogenic bacteria from water produced from the Madison aquifer of Montana. The bacteria that Olson isolated grew well at 50°C, the temperature of the water at the wellhead (which is lower than the in situ temperature), but could not grow at 22°C. Because the formation waters were recovered within three weeks of drilling, Olson realized that this was almost certainly insufficient time for introduced organisms to acclimate to higher temperatures. This, in turn, was good evidence that the bacteria recovered were indeed part of the indigenous flora of the Madison aquifer and had not been introduced during drilling procedures.

Petroleum geologists often consider that 100°C to be the maximum temperature at which bacteria can be active and thus be involved with petrogenesis or methanogenesis. This assumption appears to be based more on convention than on actual data. The destruction of bacteria at 100°C (i.e., by boiling) is more related to the coexistence of two phases, liquid and vapor, that occurs at "normal" (i.e., one atmosphere) pressures. Because water in the deep subsurface is under pressure and hence does not boil as easily, there is no a priori reason why bacteria cannot be as active at 150°C as at 50°C. The only constraint is that the organisms would have to be equipped with enzymes and nucleic acids capable of efficient operation at these temperatures. Thus, while hard evidence is lacking to show that bacteria in the deep subsurface are capable of activity at temperatures above 100°C, it is highly likely that such microorganisms exist. In fact, such thermophilic bacteria may be instrumental in such important processes as petrogenesis, methanogenesis, and reservoir porosity enhancement.

3.3.2 Water

All microorganisms require water in order to carry out life processes, and all but a few require liquid water in order to grow. Many microorganisms, however, are especially designed to resist drying. The thick cell wall of *Staphylococcus,* which is adapted to living on human skin, is very efficient at preserving moisture in the cell and allows it to thrive in that relatively dry environment. Similarly, many soil

microorganisms are highly resistant to drying, an adaptation crucial in an environment that is subject to successive wet and dry periods.

At first glance, it might seem that in ground-water systems drying would not be a limiting factor to microbial growth. However, there are several ways in which moisture availability may be a problem for bacteria in aquifer systems. The most obvious one is the unsaturated zone—the zone that exists between the soil zone and the saturated water table—where water between pore spaces coexists with gases. In these gas-filled interstices, cell drying is at least a possibility, and one that microbes living in them must address. Another way that drying may be a problem in ground-water systems is in the very fine-grained sediments that comprise confining beds. Because of the high surface area of clay minerals in these sediments, their generally compact arrangement, and the affinity for water to clay mineral surfaces, water actually available to bacteria may be limited. It is commonly observed that clayey confining beds exhibit much less microbial activity than adjacent sandy aquifers (Phelps et al., 1989a; Chapelle and Lovley, 1990). One of the possible causes of this phenomenon is the nonavailability of water due to the high clay content.

3.3.3 Molecular Oxygen

The presence of molecular oxygen, either in the gaseous or dissolved form, is an absolute requirement for some microorganisms that are termed obligate aerobes. These microorganisms produce energy by removing electrons from organic carbon or inorganic electron donors, and by transferring these electrons to molecular oxygen in order to produce energy. Other microorganisms, called facultative anaerobes, prefer to use molecular oxygen if it is available, but if it is not available, they are able to use alternative electron acceptors for metabolism. Some kinds of obligate aerobes, while requiring oxygen in order to grow, cannot tolerate oxygen in high concentrations. These are termed microaerophilic bacteria.

While oxygen is the preferred electron acceptor for many kinds of bacteria, oxygen—or rather its by-product, hydrogen peroxide—is toxic to many other bacteria. For these obligate anaerobes, the presence of molecular oxygen either inhibits growth or actually kills the microorganism. Obligate anaerobes vary widely in their tolerance to oxygen. Some kinds of anaerobes, such as methanogens, tolerate oxygen very poorly and must be cultured under strict anaerobic conditions. Other kinds of anaerobes, such as some sulfate reducers, can tolerate exposure to oxygen but generally cannot grow in its presence.

In many ground-water systems, oxygen is present in and near recharge areas, where oxygen is delivered with infiltrating rainwater. In these systems, aerobic microorganisms are the predominant species present. However, because of the heterogeneity of subsurface sediments, particularly the discontinuous distribution of organic carbon, small pockets, termed microenvironments, may be present that lack molecular oxygen. These anaerobic microenvironments may then become the focus of anaerobic metabolism. Thus, the presence of dissolved oxygen in ground-water systems does not generally exclude the presence of anaerobic metabolism.

3.3.4 pH

Bacteria in nature are capable of living in a surprisingly wide range of pH. Some bacteria, notably sulfide-oxidizing bacteria, are perfectly at home in an acidic pH

range of 3.0 to 4.0. This is not terribly surprising, in that sulfide-oxidizing bacteria produce sulfuric acid (H_2SO_4) as a by-product of their metabolism. Clearly, the ability to tolerate acid is a requirement of that sort of lifestyle. Other bacteria are quite capable of growth at alkaline pH of 10.0 or above. Many of these bacteria live in unusual environments, such as alkaline lakes, where such pH's occur naturally.

Regardless of the pH that they are adapted for, bacteria usually maintain an intercellular pH in the 7.5 range. Thus, in order for bacteria to live in environments where pH conditions are significantly different from 7.5, they must employ specialized proton-transporting mechanisms in order to remain viable. The nature of these mechanisms is of considerable interest to microbiologists, because, as we will see later, bacteria utilize proton gradients across their cell membranes in order to harness energy.

Actively growing bacteria often produce a variety of waste products that can significantly affect the pH of the surrounding environment. When laboratory media for growing bacteria are prepared, this fact is taken into account by providing pH buffers, such as carbonates or phosphates, that tend to stabilize the pH of the media.

Extremes of pH are not common in pristine ground-water systems. In systems in which there is active oxidation of sulfides, natural pH's may be as low as 4.0 or 5.0. But pH's that are lower than this are extremely rare. Similarly, very high pH's are not common in ground-water systems. In carbonate-buffered systems, pH's may be found as high as 9.0 but rarely higher. The relatively restricted range of pH's found in ground-water systems generally reflects the buffering effects of the framework carbonate or silicate minerals that make up the aquifers. Because of the relatively restricted pH range typical of ground-water systems, it seems unlikely that pH presents a significant problem to bacterial growth in pristine systems.

In ground-water systems contaminated by toxic wastes, more extreme pH conditions may be found. In aquifers contaminated by municipal waste leachates, which often contain elevated concentrations of organic acids, pH's as low as 3.0 have been reported (Baedecker and Back, 1979). Similarly, ground-water systems contaminated by some industrial wastes, such as sludges from cement manufacturing, may exhibit pH's as high as 11.0. In these cases, pH stress may indeed present a significant environmental problem for indigenous bacteria.

3.3.5 Osmotic Pressure

Cells can gain or lose water by osmosis, depending on the relative concentrations of dissolved constituents inside or outside the cell. If the outside of the cell has higher concentrations, the environment is said to be hypertonic. If the outside of the cell has lower concentrations, the environment is said to be hypotonic. If the concentrations are the same inside and outside, the conditions are said to be isotonic.

A cell in a hypertonic environment will tend to lose water into its surroundings, which could cause it to shrink and dehydrate. Conversely, a cell in a hypotonic environment will tend to gain water, which could cause it to lyse. Clearly, osmotic pressure is a critical condition that the cell must deal with in order to live and grow.

As discussed earlier, a primary function of the cell wall is to regulate the osmotic flow of water out of or into the cell. Not surprisingly, microorganisms that live in

exceedingly saline environments—the halophiles, for example—have cell walls that have much different properties than those found in more dilute aqueous environments.

Because subsurface environments include a wide range of salinities (from practically rainwater to 30% brine solutions), subsurface bacteria exhibit a wide range of tolerance to osmotic conditions. It is very important for investigators to take such conditions into account when they are designing the growth conditions of enrichment media. One particularly good example of why it is important to consider osmotic sensitivity was given by Wunsch (1988). Wunsch was studying water quality problems associated with coal seam aquifers in Kentucky. One common problem was abnormally high concentrations of dissolved barium in the ground water. In addition, there was often a characteristic "rotten egg smell" associated with the ground water. Wunsch knew that concentrations of barium in water are largely controlled by concentrations of sulfate. Because sulfate tends to precipitate barium as the mineral barite, any process that actively removed sulfate from the system would tend to increase concentrations of barium in the ground water. These two bits of evidence suggested to Wunsch that sulfate-reducing bacteria were present and active in the coal seam aquifers and that their activity was the root cause of the high barium concentrations in ground water. However, in order to show that this was the case, Wunsch needed proof that such bacteria were, in fact, present.

Wunsch approached a colleague who had worked extensively on sulfate-reducing bacteria in oil fields. The colleague assured him that the presence of such bacteria was easily shown simply by inoculating an appropriate growth medium with samples of the water in question. Furthermore, the colleague volunteered to prepare the media. Some weeks later, armed with 20 or 30 vials of the media, Wunsch sampled the wells in question, inoculated the sulfate-reducing media, and allowed them to incubate. After several weeks of incubation, not one of the vials showed any evidence of bacterial growth.

Puzzled, Wunsch returned to his colleague to inquire if he had perhaps inoculated the media improperly. During the course of the conversation, the colleague mentioned that the media contained about 100,000 mg/L total dissolved solids (TDS), which approximated the composition of the oil field brines that he was accustomed to. The water that Wunsch was studying, however, had chloride concentrations of less than 1,000 mg/L TDS. Could it be that the bacteria in Wunsch's system simply couldn't cope with the osmotic stress when transferred to the high-TDS media? In order to find out, the colleague made up a new batch of media and Wunsch dutifully sampled his wells again. This time, much to Wunsch's satisfaction, the sulfate-reducing bacteria *Desulfovibrio* grew from all but two of the wells that he sampled. Furthermore, the two wells recently had been chlorinated in order to minimize the hydrogen sulfide smell problem. The reason that *Desulfovibrio* hadn't grown before was that the microorganisms were incapable of dealing with the osmotic shock dealt them when they were placed in the high-TDS media. This incident clearly demonstrates the importance of matching the osmotic conditions of growth media to the osmotic conditions prevailing in the ground-water system under study.

3.4 TECHNIQUES FOR CULTURING BACTERIA

Suppose a fisherman goes to his favorite lake for a nice relaxing day of fishing. When he gets there, he decides that he'd like to bring home some nice fat bass for

his family to have for supper. Accordingly, he opens his tackle box and selects a lure that looks, acts, and moves like a crawfish—a favorite food of bass. Using this lure, he promptly gets a strike and in due course reels in a "lunker" bass. This is repeated until he has as many bass as his family can use.

Having accomplished his goal of catching bass, he decides that now what he needs is a stringerfull of bluegills in order to make a succulent fish chowder. So he removes the crawfish lure and replaces it with a lure that simulates a swimming insect—a favorite food of bluegills. Sure enough, as soon as he changes lures, he stops catching bass and begins catching bluegills.

The point here is that both types of fish were present in the lake, but in order to catch one or the other, the fisherman needed to use the proper bait for the particular fish he wanted to catch. This is very similar to the problem that microbiologists face when they are attempting to isolate a particular bacterial species from an environmental sample. One difference is that, instead of trying selectively to catch one species out of four or five like our fisherman, the microbiologist must typically differentiate between hundreds of microbial species. Culture media are the microbiologist's "lure" for "catching" (i.e., isolating) the particular species of interest.

3.4.1 Design of Growth Media

The first step in isolating a particular type of bacteria from an environmental sample is to select the appropriate culture medium. Culture media consist of at least five separate functional components. First, there must be a carbon source provided in a form that the bacteria are capable of assimilating. Second, the medium must contain a source of nitrogen so that the growing cells can synthesize proteins. Third, inorganic ions—phosphate, potassium, sulfur, trace metals, etc.—needed by the organism must be present. Fourth, the appropriate vitamins must be supplied. Fifth, the medium or the surroundings of the medium must contain an appropriate electron acceptor to allow the microorganism to respire (if it is a respiring microorganism).

Carbon Sources Microorganisms are capable of using a wide variety of carbon-bearing compounds as carbon sources for cell growth. Simple sugars, polysaccharides, organic acids, and proteins are the most common carbon sources that are utilized in preparing growth media. Glucose, the most common hexose (six-carbon sugar) in both the cell and in nature, is very commonly used as a carbon source in growth media. Glucose is a monosaccharide (Fig. 3.5a) and can be used by the cell as a biosynthetic precursor for many other sugars. Thus, by providing glucose in the media, the cell is able to synthesize many of the other compounds necessary for growth. Because glucose and other saccharides can be partially oxidized by fermentative bacteria without the reduction of external electron acceptors, they are sometimes referred to as fermentable substrates.

It is also common to provide disaccharides, sugars composed of linked monosaccharides, as fermentable substrates in growth media. For example, the disaccharide lactose (milk sugar) is composed of glucose and galactose connected by glycosidic linkages (Fig. 3.5b). In order to utilize dissacharides, the cell must first cleave the linkage connecting the monosaccharides, using specialized enzymes. For example, bacteria that are able to utilize lactose produce the enzyme beta-galactosidase. A simple method for discriminating strains of bacteria that produce

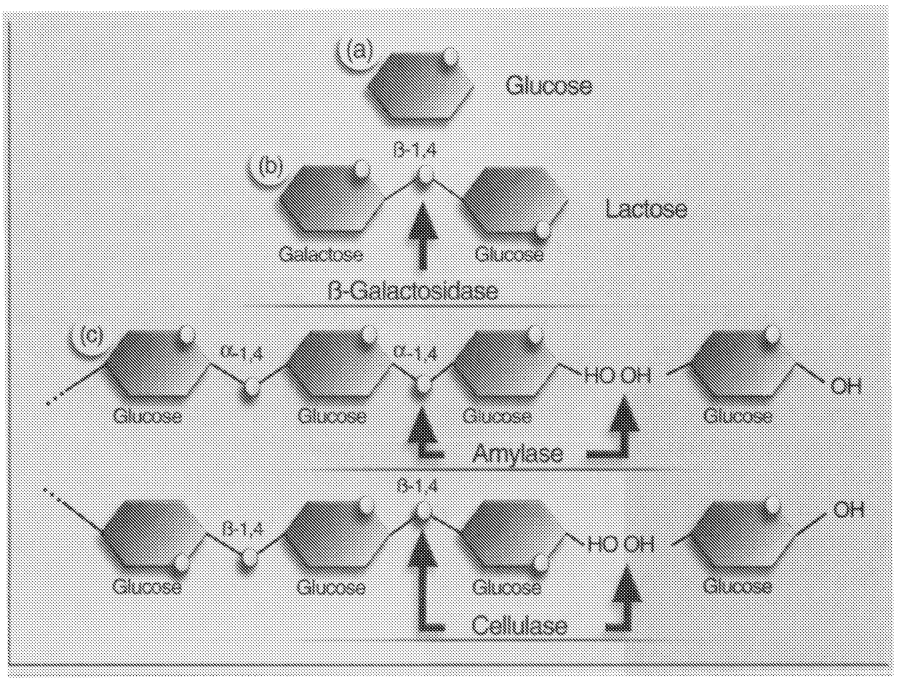


Figure 3.5. Structures of some common sugars, and the site of attack of particular degradative enzymes.

beta-galactosidase from strains that do not is to grow them alternatively on media containing glucose as a carbon source and then transfer them to media containing lactose as a carbon source.

Polysaccharides, or compounds formed by linking many monosaccharides (Fig. 3.5c), are a common substrate for microorganisms in nature and may be used in growth media. Bacteria capable of producing the enzyme cellulase, an enzyme that is specific for beta linkages between sugars, can easily be distinguished from those that produce amylase, an enzyme specific for alpha linkages, by using different polysaccharides in media. Because of their insoluble nature, polysaccharides are somewhat more difficult to work with and are not commonly used in preparing growth media.

Organic acids, such as acetate or formate, may also be used as carbon sources in growth media. Because fermentative bacteria cannot partially oxidize these compounds, they are sometimes referred to as nonfermentable substrates. Amino acids and proteins are other forms of carbon commonly used in growth media. Many pathogenic strains of *Streptococcus* have the capability of hydrolyzing proteins, whereas nonpathogenic strains of the same genus lack this capability. Growth on media containing proteins as a carbon source is sometimes used as a criterion for differentiating between these pathogenic and nonpathogenic strains.

For many environmental applications, it is of particular interest to determine if indigenous bacteria are capable of utilizing xenobiotics, or carbon compounds not commonly found in nature, as carbon sources. For example, carbon sources, such as petroleum hydrocarbons, phenolic compounds, or chlorinated hydrocarbons, may

be used in preparing growth media. This method has been used to demonstrate the presence or absence of petroleum hydrocarbon-oxidizing bacteria in field studies (Ehrlich et al., 1984). A more common method of demonstrating xenobiotic utilization, however, is through the use of microcosm experiments, which will be discussed later.

Nitrogen Sources Nitrogen is needed by microorganisms for protein and nucleic-acid synthesis. Nitrogen can be obtained from either organic or inorganic sources. The most common inorganic sources are ammonia and nitrate. When nitrate is used as a nitrogen source, it must first be reduced to ammonia. This reduction is catalyzed by the enzyme nitrate reductase, a molybdenum-containing flavoprotein. Ammonia is assimilated into the cell's metabolism with the mediation of glutamic dehydrogenase, which forms glutamic acid. Nitrogen for cell growth can also be provided in growth media from organic sources, such as amino acids or proteins.

Phosphorus and Inorganic Nutrients Phosphate is utilized by microorganisms for synthesizing nucleic acids. In addition, the energy currency of the cell is largely controlled by adenosine triphosphate (ATP). Thus, phosphate is an important component of growth media and is generally provided as a potassium salt (K_2HPO_4 or KH_2PO_4).

Potassium is universally required by microorganisms. Many enzymes involved with protein synthesis are specifically activated by potassium. As just mentioned, potassium is often provided in growth media as salts of phosphate.

Magnesium is required in order to activate certain enzymes. Magnesium also is involved in the production and activity of ribosomes, nucleic acids, and cell membranes. Gram-positive bacteria require about ten times more magnesium then typical gram-negative bacteria, an observation that probably reflects differences in cell membrane function. Although calcium and magnesium are closely related in the periodic table, calcium cannot replace magnesium for many functions. Calcium ions are important in the function of cell walls and in the production of bacterial endospores.

Sodium is not required by all bacteria, particularly bacteria adapted to freshwater environments. Many species of marine bacteria seem to have a requirement for sodium, however, and it is typically provided in culture media for marine environmental samples.

Iron is an essential trace nutrient for all microorganisms. Iron can exist in either metallic form, with an oxidation state of zero (Fe^o); in the ferrous state, with an oxidation state of plus two (Fe(II)); or in the ferric state, with an oxidation state of plus three (Fe(III)). Because iron is readily oxidized from Fe(II) to Fe(III) and reduced from Fe(III) to Fe(II), it is widely used as an electron carrier in cytochromes. It is therefore a critical component of the electron transport system of many microorganisms.

Other trace elements necessary for bacterial growth and therefore typically provided in culture media include copper, zinc, cobalt, manganese, and molybdenum. Molybdenum is a component of some important metal protein complexes, such as nitrate reductase. Cobalt is needed for the synthesis of vitamin B_{13}. Zinc is a structural component of certain enzymes in that it helps hold protein subunits together.

Manganese is an activator of some enzymes that act on phosphate-containing compounds and is a functional component in other enzymes.

Many microorganisms are not capable of synthesizing organic compounds necessary for their growth. These compounds, known as growth factors or vitamins, must be supplied from external sources. These growth factors are highly species-specific. For example, the fermentative bacteria *Clostridium butyricum* cannot synthesize biotin, which is necessary for carboxylation reactions. Similarly, the lacctobacilli and many other bacteria require riboflavin for their electron transport systems and vitamin B_{12} to assist with molecular rearrangement reactions. Metabolizing organic acids as an energy and carbon source requires pantothenic acid, which must be supplied externally for many bacteria. Microorganisms that require particular growth factors to be successfully cultured are termed fastidious. In culturing fastidious microorganisms in the laboratory, growth factors must be supplied in the culture media.

Electron Acceptors Microorganisms obtain energy by transferring electrons from electron donors, such as organic carbon, or reduced inorganic compounds, such as sulfides, to compounds that accept electrons. In fermentations, organic carbon acts as both the electron donor and the electron acceptor, producing a number of incompletely oxidized by-products. In respiration, electrons are transferred directly to inorganic compounds that act as terminal electron acceptors. Electron acceptors are compounds that are relatively oxidized and include molecular oxygen, nitrate, Mn(IV), Fe(III), sulfate, or carbon dioxide. Thus, in order for respirative microorganisms to grow in culture, one or more of these compounds must be provided in the culture media.

In order to grow aerobic or facultatively anaerobic microorganisms, oxygen is provided simply by culturing in a petri dish or a test tube that is open to the air. However, the presence of molecular oxygen often inhibits the utilization of other potential electron acceptors. To promote the growth using electron acceptors other than oxygen, media must be prepared under anaerobic conditions, or under conditions from which oxygen has been excluded. Nitrate and sulfate are generally provided to culture media as electron acceptors from salts, such as sodium nitrate or sodium sulfate. However, Mn(IV) and Fe(III) compounds are relatively insoluble and are often provided in a suitable solid form, such as colloidal hydroxides. Some soluble Fe(III) compounds, such as ferric citrate, may also be used.

Selective Growth Using Culture Media A culture medium that is designed to grow one particular type of microorganism to the exclusion of others is called a selective medium. Selective media are extensively used in medical microbiology in order to obtain the causative agents of disease. For example, sodium tetrathionate is toxic to the normal flora of the human intestinal tract, but it is not toxic to bacteria of the pathogenic genera *Salmonella* or *Shigella*. Thus, if these pathogens are suspected of causing intestinal disease, sodium tetrathionate is added to media used to culture stool specimens—a procedure that selectively excludes growth of the more numerous normal flora.

Another technique for separating bacterial species is by the use of differential media. Differential media contain dyes, pH indicators, or other components that make a colony of a particular bacterial strain look different from other strains. This

colony can then be selectively transferred to fresh media, effectively separating it from other strains that might be present. A common method of differentiating pathogenic from nonpathogenic strains of *Streptococcus,* for example, is to grow the suspect strains on a medium that contains red blood cells. Because pathogenic *Streptococcus* stains typically destroy red blood cells, a colony of pathogenic *Streptococcus* will form a clear zone around itself that can be readily distinquished from the red background of the blood agar.

Numerous other techniques can be used in order to grow selectively one kind of microorganism to the exclusion of others. Salts, antibiotics, specific enzyme inhibitors, the presence or absence of electron acceptors, and the use of different carbon sources are all tools that can be used in designing selective media.

Table 3.1 lists the formulas for two types of selective media commonly used in investigations of subsurface bacteria. Both media use acetate as a sole carbon and energy source. Because acetate is a nonfermentable substrate, these media will tend to exclude growth of strictly fermentative bacteria. However, medium S contains sulfate as a sole electron acceptor, whereas medium F contains poorly crystalline ferric oxyhydroxide as a sole electron acceptor. Thus, medium S will systematically exclude growth of microorganisms that require molecular oxygen, nitrate, or Fe(III) as terminal electron acceptors. Similarly, medium F will systematically exclude growth of microorganisms that require oxygen, nitrate, or sulfate for growth. A common use of such selective media is to evaluate the metabolic potential of bacteria in subsurface sediments (Lovley et al., 1990).

Another difference between these two types of media is the manner in which trace minerals and vitamins are provided. In medium F, which is known as a defined medium, each trace mineral or vitamin is added in known amounts. In medium S, vitamins and trace minerals are provided by adding yeast extract, a compound known to contain essential nutrients but in undefined amounts. In general, defined media are used in order to delineate the exact nutritional requirements of a microorganism. When microbiologists are dealing with microorganisms that have unknown nutritional requirements, they often use yeast extract as a catchall ingredient.

Finally, the two media differ in the manner in which they establish redox conditions. Medium F is flushed with a mixture of N_2:CO_2 from which all traces of oxygen have been removed. This effectively removes all oxygen from the medium, thereby establishing strict anaerobic conditions. In medium S, redox buffers (ascorbic acid, thioglycolate) are added to lower the redox potential of the medium.

3.4.2 Isolating Bacteria from Environmental Samples

Environmental samples, such as water from lakes, aquatic sediments, ground water, or sediments from aquifer systems, almost never contain just a single type of microorganism. Such environments contain mixed populations, of which each species fills a particular niche. The first problem in isolating bacteria from samples of these environments is to physically separate the strains present.

In practice, this separation is generally accomplished through a multistep procedure. First, a selective medium is used that allows or encourages growth of the particular kind of microorganism sought. Growth on the selective medium implies that such microorganisms are indeed present. However, since multiple strains may be present, a dilution procedure is used to separate the strains. It is important to

TABLE 3.1 Formulas for a Defined (Medium F) and Non-defined (Medium S) Media.

	Medium F (Selective for Fe(III) Reducers)	Medium S (Selective for Sulfate Reducers)
Electron donor (g/L)	Na · Acetate, 6.8	Na · Acetate, 6.8
Electron Acceptor (g/L)	$Fe(OH)_3$, 100	$NaSO_4$, 1.0 $MgSO_4$, 2.0
Minerals (g/L)	NH_4Cl, 1.5 NaH_2PO_4, 0.6 KCl, 0.1 $NaHCO_3$, 2.5 $CaCl_2$, 0.1 $MgSO_4$, 3.0 $MnSO_4$, 0.5 NaCl, 1.0 $FeSO_4$, 0.1 $CaCl_2$, 0.1 $CoCl_2$, 0.1 ZnCl, 0.13 $CuSO_4$, 0.001 $AlK(SO_4)_2$, 0.01 H_3BO_2, 0.01 Na_2MoO_4, 0.025 $NiCl_2$, 0.024 $NaWO_4$, 0.025	NH_4Cl, 1.0 KH_2PO_4, 0.5 $CaCl_2$, 1.0
Vitamins (mg/L)	biotin, 2.0 folic acid, 2.0 pyridoxin, HCl, 10.0 riboflavia, 5.0 thimaine, 5.0 nicotinic acid, 5.0 pantothenic acid, 5.0 B_{12}, 0.1 p-aminobenzoic acid, 5.0 Thioctic acid, 5.0	yeast extract, 3.5 g/L
Redox buffers (g/L)	none, purge with N_2:CO_2 to remove oxygen	Ascorbic acid, 1.0 Na · thioglycolate, 0.1
pH	Adjust to 7.0	Adjust to 7.0

remember that all culture media are selective to some degree, and that the lack of growth of particular microorganisms does not imply that those microorganisms are absent from the sample. It may simply mean that the culture medium is not appropriate for that organism.

For aerobic and facultatively anaerobic bacteria, by far the most common dilution procedure is the streak-plate method. In this method, the growth medium is solidified by addition of about 2% agar to the media. Agar is a polysaccharide gelling agent that is derived from certain kinds of algae. The agar medium mix is heated to dissolve the agar and then autoclaved, that is, sterilized by heating at 250°F under 15 atmospheres of pressure for 15–30 minutes, and then poured into

sterile petri dishes. When the medium is cooled it solidifies, providing a surface of nutrients on which aerobic bacteria may be grown.

Mixtures of bacterial strains can be diluted by taking a small amount of the inoculum, or sample to be diluted, and streaking it onto the agar medium using an inoculating loop (Fig. 3.6). The process of streaking the sample spreads individual cells over the medium. As these individual cells reproduce, they form colonies, in which each cell is identical to the first cell. This streak dilution procedure may be repeated several times in order to make sure that the isolate is indeed pure. The purity of the isolate is generally checked by making a gram stain and examining it under the microscope.

Mixtures of bacterial strains can also be separated by serial dilution in liquid media (Fig. 3.7). In this procedure, water or sediment is added to a tube of liquid medium, mixed, and then 1 milliliter (ml) of the mixture is transferred to 9 ml of fresh medium, making a 1:10 dilution. This dilution procedure is repeated until the original sample has been diluted as much as $1:10^{10}$. The goal is to dilute the sample until just a single cell is transferred to the next tube, resulting in a pure culture. In practice, this procedure of successive dilution must be repeated numerous times in order to obtain a pure culture. It is also common practice to combine the serial dilution with the streak dilution procedure in order to obtain pure cultures.

An example of how these techniques have been used to study bacteria from subsurface environments was given by Balkwill and Ghiorse (1985) in a study of a shallow water table aquifer in Lula, Oklahoma. Because the nutritional requirements of indigenous bacteria were largely unknown, three types of culture media were prepared. The first medium consisted of 10 grams (g) peptone, 5 g trypticase, 10 g yeast extract, 10 g glucose, 5 g $MgSO_4$, 0.6 g CaC_{12}, and 15 g agar per liter of distilled water. This medium is known as PTYG (peptone, trypticase, yeast extract, glucose) agar and is fairly rich in available nutrients. It contains several

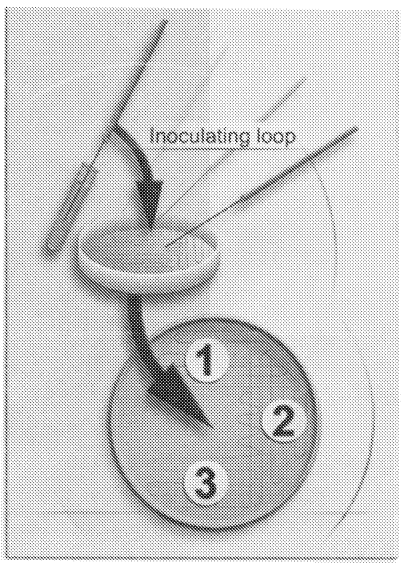


Figure 3.6. The streak dilution method of separating bacterial cells on solid growth media.

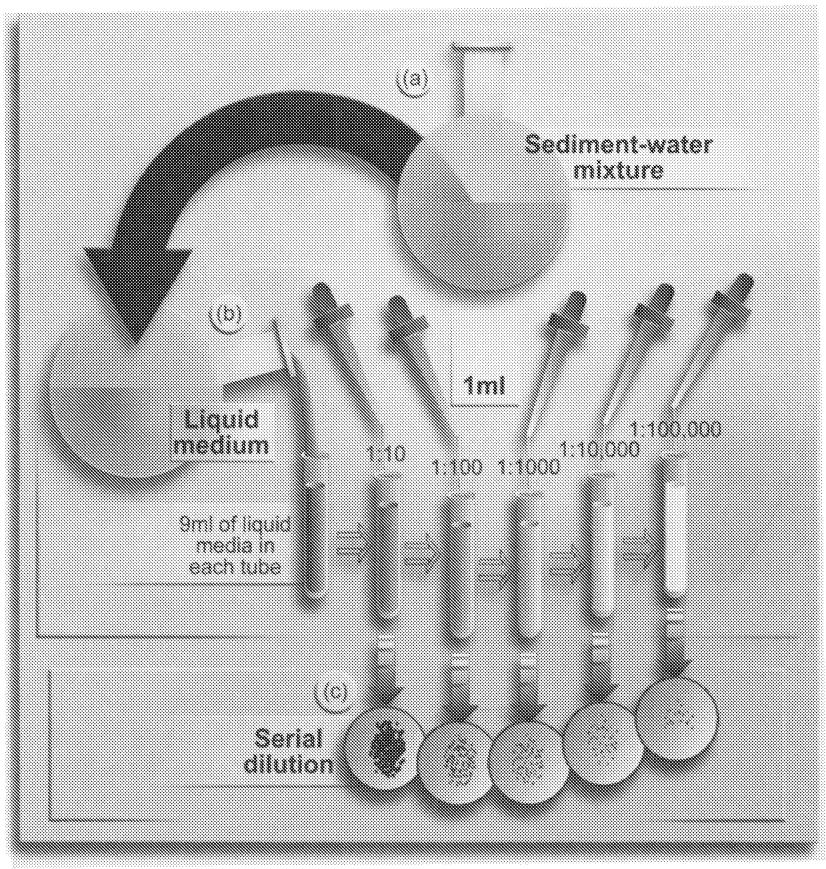


Figure 3.7. Serial dilution of bacterial cells in liquid media.

different carbon and energy sources, as well as trace nutrients. However, because the subsurface environment was thought to be relatively nutrient deficient, a 1:100 dilution of the PTYG agar was also prepared. Finally, because it was possible that the microorganisms were adapted specifically for the carbon sources available in the soil at that site, a growth medium was prepared in which the extract of the local surface soil (the liquid supernate left over after autoclaving a water soil suspension) was used as a carbon source.

These media were inoculated by making serial dilutions of the subsurface sediments and spreading them onto each type of medium. This procedure was performed in triplicate, and the inoculated plates incubated aerobically at about 25°C (the temperature of the local ground water). After two weeks, the plates were examined for colony growth, and selected colonies were transferred to fresh media. After several transfers, the bacteria were gram-stained in order to verify culture purity, and the strains subsequently were characterized.

This general procedure, with minor variations imposed by local conditions, has been widely used in order to characterize the microflora of subsurface environments (Chapelle et al., 1988; Sinclair and Ghiorse, 1987; Balkwill, 1989).

Rich Versus Dilute Media The components of culture media are just one of several important attributes that must be considered when microorganisms are cultured from environmental samples. Because subsurface environments are typically oligotrophic (nutrient deficient) compared with many surface environments, many investigators have suggested that dilute media are more effective for growing indigenous microorganisms than the rich media often used in the laboratory.

There is experimental evidence that this is indeed the case. In the Lula, Oklahoma, study just mentioned, sediments from the deeper saturated zone (~15 feet depth) contained bacteria that grew more readily on dilute PTYG agar and on soil extract agar than on the nutritionally rich full-strength PTYG agar. And, as before, this general pattern has been observed in other studies of subsurface microorganisms. In conditions of extreme nutrient deprivation, having cell walls and membranes that are very permeable to potential substrates would be a considerable advantage. Selection for that trait would therefore be expected. However, if these cells were transferred to very rich culture media, the permeable cell walls might be unable to protect effectively against osmotic shock, resulting in cell lysis or death.

The mechanisms leading to more efficient growth of some subsurface bacteria on dilute media have yet to be systematically explored. However, this trait must be taken into consideration by microbiologists when designing culture media for studies of subsurface bacteria.

3.5 ENUMERATING BACTERIA

Techniques for enumerating bacteria are widely used in laboratory studies of bacterial population growth. These techniques enable microbiologists to quantify rates of population growth under different environmental conditions. For example, the optimum pH range of a particular isolate may be delineated by setting up multiple tubes of liquid media that are identical in all respects except for pH. The tubes are inoculated at the same time, and concentrations of cells are enumerated over time. The optimum pH range for that particular organism is then clearly indicated by the observed rates of cell growth. In field studies of subsurface microorganisms, considerable resources have been applied to counting the number of bacteria present. These studies have generally borrowed techniques from soil microbiology for making cell counts.

There are two main types of cell-counting procedures. There are viable counts, which use culture techniques to estimate the number of cells capable of growth and reproduction. There are also direct counts, which attempt to enumerate all of the cells that are present, whether they are viable or not. As we shall see, each of these procedures gives different types of information, and neither one can give a "total" count of the microorganisms actually present. It is critical, when cell count data are evaluated, that the types of techniques used be carefully considered.

3.5.1 Viable Counting Procedures

By far, the most widely applied procedure for counting viable cells is the plate count. This procedure is applicable to both sediment samples and water samples,

although the details differ somewhat. For sediment samples, the most common protocol is to place sediment in a test tube with sterile distilled water and to shake the tube vigorously in order to dislodge bacteria attached to sediment surfaces. Sometimes chemicals, such as sodium pyrophosphate, are added to the sediment slurries in order to encourage bacteria to desorb from sediment surfaces, although the effectiveness of such chemicals is not well documented. Once the slurry has been prepared, it is serially diluted, and aliquots of each dilution are spread onto agar-based culture media and incubated. If the sediment contains large amounts of bacteria, then plates inoculated with a concentrated slurry will grow hundreds of colonies. Such high numbers of colonies cannot be reliably counted, and plates inoculated with more dilute slurry are enumerated instead. Generally, an ideal number of colonies to count on each plate is between 30 and 300. Because of sample nonhomogeneity, it is important to inoculate more than one plate for each sediment dilution. Typically, plates are inoculated in triplicate. Once the colony counts are made, the original sediment slurry is dried, weighed, and results reported in cells per gram of dry sediment plus or minus one standard deviation.

Enumerating cells in water samples using the plate count technique is identical to that just described, except that, of course, the bacteria do not need to be desorbed from sediment surfaces. A variation of the plate count technique that is often applied to water samples is called the membrane filtration method. In this method, the water sample to be counted is passed through a membrane filter under vacuum. The filter is then placed on an appropriate medium, and the trapped microorganisms are allowed to grow into colonies. These colonies are then counted. This technique is particularly well suited for water samples that contain low concentrations of bacteria because relatively large volumes of water can be filtered.

Another viable counting technique that is widely applied to sediment and water samples alike is the most-probable-number (MPN) technique. In this method, one milliliter of water sample is diluted into 99 milliliters of liquid growth media, giving a 1:100 dilution. The 1:100 dilution is itself then diluted 1:100 giving a 1:10,000 dilution. This procedure is repeated as many times as is appropriate, and the tubes are incubated. The media in tubes that received viable cells soon turn cloudy due to cell growth. However, the media in tubes that were diluted to the point that no cells were received remain clear. Thus, if growth is observed in the 1:10,000 tubes but not in the 1:1,000,000 tubes, there is a high probability that the concentration of cells in the original sample was greater than 10,000 cells per ml but less than 1,000,000 cells per ml. When this procedure is performed with an appropriate number of replicate tubes for each dilution (usually three), statistical procedures can be used to give a probable cell concentration in the original sample.

Several drawbacks to these viable procedures are immediately obvious. First, not all bacteria may be dislodged from sediment surfaces when samples are slurried. In fact, there is good evidence that many bacteria are not dislodged. Second, the only bacteria counted with these procedures are those that are capable of growth on the media and under the incubation conditions provided. Fastidious or slow-growing bacteria will be undercounted relative to nutritionally diverse and fast-growing microorganisms. Also, if the incubations are carried out under aerobic conditions, obligate anaerobes will not be counted with this procedure. Given these problems, which may differ from sample to sample, it is clear that plate counts give a minimum estimate as to the number of bacteria present.

3.5.2 Direct Counting Procedures

A widely used technique for counting bacteria in environmental samples is the direct count. This involves placing a known volume of a microorganism-containing liquid on a microscope slide and individually counting the number of cells present. This technique was developed for enumerating cells in water. However, it has been widely applied to sediment samples as well by attempting to desorb cells from sediment particle surfaces.

The most commonly applied direct-count method in subsurface microbiology studies is the acridine orange direct count, or AODC, method. When it is applied to sediment samples, cells are desorbed from sediment particles, as discussed earlier, and the cell-containing supernate is stained with acridine orange. Acridine orange is a fluorescent stain, so called because it fluoresces when subjected to ultraviolet light of the appropriate wavelength. The cells are then passed through a membrane filter and are enumerated using an epifluorescent microscope, which illuminates the sample with ultraviolet light so that the fluorescing cells can be observed visually.

In addition to allowing enumeration of cells, the AODC method gives some information as to the growth status of individual cells. Acridine orange binds to nucleic acids, both RNA and DNA, in the cell. When the cell binds to RNA, it emits a characteristic orange light—hence the name "acridine orange." When the cell binds to DNA, the emitted light tends to be somewhat greenish in color. Rapidly growing cells contain more RNA relative to DNA than inactive cells and therefore appear orange in color. Cells that are not growing rapidly contain more DNA than RNA and appear greenish in color. Thus, the AODC method can help the investigator judge if cells in environmental samples are rapidly growing or not.

A modification of the AODC method combines some of the features of viable counts with direct-counting procedures. In this method, cells in the sample to be counted are treated with nalidixic acid, an antibiotic that allows cellular growth but inhibits cellular division, then treated with nutrients to encourage cell growth, and incubated. Because viable cells can grow but not divide, they tend to enlarge enormously. For example, a micron-long cell might grow to be 10 microns in length under these conditions. Viable cells—those cells capable of active growth—therefore enlarge, whereas nonviable cells do not. This technique, which is called the direct viable count method (DVC), can be used to estimate what portion of a bacterial population is viable and what portion is nonviable. As with the viable cell-counting methods discussed earlier, however, this method can count only those "viable" cells that are capable of growth on the nutrients offered and under the given incubation conditions.

There are several practical limitations to the effectiveness of AODC and DVC procedures. The most serious has to do with separating the cells from the sediment. Because acridine orange is a cationic dye—that is, it has a net positive charge—it binds readily to negatively charged clay particles. If sediment samples contain a large amount of clay, staining of the suspended clays can render a sample uncountable. Just as troublesome, it is often difficult to distinguish a fluorescing clay particle from a bacterial cell, and so inaccuracies in the counts can be a significant problem. Finally, this procedure works best on sediment samples that contain anywhere from 10^6 to 10^8 cells per gram. Many sediments from the deep subsurface

exhibit cell abundances that are less than 10^6 cells per gram. Thus, these direct-counting procedures do not work well on many sediments. Most investigators who are working with subsurface sediments consider direct cell counts to be highly qualitative and not an unequivocal measure of microbial abundance.

Other direct-counting procedures that are widely used in microbiology laboratories but that have seen only limited use in studies of subsurface bacteria include electronic cell counters and turbidity measurements. Electronic cell counters were devised for routine counting of red blood cells in hospital laboratories. They are very fast and convenient, although somewhat expensive. Also, they tend to clog at high cell densities. Electronic counters measure the electrical resistance of fluid passing through a small (~5 micron) hole. As a cell passes through the hole, the resistance increases sharply, and this increase is recorded on a counting device. Given the volume of liquid passing through the instrument, and given the electrical "pulses" recorded, the number of cells can be quickly computed. As with most direct-counting methods, the counter cannot distinguish between mineral particles and cells. Thus, this method is not easily applied to counting cells in sediment suspensions.

Turbidity measurements are widely used in cell growth studies because they are simple, direct, and nondestructive. A cell suspension looks turbid because light is scattered by the cells in the liquid. The amount of light scattered is directly proportional to the number of cells in suspension. Thus, light passing through a cell suspension can be measured with a photometer and calibrated to cell numbers. However, as turbidity measurements are specific to the kind of medium being used and are specific to bacteria in pure culture, this technique is not generally applicable to environmental samples. It is used, however, to characterize the growth behavior of microorganisms isolated in pure culture from environmental samples.

3.6 CHAPTER SUMMARY

This chapter has described how individual bacterial cells and populations of cells grow. Most bacterial cells reproduce by binary fission, thus producing an exponential increase in cell numbers in the absence of limiting conditions. In nature, however, there are always limits on bacterial growth. These limits may be the lack of carbon, nitrogen, phosphorus, or inorganic nutrients in the environment. Similarly, environmental conditions, such as temperature, pH, or osmotic conditions, may be more or less favorable for growth of a given bacterial strain.

Techniques for culturing bacteria from environmental samples are based on reproducing optimum growth conditions for the strains of interest. Bacteria present in shallow ground-water systems are most efficiently grown on relatively dilute culture media, reflecting the oligotrophic conditions of most aquifers. A variety of techniques are available for enumerating bacteria in subsurface environments. Culture media techniques are most commonly used to estimate numbers of viable cells present, whereas direct microscopic techniques are generally used for obtaining total counts of cells.

QUESTIONS TO CONSIDER

1. Is the growth of bacteria in most ground-water systems in (a) exponential, (b) stationary, or (c) death phase? How could one tell the difference between stationary and death phase?
2. Do psychrophilic microorganisms respire more slowly at their temperature optimum than do mesophilic microorganisms at their temperature optimum? How could the answer to this question affect bioremediation in cold climates?
3. What are the differences between selective media and differential media for growing bacteria?
4. Why is it that plate counts or most probable number counts of microorganisms are generally lower than direct counts of bacteria?
5. How can acridine orange direct counts give a qualitative indication of how fast individual cells are growing?

CHAPTER 4

BACTERIAL METABOLISM

A reasonably precise definition of a living entity is "an unstable, organized complex, which maintains a steady-state condition through the constant utilization of energy." The ability to utilize energy in an organized way, therefore, is the hallmark of life processes.

The term metabolism denotes the complex series of energy-utilizing chemical reactions carried out by the cell. There are two broad types of metabolic processes. First, catabolism extracts energy from organic compounds by breaking them down into component parts, thereby releasing energy. Anabolism does just the reverse, using energy to build organic compounds by fitting the component parts together in the proper sequence. The metabolic functions of the cell include extracting energy from certain compounds, storing that energy temporarily within the cell, using the energy for construction of cell parts—synthesis of enzymes, nucleic acids, and polysaccharides—repairing damage, and generally maintaining the status quo. An enormous amount of energy is necessary in order support such extensive functions. Some bacteria, for example, are capable of metabolizing an amount of nutrients equivalent to their own weight in just a matter of seconds. In this chapter, the basic principles of energy utilization in bacterial cells are reviewed.

4.1 THERMODYNAMICS AND BACTERIAL METABOLISM

All energy transformations, in either living or nonliving systems, must conform to the laws of thermodynamics. The first law of thermodynamics is simply one of energy balance: In any chemical reaction, the amount of energy present in the reactants must equal the amount of energy in the products. For example, if one mole of glucose is completely oxidized, 686 kilocalories (kcal) of energy are produced along with carbon dioxide and water:

$$C_6H_{12}O_6 + 8O_2 \rightarrow 6CO_2 + 12H_2O + 686 \text{ kcal} \quad (1)$$

Thus, by the first law, a cell using one mole of glucose as an energy source can obtain not more than 686 kcal of energy.

This does not mean, however, that all of that 686 kcal of energy is available to the cell. The second law of thermodynamics states that some portion of the energy, called free energy, will be available to do work, whereas another portion, called entropy, will be unavailable to do work. Studies of energy utilization in living cells show that, of the 686 kcal released by oxidizing one mole of glucose, a maximum of only about 280 kcal is actually available as free energy and can be utilized by the cells. In other words, at least 60% of the energy released by glucose oxidation goes toward increasing entropy and is lost as heat.

The glucose oxidation reaction just discussed has a net release of free energy and is known as an exergonic reaction. Reactions that must absorb free energy in order to go to completion, such as ATP synthesis, are known as endergonic reactions. The amount of useful energy liberated or taken up during a reaction is called the Gibbs free energy change (ΔG) of the reaction. At standard conditions (i.e., all reactants present at concentrations of 1.0 M, pH equal to 7.0, one atmosphere of pressure, and temperature of 25°C), the Gibbs free energy of the reaction is denoted as ΔG_o. Energy-releasing exergonic reactions have a negative ΔG, whereas energy-consuming endergonic reactions have a positive ΔG.

Living cells combine, or couple, exergonic and endergonic reactions in order to drive useful life functions. This is accomplished by employing intermediate reactants that temporarily store energy obtained from an exergonic reaction. These intermediate reactants then transfer the energy to the site where the endergonic reaction takes place. For example, consider these two reactions:

$$A \rightarrow B \qquad \Delta G_0 = -100 \text{ kcal} \quad (2)$$

$$C \rightarrow D \qquad \Delta G_0 = 50 \text{ kcal} \quad (3)$$

Reaction (2) is exergonic and can proceed spontaneously. Reaction (3), however, requires energy input in order to proceed. By adding two intermediate reactants (X and Y), the energy released from reaction (2) can be used to drive reaction (3).

$$A + X \rightarrow B + Y \qquad \Delta G_0 = -20 \text{ kcal} \quad (4)$$

$$C + Y \rightarrow D + X \qquad \Delta G_0 = -30 \text{ kcal} \quad (5)$$

In reaction (4), 80 of the 100 kcal of free energy available from reaction (2) is used to convert X to Y. This results in an exergonic reaction that can freely proceed but also effectively stores 80 kcal in compound Y. When compound Y is added to reaction (3), the overall free energy of reaction becomes (−80 + 50), or −30 kcal, again creating another exergonic reaction, but one that synthesizes compound D. Thus, by coupling exergonic and endergonic reactions through the use of energy-storing intermediate reactants, it is possible to synthesize compounds that otherwise could not be synthesized.

This reaction-coupling strategy is the basis for metabolism in living cells. Energy is derived from catabolic (exergonic) reactions, stored in high-energy intermediate compounds, and used in anabolic (endergonic) reactions that allow the cell to grow. Clearly, the energy-storing intermediate reactants are fundamentally important to the cell, and these will be considered next.

4.2 ATP SYNTHESIS—STORING ENERGY

Living cells use a number of compounds for temporarily storing energy and for using this energy to couple exergonic and endergonic reactions. These energy-storing compounds include adenosine triphosphate (ATP), guanosine triphosphate (GTP), acetyl-coenzyme A, and others. By far, the most important of these is ATP. ATP performs very much the same function in the cell that money plays in an economy. It is the "currency" by which all or most metabolic "transactions" are valued. The formation of ATP stores energy, and the hydrolysis of ATP releases energy.

The basic reaction in forming ATP is shown schematically in Figure 4.1. In this reaction, adenosine diphosphate (ADP) acquires an additional phosphate group to form ATP. This type of reaction is called phosphorylation and requires energy input. This energy is stored in the phosphate bond, which is sometimes referred to as a high-energy bond. The reverse of this reaction, which involves breaking the high-energy bond, liberates energy.

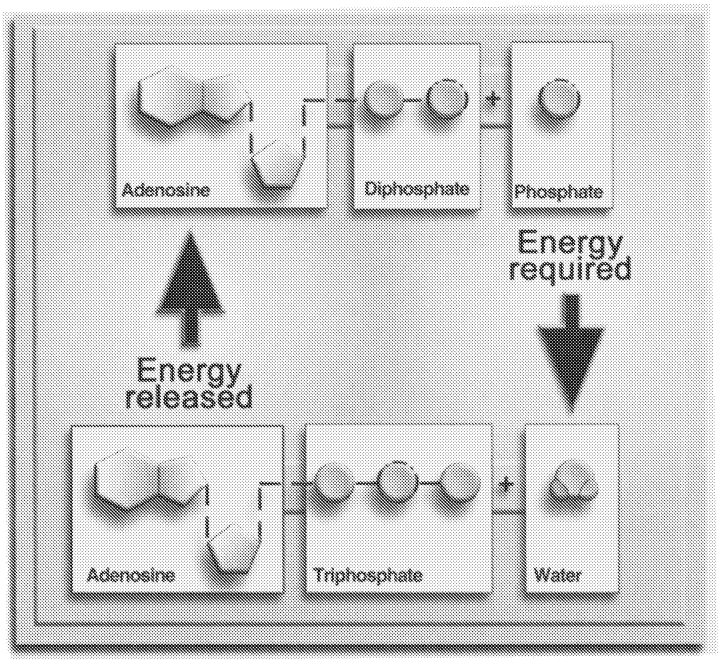


Figure 4.1. Formation of ATP from ADP with the storage of energy.

In addition to using intermediate compounds for storing energy, living cells also use intermediate compounds for storing electrons that are generated in energy-releasing oxidation reactions. Enzymes that remove electrons from energy-rich compounds (called dehydrogenases) often have electron-storing intermediate compounds as their coenzymes. One of these coenzymes is called nicotine adenine dinucleotide (NAD). NAD can exist in a reduced ($NADH + H^+$) or oxidized (NAD^+) form, with the electron-transferring reaction written as

$$NAD^+ + 2H^+ + 2e^- = NADH + H^+ \tag{6}$$

Because ATP is an intermediate compound in an overall energy-releasing oxidation reaction, actual phosphorylation of ADP occurs only in the larger context of electron-transferring, energy-releasing oxidation reactions. Consider, for example, the oxidation of glucose to ethanol and carbon dioxide:

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2\ CO_2 \tag{7}$$

This overall reaction is exergonic and releases 57 kcal of energy per mole of glucose oxidized. How does the cell store and utilize this energy? The cell performs this process in stepwise fashion, using ATP and reduced NAD as intermediates.

The steps involved in one pathway, or sequence of biochemical processes, used by cells to oxidize glucose are shown in Figure 4.2. First, glucose is phosphorylated by ATP to form glucose-6-phosphate. This initial phosphorylation activates the glucose so that the subsequent reactions become possible. Glucose-6-phosphate undergoes an isomerization, or rearranging, reaction to form fructose-6-phosphate. This molecule is again phosphorylated to form fructose-1-6-diphosphate. This molecule is then split, through the mediation of the enzyme aldolase, forming two glyceraldehyde-3-phosphate molecules. Note that, up to this point, there has been no oxidation and no energy produced. In fact, two molecules of ATP have been spent. Now the first oxidations take place, with glyceraldehyde-3-phosphate being converted to 1,3-diphosphoglyceric acid and with NAD accepting two electrons. In this step of the reaction sequence, a new high-energy phosphate bond is produced. The energy in this bond is then transferred and stored in ATP by forming 3-phosphoglyceric acid. Another ATP molecule is synthesized later in the reaction sequence when phosphoenol pyruvic acid is converted to pyruvic acid.

This sequence of reactions, ending with the synthesis of pyruvic acid (Fig. 4.2) is called glycolysis or the Embden-Meyerhof-Parnas (EMP) pathway, and it is basic to the metabolism of many microorganisms. The overall reaction of the glycolytic pathway is

$$\begin{aligned} \text{glucose} + 2\text{phosphate} + 2\text{ADP} + 2\ \text{NAD} \rightarrow \\ 2\ \text{pyruvate} + 2\ \text{NADH} + 2CO_2 + 2\text{ATP} \end{aligned} \tag{8}$$

Note that even though two ATPs were needed to initiate the reaction sequence, the cell receives a net gain of 2 ATPs. This mode of generating ATP is fermentative—that is, electrons are not transferred to a mineral electron acceptor (such as oxy-

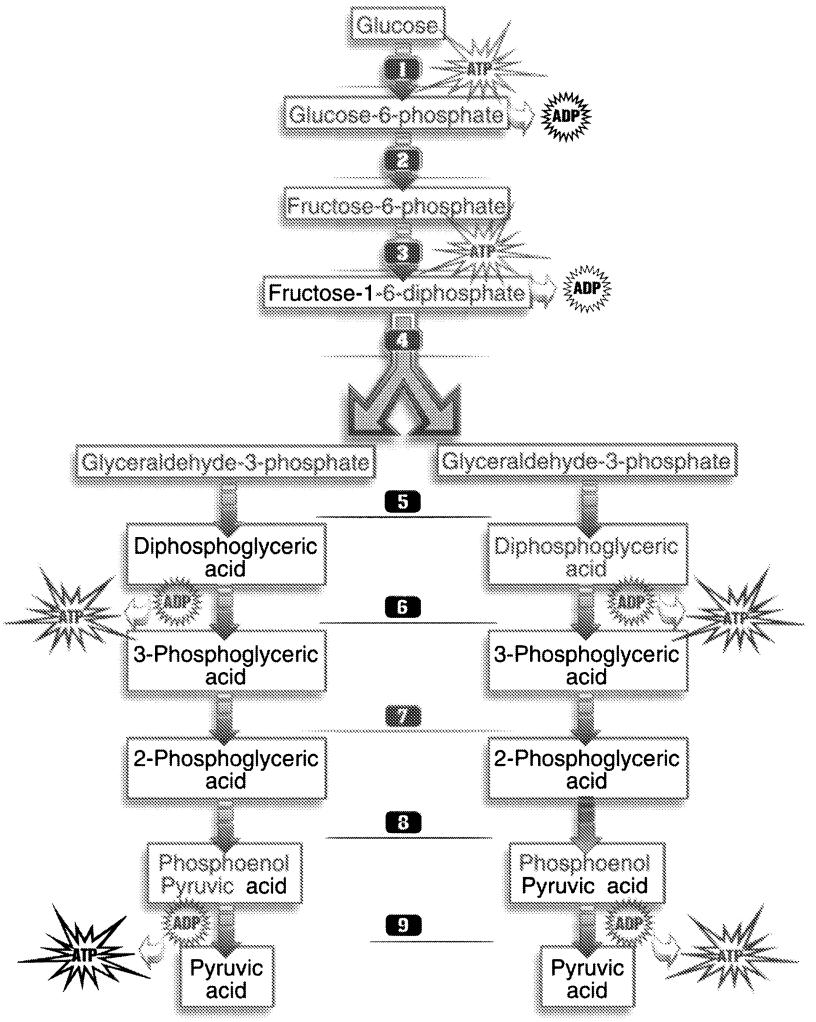


Figure 4.2. The Embden-Meyerhof-Parnas (EMP pathway) for the partial oxidation of glucose.

gen)—and is referred to as substrate-level phosphorylation. This distinguishes it from respirative pathways that employ electron transport systems in ATP synthesis. Electron transport systems will be discussed in the next section.

4.3 ELECTRON TRANSPORT SYSTEMS—RELEASING ENERGY

In the glycolysis pathway discussed earlier, 1 molecule of glucose yields 2 molecules of ATP. This is only a fraction of the free energy actually available in glucose. If all of the free energy available from glucose oxidation is used, 38 molecules of ATP could be synthesized. However, in order for complete oxidation to occur, there

must be materials available to accept the electrons generated. Inorganic compounds that can accept electrons and can allow complete oxidation of organic substrates are called electron acceptors. The most common electron acceptors used by microorganisms in natural environments are oxygen, nitrate, ferric iron, sulfate, and carbon dioxide. Other oxidized compounds that are abundant in unusual natural environments, such as manganate, selenate, and uranyl anions, also can act as electron acceptors for microorganisms. The process of coupling oxidation of organic chemicals with the reduction of external electron acceptors is called respiration.

In order to transfer electrons from organic molecules to electron acceptors, cells employ electron transport systems. These systems, as the name implies, physically carry electrons. More important, however, electron transport systems are able to conserve some of the energy released in oxidation-reduction reactions by synthesizing ATP.

Electron transport systems in microorganisms consist of hydrogen carriers, such as flavoprotein and coenzyme Q, and electron carriers, such as cytochromes and iron-sulfur proteins. There are several different cytochromes, designated cytochrome a, b, c, and d, respectively. Each of these cytochromes have slightly different electronegativities (affinity for electrons) so that electrons can be passed from one to the other sequentially. Cytochromes and flavoproteins are associated with each other within the cell at sites called electron transport particles. At these sites, electrons are efficiently passed from molecule to molecule in an organized fashion.

An example of an electron transport chain, that utilized by *E. coli,* is shown in Figure 4.3. In the cytoplasm, an NADH molecule is oxidized to NAD with the concurrent reduction of a flavoprotein in the cell membrane. The flavoprotein then

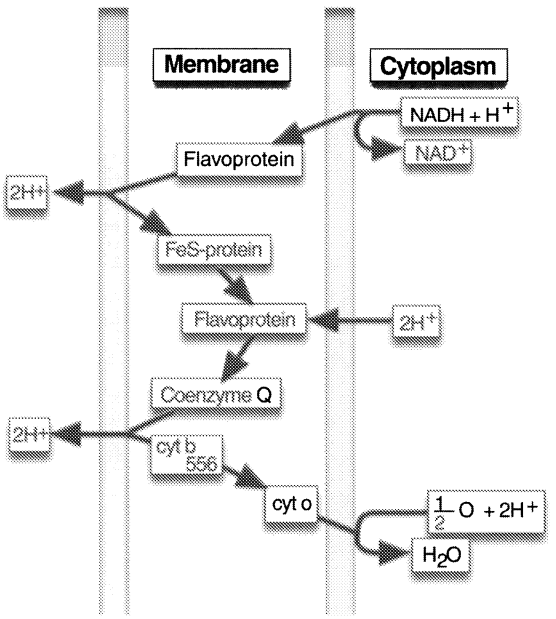


Figure 4.3. The electron transport system of *E. coli.*

transfers two H^+ ions out of the cell and reduces an FeS protein. The FeS protein transfers the electrons back to a flavoprotein, which then picks up two more H^+ ions from the cytoplasm. The electrons and H^+ are then carried out of the cell membrane by coenzyme Q with the reduction of cytochrome b556. Cytochrome b556 transfers its electrons to cytocrome o, which carries the electrons back to the cytoplasm. The last step in this chain of processes occurs in the cytyoplasm, with the transfer of electrons from cytochrome o to molecular oxygen, forming water. The energy released by this series of oxidation-reduction reactions performs work by transporting H^+ ions out of the cell. The potential energy stored by these H^+ transport processes is then converted into ATP by the cell using chemiosmosis, which will be discussed in the next section.

When NADH is the electron donor and oxygen is the electron acceptor, about three ATPs are generated for each oxygen atom and each NADH molecule. Thus, processing NADH through an electron transport system enables the cell to capture and to use far more of the energy present in glucose than would be the case with substrate-level phosphorylation.

4.4 CHEMIOSMOSIS—HARNESSING ENERGY FROM ELECTRON TRANSPORT

The electron transport mechanisms involved in energy production (Fig. 4.3) show schematically how energy is released by moving electrons in sequences of oxidation reduction reactions. However, the electron transport diagram does not explain the mechanism by which energy thus released is utilized by the cell. This question—perhaps the most important in microbial metabolism—took more than 40 years of determined investigation to answer. By the mid-1980s, however, it became clear that cells employ a mechanism called chemiosmosis to convert electrical energy (i.e., electrons moving through the electron transport system) to chemical energy (ATP).

Figure 4.4 shows the essential characteristics of the chemiosmosis model. As electrons move through the electron transport chain, their energy is used to transport protons from inside the cell membrane to outside the cell membrane. This active transport of protons sets up a proton gradient between the outside and inside of the cell membrane. Like any osmotic gradient, this proton gradient has potential energy associated with it that is termed the proton motive force. The cell captures this potential energy through the use of complex membrane-bound enzymes that are collectively referred to as the ATP synthase complex or, simply ATP-ase. The ATP-ase complex acts much like a water gate through which running water is used to drive a waterwheel. As protons diffuse through the ATP synthase complex, the potential energy is captured and stored chemically as ATP. In the case of aerobic respiration, the electrons are transferred to molecular oxygen, forming water.

4.5 THE ROLE OF ENZYMES

We have seen that microorganisms must conform to the laws of thermodynamics when they are carrying out metabolic processes. Only reactions with a negative

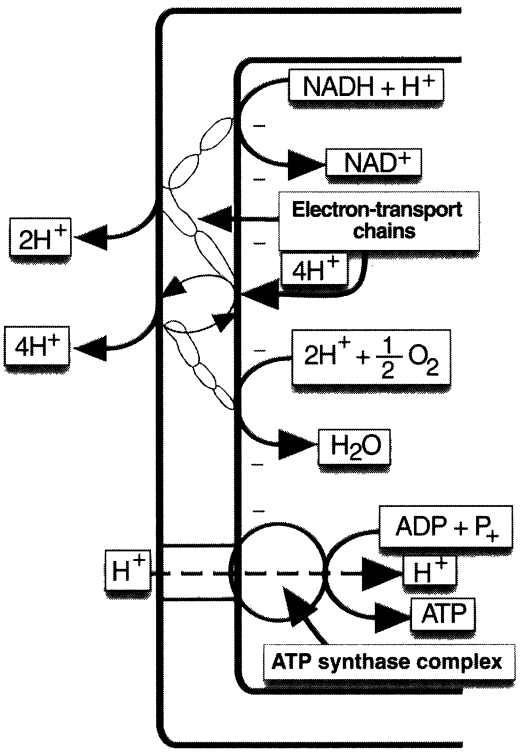


Figure 4.4. The chemiosmosis model of ATP synthesis. (Modified from Gottschalk, 1986, with permission courtesy of Springer-Verlag).

free energy occur spontaneously and can be used by the cell. Reactions with a positive free energy can be made to occur spontaneously, however, by coupling them with energy-releasing reactions through energy-storing intermediate compounds such as ATP.

Thermodynamics determines which reactions are energetically favorable and which are not. However, the rate at which the reactions are actually carried out does not depend on free energy change but, rather, on reaction mechanism. For example, if one were to mix glucose with oxygenated water in the laboratory, these chemicals would not immediately react to form CO_2 and water, even though that reaction is energetically favorable. At the low temperatures in the laboratory (25°C), collisions of oxygen and glucose molecules occur too infrequently and without enough force for the reaction to proceed very quickly. At a higher temperature, say 1000°C, this reaction proceeds very quickly. But, at the temperatures favored by living cells, glucose oxidation simply does not occur quickly enough by itself to be of much use in energy production.

Living systems, which depend on hundreds of chemical reactions per second in order to sustain life, solve this problem through the use of enzymes. Enzymes are proteins that catalyze chemical reactions in living cells. They work by combining chemically with a substrate or a combination of substrates and by bringing them into a configuration so that a particular reaction may occur. For example, we've

seen that, if glucose and oxygen are chemically mixed in the laboratory at room temperature, oxidation proceeds at an immeasureable rate. However, if glucose oxidase, an enzyme that brings glucose and oxygen together so that they can react, is added to the solution, glucose is quickly oxidized to gluconic acid with the production of hydrogen peroxide. The mechanism of this reaction is similar to many enzymatically catalyzed reactions. First, glucose, oxygen, and water combine on the surface of glucose oxidase to form a complex:

$$\text{glucose} + O_2 + H_2O + \text{glucose oxidase} \rightarrow$$
$$\text{glucose-}H_2O\text{-}O_2\text{-glucose oxidase complex}$$

By binding with the glucose oxidase, the three reactants are brought together in a configuration that allows them to combine chemically. Once they have combined, they no longer "fit" on the surface of the enzyme, and the products are released:

$$\text{glucose-}H_2O\text{-}O_2\text{-glucose oxidase complex} \rightarrow$$
$$\text{glucose oxidase} + \text{gluconic acid} + H_2O_2$$

This general behavior is common to many enzymatically catalyzed reactions and is often summarized by the equations

$$\underset{\text{enzyme}}{E} + \underset{\text{substrate}}{S} \rightarrow \underset{\text{enzyme-substrate complex}}{ES}$$

and

$$ES \rightarrow \underset{\text{enzyme}}{E} + \underset{\text{products}}{P}$$

Inspection of these equations suggests that the rate of reaction will be sensitive to a number of factors. First of all, if the concentration of substrate increases, one would expect that the rate of reaction would increase. However, because the reaction rate is also dependent on the amount of enzyme present, it can only increase until all of the available enzyme is fully employed. This behavior is called saturation kinetics, referring to the fact that as substrate concentrations become large, all of the enzyme is fully utilized (saturated) and reaction rates can no longer increase (Fig. 4.5).

This behavior can be expressed mathematically by the Michaelis-Menton equation, which may be written in the form

$$v = V_{max}S/(K_m + S)$$

where K_m is the substrate concentration at which reaction rate (v) is half of the maximum rate (V_{max}). This equation states that the reaction rate will increase in proportion to increasing S up to a particular point, at which saturation is achieved and the reaction rate becomes constant (Fig. 4.5).

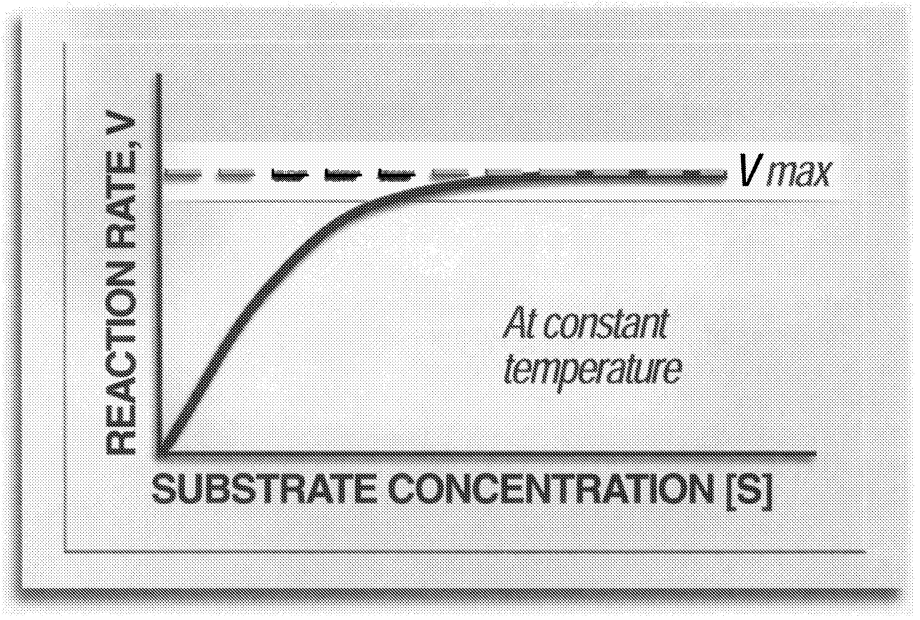


Figure 4.5. The dependence of enzymatic reaction rates on substrate concentration.

Enzymes are classified according to the kinds of reactions that they catalyze. Oxidases and reductases catalyze oxidation reduction reactions, lyases remove functional groups from organic compounds, and transferases move functional groups from molecule to molecule. Hydrolases hydrolize polymers that are linked together by covalent bonds. Ligases perform the reverse of this function by combining monomers together into polymers. Ligases may also be called polymerases.

Enzymes are also sometimes classified on the basis of where in the cell they are used. Enzymes that are used in the cytoplasm are termed cytoplasmic enzymes. Similarly, enzymes that occur in the cell membrane may be referred to as membrane-bound enzymes. Many cells, particularly gram-positive cells, produce exoenzymes, which are passed out of the cell to help break up potential substrates into components that can then be transported into the cell.

Many enzymes require a nonprotein component, called a coenzyme, in order to become active. These coenzymes often contain vitamins or nucleotides, and their special function is often to transfer components from the enzyme to the substrate. NAD, which we've discussed earlier, is a coenzyme that transfers electrons and protons. ATP (in addition to storing chemical energy) is a coenzyme that transfers phosphate groups, and coenzyme A transfers acetyl groups. Some enzymes also require the presence of cofactors, which are inorganic ions, such as iron or magnesium. Such cofactors often form the reactive center of the enzyme and assist in bringing the substrates together in the proper configuration so that reaction occurs.

The regulation of enzymatic activity is crucial to the cell, and this is accomplished by a number of mechanisms. If the cell encounters a potential substrate, enyzmes specific for that substrate can be induced, or newly manufactured, to utilize that substrate. The classic example of enzyme induction is that of the lactose

operon of *E. coli*. An operon is that portion of the cell's DNA that codes for specific proteins. In the absence of lactose in the cell, a protein molecule, called a repressor protein, covers the lactose operon and prevents the expression of three enzymes needed to break down lactose. However, in the presence of lactose, lactose binds with the repressor protein and removes it from the lactose operon. This allows expression of the genes and results in the production of the necessary enzymes. However, once lactose has been used up and the cell no longer needs the enzymes, the repressor protein recombines with the lactose operon, and enzyme production stops.

Inductive-repressive regulation of enzymatic activity is very efficient from the standpoint of optimizing the cell's resources. In the case of the lactose operon, it is inefficient for the cell to spend energy continually in order to produce lactose-utilizing enzymes, unless lactose is actually present. However, induction takes time. The characteristic lag time observed when cells are placed in a medium to which they are not adapted is due in part to time required for induction of enzymes to utilize the new substrate. In order to respond quickly to environmental changes, other enzyme-regulating strategies are also employed.

In addition to induction and repression, the activity of enzymes can be altered in the cell by a number of other strategies. For example, if the enzyme encounters a molecule that resembles its substrate, the enzyme might bind the "imposter" to its active site rather than to the normal substrate. Because the imposter molecule competes with the normal substrate, the activity of the enzyme decreases. This is termed competitive inhibition. Alternatively, an organic molecule might bind a non-active site of an enzyme in such a way as to change its shape. If this change of shape is sufficient, the enzyme may be inactivated. This is called noncompetitive inhibition.

Rates of biochemical processes in cells can also be regulated by altering the shape, and therefore the activity, of the enzyme. Binding sites on enzymes that are involved in regulating enzyme activity are called allosteric effector sites. In some cases, the end product of an enzymatic reaction sequence binds with an enzyme and acts as an allosteric inhibitor. This strategy allows the cell to shut off production of a particular product quickly without having to repress enzyme production at the genetic level. This type of regulation is called feedback inhibition and is widely used to regulate biochemical pathways. Alternatively, the allosteric binding site may serve to activate a particular enzyme. This is referred to as feedback activation. Both feedback inhibition and feedback activation are widely used as enzyme-regulating strategies in cells.

Regulating the activity of enzymatic reactions is crucial to the cell. The goal is to match the supply of enzymes to the supply of substrates in order to maximize metabolic efficiency. Enzyme activity is closely regulated by balancing enzyme production with enzyme need and by employing feedback inhibition and feedback activation strategies. Because of the dynamic nature of enzyme activity regulation, the cell is able to adjust to rapidly changing conditions in its environment.

4.6 ENERGY-RELEASING PATHWAYS OF GEOCHEMICAL IMPORTANCE

We have seen that microorganisms are able to obtain energy for cell growth by coupling endergonic and exergonic reactions into metabolic pathways. In the ab-

sence of an external electron acceptor, fermentative pathways, such as glycolysis, may be used. When electron acceptors are available, microorganisms employ electron transport systems and chemiosmosis to oxidize organic compounds completely to CO_2 and to store the energy obtained as ATP.

There are hundreds of known metabolic pathways, painstakingly worked out over the years, by which microorganisms produce and transform energy. In this section, some of these pathways of particular importance to microbial processes in ground-water systems are considered.

4.6.1 Lactate and Acetate Fermentations

As illustrated in Figure 4.2, the principal end product of glycolysis is pyruvate. This pyruvate may then be partially oxidized to a number of organic compounds with the production of additional ATP. The variety of these fermentations is virtually endless, and some of the possible end products are shown in Figure 4.6. Of these pathways, the production of formate, acetate, and lactate are important in ground-water geochmemistry, as these products are widely used by respirative bacteria.

One pathway by which both acetate and lactate are produced is known as the *Bifidum pathway* and is shown schematically in Figure 4.7. This pathway is named after the microoganism, *Bifidobacterium bifidum,* that carries out this particular fermentation. First, glucose is phosphorylated to fructose-6-phosphate, as in glycolysis. One molecule of fructose-6-phosphate is then split into acetyl-phosphate and erythose-4-phosphate. The acetyl-phosphate is then converted to acetate with the production of ATP. The remaining molecules of fructose-6-phosphate and erythose-4-phosphate are then combined and led through a number of intermediate steps to produce more acetyl-phosphate and glyceraldehyde-3-phosphate. The acetyl-phosphate produces more acetate, whereas the glyceraldehyde is oxidized to

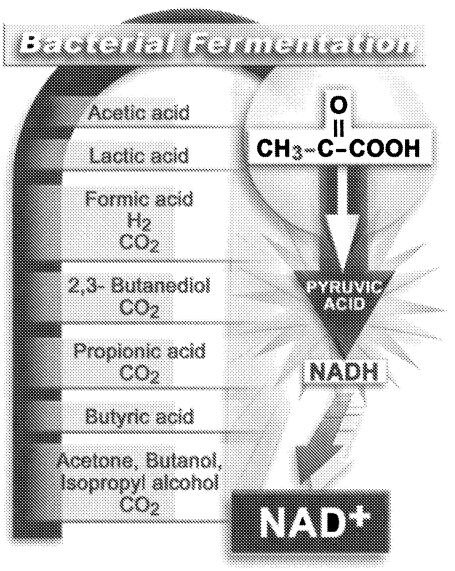


Figure 4.6. Some of the many possible end products of bacterial fermentations.

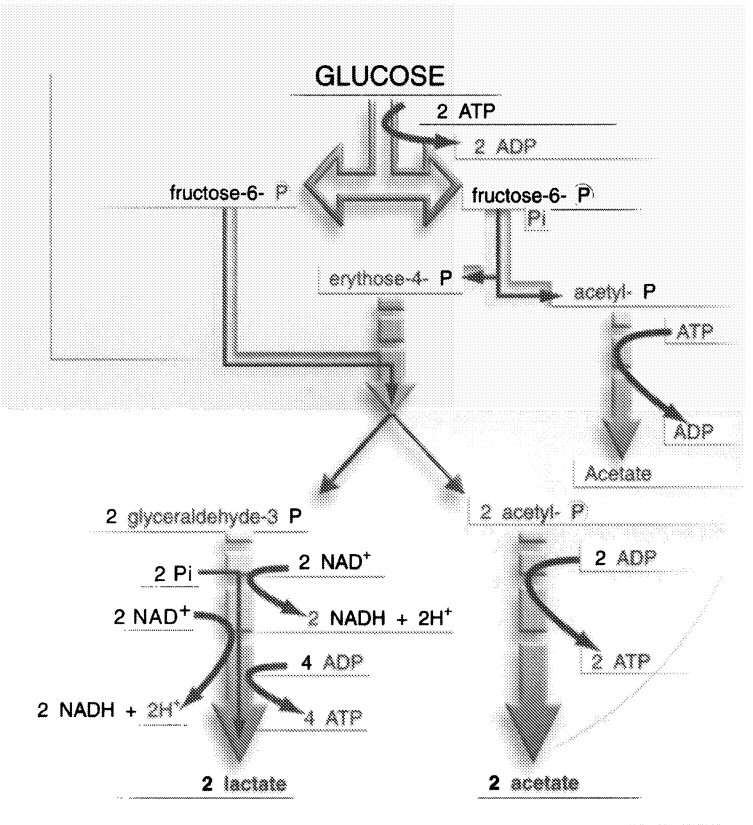


Figure 4.7. Production of acetate and lactate via the Bifidum pathway. (Modified from Gottschalk, 1986, with permission courtesy of Springer-Verlag).

lactate, both with the production of ATP. In this fermentation, therefore, three moles of acetate and two moles of lactate are produced for each two moles of glucose.

4.6.2 Ferredoxins and the Production of Hydrogen and Acetate in Fermentation

Hydrogen is an important substrate for many anaerobic bacteria, particularly Fe(III) reducers, sulfate reducers, and methanogens. Hydrogen is produced in a number of fermentations, notably by members of the genus *Clostridium*. In order to carry out these hydrogen-producing fermentations, the clostridia employ ferredoxins. Ferredoxins are iron-sulfur proteins that serve as electron carriers. They have the unusual property that the redox potential of the reduced-oxidized ferredoxin couple is virtually identical to that of the H_2/H^+ couple. Thus, even in an environment that is saturated with hydrogen gas, reduced ferredoxin can transfer electrons to hydrogenase and hydrogen can be evolved. This enables the clostridia to continue fermentations in extremely reducing environments.

The first step in these hydrogen fermentations is the production of pyruvate through glycolysis. Pyruvate then goes through a multistep process by which acetyl-phosphate and hydrogen are formed. This multistep process, shown in Figure 4.8, is known as the phosphoroclastic reaction. First, pyruvate is decarboxylated by pyruvate-ferredoxin oxidoreductase and combined with thiamine pyrophosphate-containing oxidoreductase (TPP-E) to form a hydroxyethyl-TPP-E complex (HETPP-E). HETPP-E then reduces ferredoxin with the production of acetyl-coenzyme A. Hydrogen gas is then evolved, and acetyl-coenzyme A is phosphorylated to acetyl-phosphate.

These types of hydrogen-producing fermentations are very important in the carbon and electro flow of anaerobic aquifer systems. It is common for anaerobic sediments collected from deep aquifer systems to exhibit hydrogen production when incubated in the laboratory (McMahon and Chapelle, 1991).

Some organisms produce acetate as their predominant fermentation product. These acetogenic bacteria are possibly important to anaerobic metabolism in deep aquifer systems, because much of the carbon flow goes through acetate. As with the hydrogen-producing bacteria, ferredoxins serve as important electron carriers.

An acetate fermentation pathway is shown in Figure 4.9. Again, pyruvate from glycolysis is the starting material. First, pyruvate is oxidized to acetyl-coenzyme A, which is subsequently used to produce ATP, with acetate as the end product.

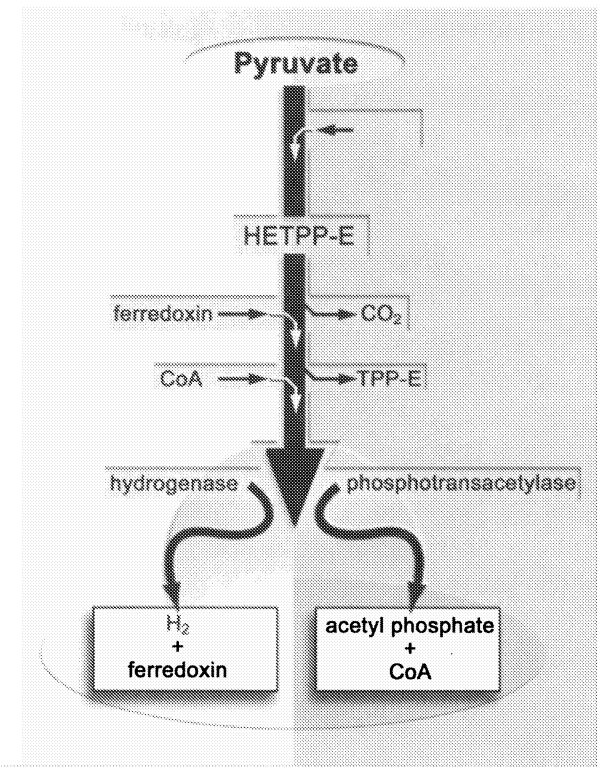


Figure 4.8. Production of molecular hydrogen from pyruvate.

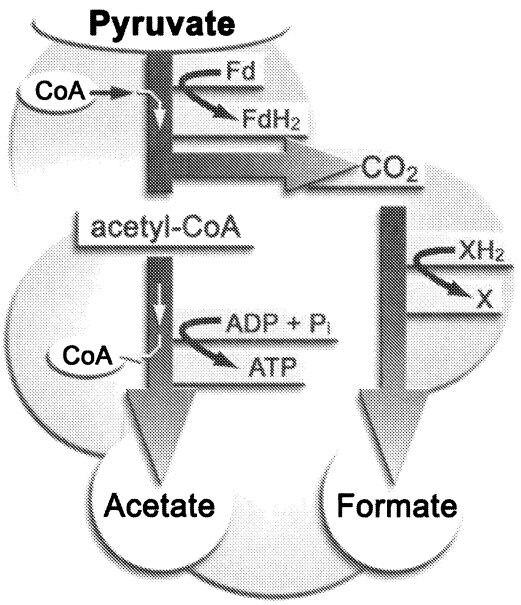


Figure 4.9. Production of acetate and formate from pyruvate.

Carbon dioxide liberated by this reaction may be combined with hydrogen to form formate as an intermediate product. The enzyme that catalyzes this reaction, formate dehydrogenase, is structured to produce formate and not just oxidize it, like the membrane-bound formate dehydrogenase found in many other organisms.

Formate may be further cycled through a number of steps with the formation of methyltetrahydrofolate. This molecule combines with a vitamin B_{12}-based enzyme to produce a substrate enzyme complex termed methyl-B_{12}-E. In a separate reaction, CO_2 may be reduced by hydrogen with the formation of carbon monoxide (CO) as the intermediate product. This CO carboxylates the methyl-B_{12}-E complex with the production of acetyl-coenzyme A and acetate. The enzyme that catalyzes CO formation, CO dehydrogenase, is unique to the acetogenic bacteria and, like the ferredoxins, is characterized by a very low redox potential.

The hydrogen and acetogenic pathways described earlier are fundamental to microbial metabolism in deep aquifer systems. When sediments from deep aquifer systems are incubated in the laboratory, the most abundant fermentation products that are produced are acetate, formate, and hydrogen. All of these are involved in the acetogenic pathway shown in Figure 4.9. Thus, the presence of acetogenic and hydrogen fermentations neatly accounts for observed fermentation products. However, studies on the production of other fermentation products, such as the higher-molecular-weight organic acids such as lactate, propionate and butyrate, have not been performed on sediment from the deep subsurface. It is possible that these fermentations are also important in some subsurface environments.

4.6.3 Methanogenic Pathways

Fermentative and respirative microorganisms in anaerobic aquatic sediments and subsurface environments live together in symbiotic association. Specifically, fer-

mentative bacteria degrade complex sedimentary organic matter using acetogenic or hydrogen-producing pathways similar to those discussed in the previous sections. The products of these fermentations, hydrogen, carbon monoxide, acetate, and formate, still contain utilizable energy. This energy is then tapped by respirative microoganisms by coupling the oxidation of fermentation products to the reduction of electron acceptors. Methanogenic respiration, in which the carbon present either in CO_2 or in acetate serves as the terminal electron acceptor, is one of the most important respirative pathways found in anaerobic subsurface environments.

There are two distinct pathways of methanogenesis that occur in natural environments. The first of these is called the CO_2 reduction pathway. As is true with many microbial processes, the overall stoichiometry of the reaction is deceptively simple and may be written as

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O.$$

$$\Delta G^\circ = -32.4 \text{ kcal}$$

In fact, methanogenic microorganisms accomplish this overall reaction in at least four steps (Fig. 4.10a) in which hydrogens are sequentially reacted with the carbon.

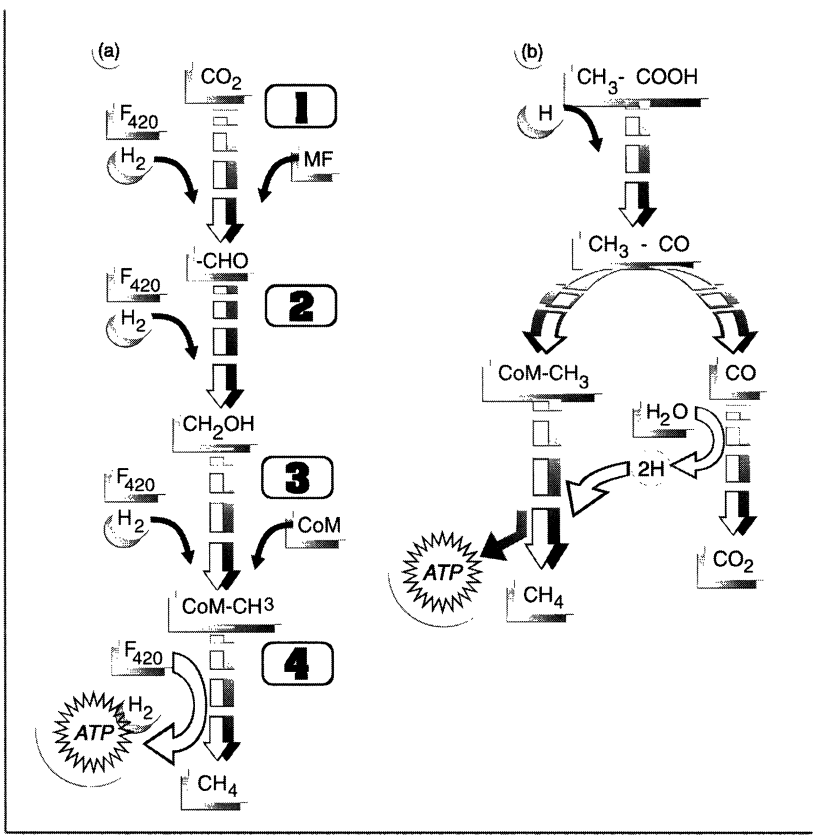


Figure 4.10. Production of methane from (a) reduction of CO_2 and (b) from acetate.

In each hydrogen transfer, a specialized protein factor, F420, acts as the hydrogen carrier. In the final step, a methyl-coenzyme M methylreductase (CoM—CH_3) complex is formed and the carbon is reduced to methane. The fourth step in this sequence is coupled to the generation of a proton motive force and subsequent synthesis of ATP.

A second methanogenic pathway is reduction of acetate according to the stoichiometry:

$$CH_3COOH \rightarrow CH_4 + CO_2.$$

$$\Delta G^\circ = -31 \text{ kcal}$$

This process is sometimes referred to in the literature as "acetate fermentation" but should not be confused with the production of acetate by fermentative acetogenic bacteria, as described in section 4.6.2.

Many of the details of the acetate fermentation methanogenic pathway have yet to be worked out. One likely schematic of these processes, however, is shown in Figure 4.10b. First hydrogen combines with acetate to form a carbon monoxide (CO)–bearing complex (CH_3—CO). This complex then supplies the reducing equivalents needed to transform the methyl-coenzyme M methylreductase (CoM—CH_3) complex to methane, with the generation of ATP. An interesting feature of this reaction is that CO, and not CO_2, appears to be the primary cleavage product of the pathway.

It is clear from the free energy of these reactions (−32.4 and −31 kcal, respectively) that these are not very thermodynamically favorable reactions. In fact, methanogenic microoganisms predominate only in subsurface environments that lack other inorganic electron acceptors. However, many subsurface environments lack such electron acceptors and methanogenesis, by either CO_2 reduction or acetate oxidation, is the predominant respirative process.

4.6.4 Sulfate Reduction

Sulfate reduction, like methanogenesis, is a fundamentally important process in the microbiology of subsurface environments. Sulfate reducers, in fact, were the first kinds of bacteria that were actually isolated from deep environments, such as oil reservoirs and aquifers.

Sulfate-reducing bacteria are dependent upon fermentative bacteria to supply them with the simple organic compounds that their metabolism is designed to exploit. Formate, acetate, lactate, and hydrogen are the principal substrates upon which sulfate reducers commonly grow. Of these, the lactate-utilizing pathway is best known. The essential features of lactate metabolism by the sulfate-reducing bacteria *Desulfovibrio* are shown schematically in Figure 4.11. A particularly interesting feature of this metabolic scheme is that the hydrogenase and its electron acceptor cytochrome c_3 is located in the periplasmic space. Other electron carriers and lactate dehydrogenase occur in the membrane, and all enzymes for pyruvate oxidation, sulfate reduction, and a second hydrogenase are located in the cytoplasm.

The first step in this process is the oxidation of lactate to acetate with the liberation of hydrogen. This hydrogen is then transported through the membrane

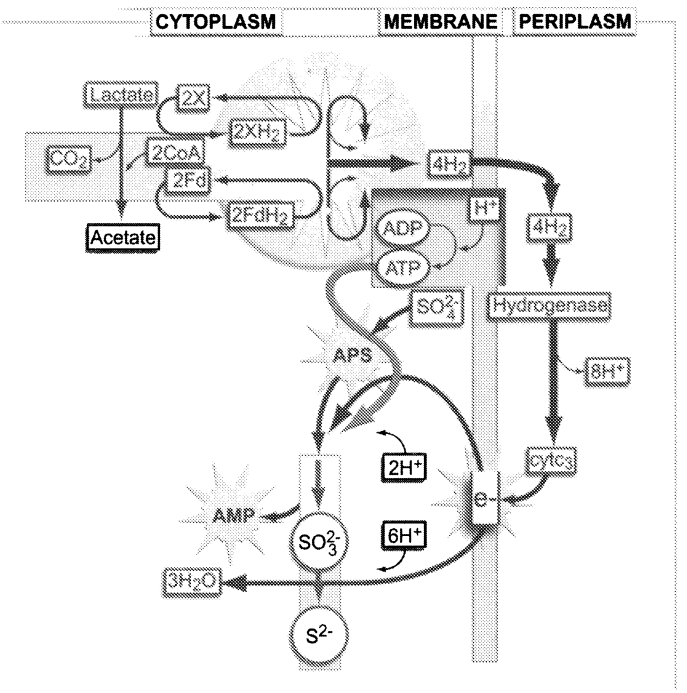


Figure 4.11. Biochemistry of sulfate reduction with lactate as a carbon source. (Modified from Gottschalk, 1986, with permission courtesy of Springer-Verlag).

to the periplasmic space. The electrons are then transferred to cytochrome c_3 and transported back into the cytoplasm. The hydrogen ions remaining in the periplasmic space form a proton motive force that is subsequently used by the cell for ATP synthesis. Once back in the cytoplasm, the electrons are transferred to sulfate. Note that, for the sulfate to act as an electron acceptor, sulfate must first be activated by ATP to form adenosine-5-phosphosulfate (APS).

4.6.5 Fe(III) Reduction

From a geochemical standpoint, Fe(III) reduction is one of the most important microbially mediated redox processes that occur in ground-water systems. Fe(III) oxyhydroxides are present in a broad range of hydrologic systems, and the reduction of these compounds, coupled with the oxidation of organic matter, is a principal cause of undesirably high concentrations of dissolved iron in ground water. In fact, producing and maintaining water-treatment facilities that are designed specifically to treat high-iron ground water is a multimillion-dollar business in the United States alone. Additionally, Fe(III) reduction is an important process in the oxidation of anthropogenic chemicals artificially introduced into the environment (Lovley et al., 1989) and is important in biogenic localization of some kinds of chemical spills.

Of all the anaerobic respiration pathways, Fe(III) reduction is the one about which the least is known. The principal reason for this is that until 1987 there were

no Fe(III)-reducing microorganisms isolated in pure culture, and therefore this metabolic processes could not be systematically explored. With the isolation of *Geobacter metallireducens* by Lovley and Phillips (1988), investigations into the metabolism of Fe(III) reduction became feasible.

At the present time, the only details known about Fe(III) reduction as carried out by *Geobacter* are that (1) there is a membrane-bound iron reductase that transfers electrons to solid Fe(III) oxyhydroxides outside the cell; (2) the electron transport mechanism involves NAD as an electron carrier, as well as at least one cytochrome of the c type; and (3) NAD^+ is reduced to NADH in the tricarboxylic acid (TCA) cycle. Details of the TCA cycle are discussed in section 4.6.7.

A possible pathway for the oxidation of acetate with the reduction of Fe(III) is shown in Figure 4.12. First, acetate is oxidized in the TCA cycle with the reduction of NAD^+ to NADH. These electrons are then transferred from NADH to cytochrome b, transported through the cell membrane, and combined with Fe(III) oxyhydroxides in contact with the cell membrane through the mediation of iron reductase.

The details of this scheme are very preliminary and will almost certainly be revised by future work. Also, it is beginning to become evident that there are many kinds of Fe(III)-reducing microorganisms in subsurface environments, and their metabolic pathways may differ substantially from that of *Geobacter metallireducens*.

4.6.6 Nitrate Reduction

A wide variety of microorganisms are able to couple oxidation of organic substrates with the reduction of nitrate in order to obtain energy for growth. This process is of great economic importance in the practice of agriculture, since it removes an important plant nutrient, nitrate, from the soil. Nitrate reduction is also an important process in the degredation of organic matter in marine sediments. In ground-water

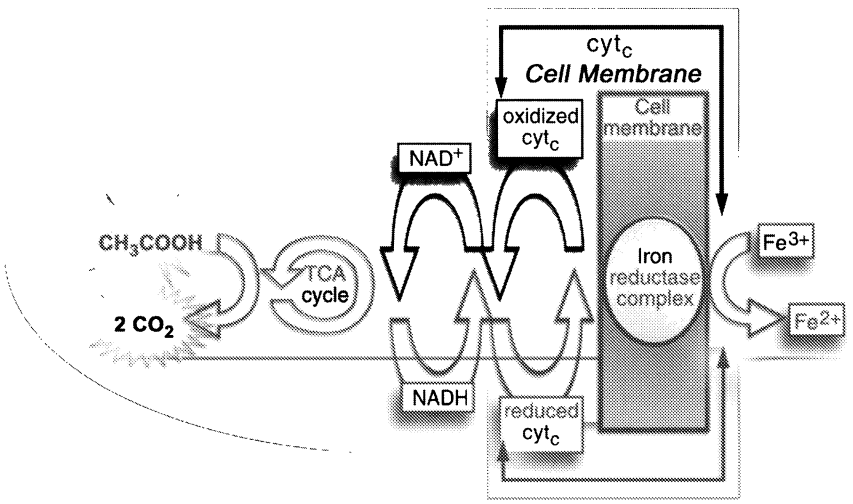


Figure 4.12. Model of electron transport in the dissimilatory Fe(III) reducer *Geobacter*.

systems, nitrate reduction tends to be an important microbial process when human influences are present (i.e. landfill leachates, agricultural chemicals, septic effluents). Nitrate reduction is rarely an important respirative process in pristine groundwater systems.

When molecular nitrogen (N_2) is the major product of nitrate reduction, as in

$$\text{glucose} + 4.8\ NO_3^- + 4.8\ H^+ \rightarrow 6\ CO_2 + 2.4\ N_2 + 8.4\ H_2O$$

the process is called denitrification. Many different genera of bacteria, including particular species of *Alcaligenes, Bacillus, Pseudomonas,* and *Thiobacillus,* are capable of denitrification. Many bacteria reduce nitrate only as far as nitrite

$$\text{glucose} + 12NO_3^- \rightarrow 6CO_2 + 6H_2O + 12NO_2^+$$

or reduce nitrate to ammonia

$$\text{glucose} + 3NO_3^- + 3H^+ \rightarrow 6CO_2 + 3NH_3 + H_2O.$$

A schematic diagram showing a possible reaction sequence in denitrification is shown in Figure 4.13. According to this sequence, nitrate is reduced to nitrite by means of a specialized enzyme, nitrate reductase. This nitrite is then bound to an enzyme-ferrous complex ($X\text{-}Fe^{2+}\text{—}N{=}O^+$). An additional nitrite molecule is added to this complex, which is then reduced to oxyhyponitrate through the mediation of nitrite reductase, and then reduced again to nitrous oxide. Finally, nitrous oxide is reduced to N_2 by nitrous-oxide reductase.

Nitrate reductase is a particularly interesting enzyme. It contains a molybdenum cofactor that is noncovalently bound to the protein portion of the molecule. Nitrate reductase is membrane-bound and contains cytochromes of the c and b type. The activity of nitrate reductase is inhibited by the presence of molecular oxygen. For this reason, nitrate-reducing microorganisms are restricted to anaerobic or mi-

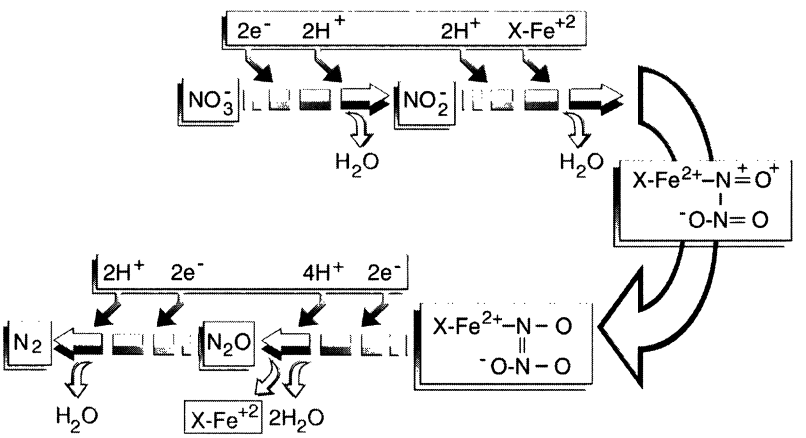


Figure 4.13. Model of electron transport in nitrate reduction. (Modified from Gottschalk, 1986, with permission courtesy of Springer-Verlag).

croaerophilic (oxygen <2%) environments. This inhibition of nitrate reduction by oxygen is the principal reason that nitrate accumulates in aerobic ground water that receives recharge from fertilized farmland, sometimes to the point of becoming dangerous for human consumption. In anaerobic ground-water systems, denitrification rarely allows the accumulation of nitrate in solution.

4.6.7 Oxygen Reduction—Aerobic Metabolism

The most energetically favorable mechanism by which microorganisms oxidize organic carbon material is aerobic metabolism, in which molecular oxygen is used as a terminal electron acceptor. There are dozens of known pathways that produce ATP with the reduction of molecular oxygen, but the most important and most closely studied is called by a variety of names, including the tricarboxylic acid (TCA) cycle, the citric acid cycle, or the Krebs cycle (named after one of the investigators involved in working out the cycle). A schematic diagram of the essential features of the TCA cycle is shown in Figure 4.14. While the TCA cycle is most common in aerobic bacteria, it also occurs in some anaerobic bacteria, such as the Fe(III) reducer *Geobacter* (Fig. 4.12).

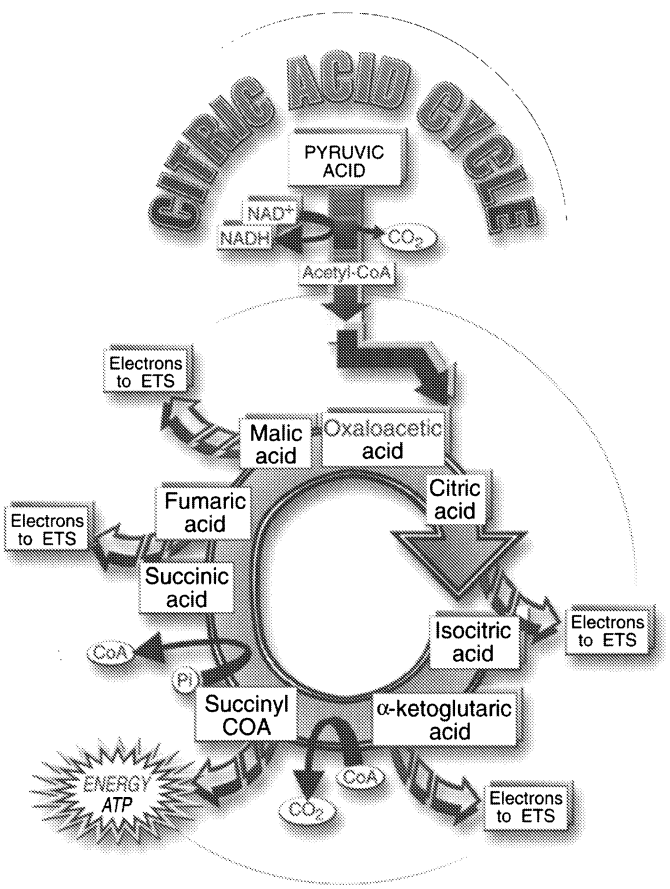


Figure 4.14. Simplified model of the tricarboxylic acid (TCA) cycle.

The starting material for the TCA cycle, as in the other respirative pathways that we've considered, is pyruvate from glycolysis. Pyruvate is decarboxylated to form acetyl-coenzyme A, which temporarily stores energy, much like ATP, and which carries a two-carbon acetyl group. This acetyl group is combined with oxaloacetic acid to form a six-carbon molecule, citric acid, after which this cycle takes one of its names. Citric acid is referred to as a tricarboxylic acid because of the three carboxyl groups that it contains, and this is the source of the term "tricarboxylic acid" (TCA) cycle.

The six-carbon compound citric acid is then oxidized with the release of CO_2 and with the reduction of NAD^+ to NADH. The five-carbon entity remaining is then oxidized, again producing CO_2 and NADH. The energy released in this oxidation is captured as GTP (guanosine triphosphate), which is used by the cell as a high-energy compound much like ATP. Continuing on, the four-carbon entity, succinic acid, is oxidized to fumaric acid, this time with the electrons being captured by FAD, a lower-energy electron carrier than NAD. The final product of the cycle is oxaloacetic acid, which initiates the entire process over again when combined with acetyl-coenzyme A. Figure 4.14 shows only an outline of the biochemistry envolved in the citric acid cycle. The net products, however, include NADH, FADH, ATP, and GTP. NADH and FADH then carry electrons to the electron transport system of the cell, where additional ATP is formed via chemiosmosis.

A comparison of the energy available from substrate level phosphorylation (2 ATPs) and oxidative phosphorylation through the citric acid cycle (38 ATPs) shows why it is so much in a cell's interest to have an electron transport apparatus: It vastly increases the amount of energy that is available for use. Some microorganisms, such as *E. coli,* are capable of both aerobic respiration and anaerobic fermentation. Not surprisingly, these bacteria much prefer to respire and are strictly fermentative only if oxygen becomes depleted in their environment.

4.7 BIOSYNTHESIS

The ultimate reason for metabolism in microorganisms is to produce and to store energy into a form (ATP) that can then be used for cellular growth. Because microbial cells are largely composed of macromolecules, such as proteins, polysaccharides, lipids, and nucleic acids, cellular growth requires the synthesis of these compounds.

Biosynthesis pathways have been closely studied in *E. coli,* and this organism serves as a convenient model for the kinds of biosynthetic processes that occur in other cells. The basic strategy employed by the cell is to use the intermediate products of glucose metabolism as the starting materials for larger macromolecules. For example, pyruvate, which is produced in glycolysis (section 4.2), can be transformed to sugar phosphates, which in turn can be linked together to form polysaccharides. Similarly, pyruvate can be transformed into fatty acids, which, when linked together, produce lipids.

All of these synthetic reactions are by themselves endergonic. Thus, in order to effect cell growth, biosynthetic reactions must be coupled with energy-producing reactions, using ATP as the intermediate energy-storing compound. As might be expected, some macromolecules require more energy for biosynthesis than others. Table 4.1 shows the ATP requirement for the synthesis of 1 gram of cell material

TABLE 4.1 ATP Requirement for Synthesis of Macromolecules from Glucose

Macromolecule	ATP Required to Synthesize Macromolecule (mmol ATP/g of cells)
polysaccharide	2.1
protein	19.1
lipid	0.1
RNA	3.5
DNA	0.9

broken down by cellular component. This illustrates that much of the total energy required to produce 1 gram of cell material (34.8 mmols ATP) is used just to make proteins. This makes intuitive sense if one considers the variety of enzymes used by the cell and the architectural complexity of each of these. The synthesis of nucleic acids and proteins is discussed in Chapter 5.

4.7.1 Amino Acids

Amino acids form the building blocks of protein molecules used by microbial cells. Many amino acids are taken up directly by the cell as substrates and used directly to build proteins. However, not all necessary amino acids are available in the environment, particularly the nutrient-poor environment of ground-water systems, and must be synthesized by the cell.

There are numerous pathways for the synthesis of amino acids that have been worked out by biochemists over the years. An example of this kind of pathway is shown in Figure 4.15, which shows the synthesis of several amino acids from oxaloacetate and pyruvate. Recall that pyruvate is synthesized in gycolysis (Fig. 4.2) and that oxaloacetate is synthesized in the tricarboxylic acid cycle (Fig. 4.14). Thus, those metabolic pathways not only provide energy production for the cell but also may be used to provide materials needed for amino acid production and, ultimately, protein synthesis.

4.7.2 Carbohydrates

Microbial cells use carbohydrates for a number of important life functions, most notably in the synthesis of the lipopolysaccharides and peptidoglycan that make up the cell wall. Polysaccharides built from carbohydrates are also used by bacterial cells to attach and adhere to surfaces in their environment, an important characteristic of bacteria that inhabit subsurface environments. Pathways of carbohydrate biosynthesis are therefore anabolic processes that are fundamentally important to the cell.

We have seen how cells are able to extract energy from carbohydrates (glucose) via glycolysis (Fig. 4.2), with a net production of pyruvate. The cell is also able to perform the reverse of this net process—that is, begin with pyruvate and synthesize glucose. The production of glucose from pyruvate, termed gluconeogenesis, involves many of the same intermediate products found in glycolysis. Gluconeo-

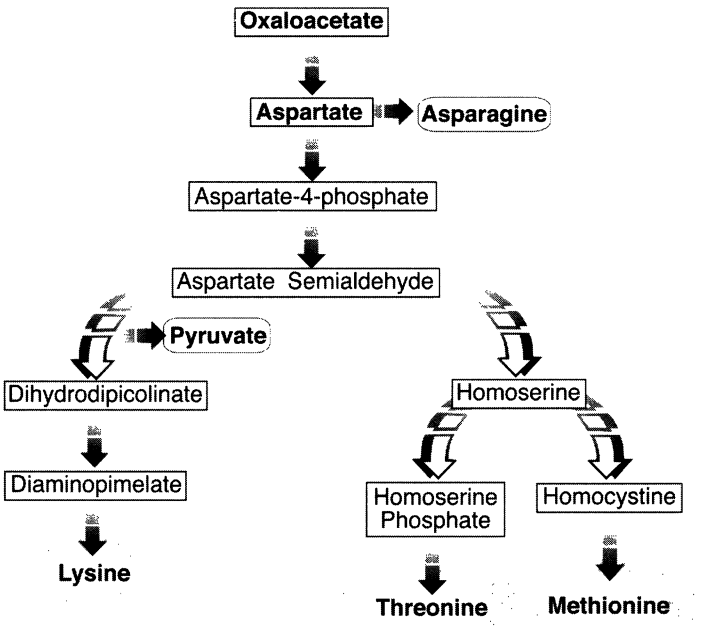


Figure 4.15. An example of amino acid synthesis, with pyruvate and oxaloacetate as starting materials (Modified from Gottschalk, 1986, with permission courtesy of Springer-Verlag).

genesis does not, however, simply involve reversing each step of glycolysis. Rather, several specialized enzymes are involved.

Pyruvate, obtained from amino acid or fatty acid metabolism, forms the starting material for gluconeogenesis (Fig. 4.16). This pyruvate is then converted to phosphoenolpyruvate (PEP) via the mediation of phosphoenolpyruvate carboxykinase.

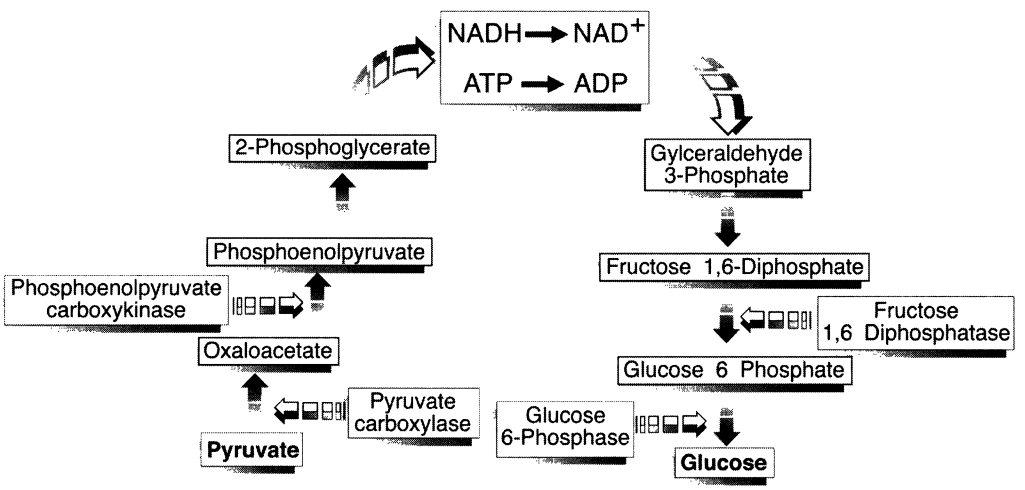


Figure 4.16. Synthesis of glucose from pyruvate via gluconeogenesis.

Recall that, in glycolysis, the reverse of this process is catalyzed by a different enzyme, pyruvate kinase. The steps leading from PEP to fructose 1,6-diphosphate are identical to the steps in the glycolitic pathway. However, the conversion of fructose 1,6-diphosphate to fructose-6-phosphate and the conversion of glucose-6-phosphate involve different enzymes than in glycolysis.

The different enzymes involved in the glycolytic and gluconeogenic pathways allow the cell to regulate the flow of carbon efficiently. For example, if there is a net deficit of ATP in the cell, the production of enzymes necessary for glycolysis is favored, resulting in the net production of ATP. If, on the other hand, the cell has ample ATP, the production of enzymes involved in gluconeogenesis are stimulated, and synthesis of glucose becomes possible. Thus, the cell will only try and synthesize glucose for cell growth if there is sufficient energy available. This type of feedback mechanism for the production or inhibition of particular metabolic pathways is basic to energy regulation in the cell.

The synthesis of glucose is important, because glucose forms the starting material for many important structural components of the cell. For example, glucose is involved in the production of the hexose amines UDP-N-acetylglucosamine and UDP-N-acetylmuramic acid, which in turn form the building blocks of the cell wall polymer peptidoglycan. The starting material for this biosynthetic pathway is fructose-6-phosphate, which as we have seen is itself synthesized in the glycolitic pathway (Fig. 4.2) or the gluconeogenic pathway (Fig. 4.16). Fructose-6-phosphate then has amine, acetyl, and phosphate groups attached sequentially to form UDP-N-acetylglucosamine. Addition of phosphoenolpyruvate at the third carbon atom of UDP-N-acetylglucosamine, with subsequent reduction of NADPH (a coenzyme closely related to NADH), forms UDP-N-acetylmuramate. These two molecules are then transported out of the cell membrane and linked together by peptide bridges in an alternating, cross-linked pattern to form peptidoglycan.

One interesting feature of this topic is that several antibiotics, notably penicillins and cephalosporins, derive their antibiotic properties from inhibiting peptidoglycan synthesis. Penicillin interferes with the peptide cross-linking of UDP-N-acetylglucosamine and UDP-N-acetylmuramate. This weakens the cell wall and, if the cell is actively growing, will result in cell lysis. Thus, penicillin will kill only actively growing cells.

4.7.3 Lipids

Lipids are used by microorganisms for a variety of functions in the cell. Lipids are widely used for energy storage in bacteria, and phospholipids are important components of the cell wall. The basic architecture of all lipids involves fatty acids and glycerol molecules bound together by ester linkages. The synthesis of fatty acids, therefore, is basic to lipid synthesis.

The overall scheme of fatty acid biosynthesis involves the sequential addition of carbon units derived from acetyl CoA. First, acetyl CoA reacts with CO_2 with the consumption of ATP to produce malonyl CoA. Malonyl CoA then binds with a protein known as acyl carrier protein (ACP). The malonyl-ACP complex then successively contributes two carbon units for the synthesis of fatty acids. As a general rule, fatty acid synthesis involves the consumption of ATP and the use of reducing power in the form of NADPH. Fatty acids, therefore, store considerable chemical energy.

Phospholipids, which form an important component of the outer cell membrane of many bacteria, are synthesized by the addition of fatty acids to glycerol phosphate. Acetyl CoA reacts sequentially with glycerol-3-phosphate to form phosphatidate. Molecules of phosphatidate are then linked together via the mediation of ACP to form elongated phospholipid chains.

4.8 CHEMOLITHOTROPHY

Microorganisms employ three basic strategies for obtaining energy to support life processes. Heterotrophy, or utilizing organic compounds as electron donors, is the most widespread strategy for microorganisms living in subsurface environments. Photosynthesis, which is extensively used by microorganisms in aquatic and terrestrial surface environments, is obviously not an option for microbes living in the subsurface. The third basic energy-obtaining strategy is chemolithotrophy. Chemolithotrophy, which literally means "chemical rock eater," refers to the use of reduced inorganic chemicals, such as H_2, H_2S, Fe^{2+}, or NH_3, as electron donors in metabolism.

The ability to obtain electrons from inorganic sources is a great ecological advantage to microbes in environments that are isolated from sunlight. This advantage, of course, is that chemolithotrophs need not be dependent on a photosynthetic food chain to produce the organic carbon compounds needed for energy and growth. This also presents chemolithotrophs with a problem, however. This problem is that they must be able to synthesize organic compounds from CO_2 in order to produce cellular components. Many chemolithotrophs are able to assimilate organic compounds in addition to deriving electrons from inorganic sources. Other chemolithotrophs, however, have biochemical mechanisms for fixing CO_2 (i.e., reducing it to organic carbon compounds) and are able to grow with CO_2 as their sole carbon source.

4.8.1 Hydrogen Oxidizers

Hydrogen, as discussed previously (section 4.3), is produced in a variety of microbial fermentations under anaerobic conditions. The interfaces of anaerobic and aerobic environments may therefore be characterized by the mixing of H_2- and O_2-bearing waters. The reaction

$$2H_2 + O_2 \rightarrow 2H_2O$$

yields 112 kcal of energy and thus has the potential to supply energy for microbial growth. Another important environment in which H_2 oxidizers often thrive is in tectonically active areas. In these areas, H_2 associated with deep hydrothermal activity is sometimes brought to the surface along fault and fracture traces where O_2 is available.

Most hydrogen-oxidizing microbes contain a membrane-bound hydrogenase that feeds electrons into the electron transport system, with subsequent ATP synthesis via chemiosmosis. An interesting feature of organisms that use the membrane-bound hydrogenase is that they carry out reverse electron transfer, forming NADH from NAD (Fig. 4.17). This NADH is then used by the cell to fix organic carbon

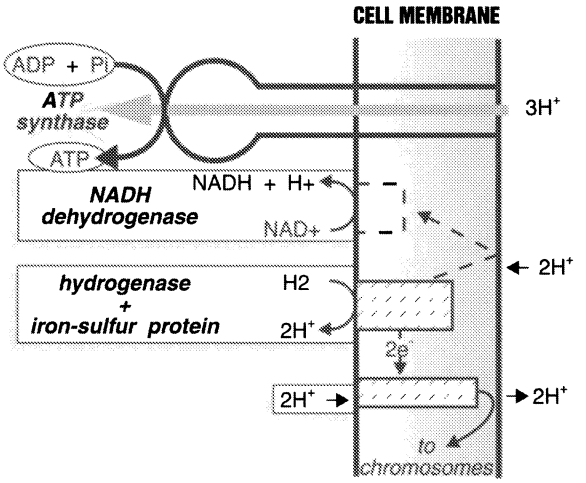


Figure 4.17. Reverse electron transfer by hydrogen-oxidizing bacteria (Modified from Gottschalk, 1986, with permission courtesy of Springer-Verlag).

compounds from CO_2. This process of CO_2 fixation will be discussed later in more detail. Other hydrogen-oxidizing bacteria, however, are unable to fix CO_2 and also require sources of organic carbon.

Examples of hydrogen-oxidizing bacteria include members of the genus Hydrogenomonas. These bacteria are gram-negative rods characterized by the presence of a polar flagella. In addition to growing on H_2, these bacteria are able to grow on some organic acids and alcohols. Hydrogen oxidizers are widely distributed in soils and surface waters. In addition, hydrogen oxidizers have been reported from deep subsurface sediments by Fredrickson et al. (1989).

4.8.2 Sulfide Oxidizers

Reduced sulfur compounds, H_2S and elemental sulfur, can be used by a variety of chemolithotrophic bacteria as a source of energy. The reaction

$$2H_2S + O_2 \rightarrow 2H_2O + 2S$$

yields 83 kcal of energy and the reaction

$$2S + O_2 + 2H_2O \rightarrow 2H_2SO_4$$

yields 236 kcal of energy. Thiosulfate ($S_2O_3^{=}$) may also act as an electron donor.

Representative examples of sulfide-oxidizing bacteria include members of the genus *Thiobacillus*. These bacteria are obligately aerobic, gram-negative, polarly flagellated rods. Many species of *Thiobacillus* are obligate autotrophs, meaning that they are unable to grow with organic carbon as an electron and carbon source. A few species, however, do grow on simple organic compounds. The biochemistry of sulfide oxidation as performed by *Thiobacillus* is summarized in Figure 4.18.

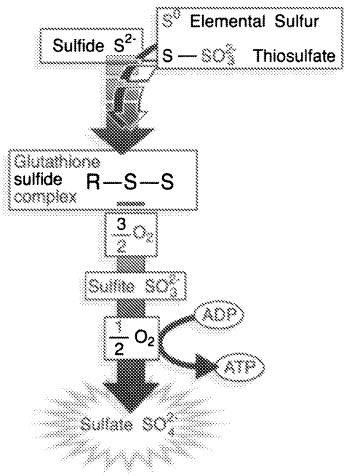


Figure 4.18. The biochemistry of sulfide oxidation in *Thiobaccillus*.

Reduced sulfur compounds are complexed with the sulfhydryl group of the tripeptide glutathione. The reduced sulfur is then oxidized to sulfite through the mediation of the enzyme sulfide oxidase. The sulfite is then further oxidized to sulfate with the generation of ATP.

Many members of the genus *Thiobacillus* grow best at approximately neutral pH, a characteristic that may become a disadvantage in some environments, since a by-product of sulfide oxidation is sulfuric acid. One species in particular, however, *T. thiooxidans,* is adapted to growth at low pH's (2–5) and thrives in the acidic conditions often produced by extensive sulfide oxidation.

Chemolithotrophic sulfur oxidation occurs in a number of important environments. For example, the metabolism of sulfide-oxidizing bacteria in coal spoil piles, with the net production of sulfuric acid, is the principal cause of acid mine drainage characteristic of strip-mined areas. The recent spectacular discovery of "black smokers" on the seafloor of tectonically active regions is another important habitat of sulfide-oxidizing bacteria. The term "black smoker" refers to the characteristic black clouds that emanate from seafloor vents. These black clouds result from the rapid oxidation of sulfides, which are present in the hydrothermal waters, by means of oxygen present in the seawater. This provides ideal conditions for the growth of sulfide-oxidizing bacteria. The growth of these sulfide oxidizers provides the base of a food chain that includes grazing protozoa, filter feeders, crabs, and fish.

Sulfide-oxidizing bacteria appear to be especially common in subsurface environments. For example, out of 45 samples of sediments cored from deep subsurface sediments (30–260 M) of the South Carolina Coastal Plain, 27 showed active growth of sulfide oxidizers (Fredrickson et al., 1989). The numbers of sulfide oxidizers present varied between about 101 and 103 cells per gram (dry weight) of sediment. Interestingly, there was evidence that sulfide oxidizers coexisted with viable sulfate-reducing bacteria in these sediments (Jones et al., 1989). It's difficult to judge from this evidence if both of these physiologic types of bacteria are active in situ, but it raises the interesting possibility of an active sulfur cycle in subsurface sediments.

4.8.3 Iron Oxidizers

The iron-oxidizing bacteria are well-known among hydrologists and are generally referred to as "iron bacteria." Iron-oxidizing bacteria are responsible for the slimy coatings of ferric iron that sometimes encrust wells, and, for this reason, they are the most notorious microbiological pests in the water well industry.

Iron-oxidizing bacteria obtain energy for growth from the exergonic reaction

$$4\ Fe^{2+} + 4\ H^{+} + O_2 \rightarrow Fe^{3+} + 2\ H_2O$$

which releases 160 kcal of energy. Iron oxidation obtains relatively little energy per mole of substrate oxidized (40 kcal/mole Fe^{2+}) compared to sulfide oxidation (150 kcal/mole H_2S) or even hydrogen oxidation (56 kcal/mole H_2). Thus, iron oxidizers are obliged to turn large amounts of Fe^{2+} to Fe^{3+} in order to obtain sufficient energy to sustain growth. Because Fe^{3+} forms highly insoluble ferric oxyhydroxides, iron-oxidizing bacteria can quickly clog a water well. This problem is particularly severe if the iron-oxidizing bacteria *Gallionella* is the principal organism present. *Gallionella* produces a sheath made of a slimy material that not only helps clog wells but also is quite unpleasent to look at. Although *Gallionella* is perfectly harmless to humans, few people would be willing to drink water from a well in which this organism was seen to be growing.

Many members of the genus *Thiobacillus,* which are principally known for their ability to oxidize sulfides, also oxidize Fe^{2+}. For example, the species *Thiobacillus* ferrooxidans is distinguished from its obligately sulfide-oxidizing cousin Thiobacillus thiooxidans by its ability to grow on Fe^{2+} as well as on H_2S.

Iron-oxidizing bacteria are widely distributed in aquatic sediments. They are particularly well suited for such environments as streambeds that receive base flow from anaerobic, Fe^{2+}-containing ground water. The mixing of this high-iron ground water with oxygen-containing surface water provides an ideal habitat for these microorganisms. Evidence for the activity of such iron-oxidizing bacteria in streambeds is often noticable as a reddish-brown floc that coats sediment grains.

In ground-water systems, the presence of iron-oxidizing bacteria in aquifer sediments is indicated by their common presence in water wells. This evidence is not unequivocal since they may be introduced with the drilling process. However, the widely observed presence of sulfide oxidizers and the fact that many sulfide oxidizers also are capable of oxidizing iron suggest that they are a common component of the subsurface microflora.

4.8.4 Ammonia-Oxidizing (Nitrifying) Bacteria

The decomposition of plant and animal remains under anaerobic conditions often results in the production of reduced nitrogen compounds such as ammonia (NH_3) and the ammonium cation (NH_4^+), by means of deamination reactions. These compounds can be oxidized to nitrate in the presence of oxygen and nitrifying bacteria. The processes leading to nitrification are of great economic importance to agriculture. It was known for centuries, for example, that composting manure under aerobic conditions increased the manure's fertilizing properties. Winogradsky was the

first to show that this resulted from the action of nitrifying bacteria that converted ammonia present in the uncomposted manure to nitrate.

Nitrification involves two distinct types of bacteria that operate in sequence. First, ammonia is oxidized to nitrite (NO_2^-) by such bacteria as *Nitrosomonas*. After this, nitrite is then further oxidized to nitrate by such bacteria as *Nitrobacter*.

Nitrifying bacteria are common in soils, and the activity of these microorganisms has been intensely studied by soil microbiologists. Fredrickson et al. (1989) showed that nitrifying bacteria are present in deep subsurface sediments of the Atlantic Coastal Plain. Out of 45 samples assayed from three different core holes in South Carolina, 13 contained nitrifying bacteria in concentrations of about 101 cells per gram of sediment. In all cases, however, numbers of nitrifiers in subsurface sediments were two to three orders of magnitude less than in the overlying surface soils. In these deep aerobic sediments, ammonia concentrations were fairly low ($<$ 0.5 mg/L), and this lack of available substrate is probably the chief limiting factor for nitrifying activity. It seems likely that nitrifying bacteria are widely distributed in aerobic ground-water systems.

4.8.5 Autotrophic CO_2 Fixation

Because many autotrophic bacteria do not or cannot obtain energy for growth from organic carbon substrates, they must have an alternative source of organic carbon to support cellular growth. In order to produce the needed organic carbon, autotrophs fix CO_2 by means of a special pathway, called the Calvin cycle. The Calvin cycle is utilized by both photoautotrophic bacteria and chemolithotrophic bacteria to convert CO_2 into carbohydrates.

The Calvin cycle (Fig. 4.19) begins with the reaction of CO_2 with ribulose 1,5-diphosphate (RuDP) to form a six-carbon compound. This exergonic reaction is catalyzed by the key enzyme ribulose 1,5-diphosphate carboxylase. The six-carbon compound formed by this reaction then splits into two molecules of 3-phosphoglycerate (PGA). Both PGA molecules are then reduced by dihydroxyacetone phosphate (NADPH), a step that also requires energy in the form of ATP. This step in the process reduces the carbon to the valence state (zero) of carbohydrates. In the Calvin cycle, the coenzyme NADPH performs electron carrier functions, as does NADH in metabolic pathways, such as glycolysis. Next, a molecule of RuDP is regenerated, and the cycle repeats. Three turns of the cycle are necessary in order to produce one molecule of glyceraldehyde-3-phosphate. In all, 12 NADPH and 18 ATP are required to synthesize 1 hexose molecule. It's evident from this that the Calvin cycle requires a considerable investment of energy by the autotrophic organism.

The coenzyme NADPH acts as an allosteric activator of RuDP in the Calvin cycle. Thus, if the cell has abundant NADPH, provided either from photosynthesis or from oxidation of inorganic compounds, the cycle will be activated and carbohydrates synthesized. On the other hand, if NADPH is not available from energy-producing reactions, the Calvin cycle is effectively shut off. This allosteric mechanism enables the cells to grow rapidly if a source of oxidizable substrate is present and allows them to conserve energy if such substrates are absent.

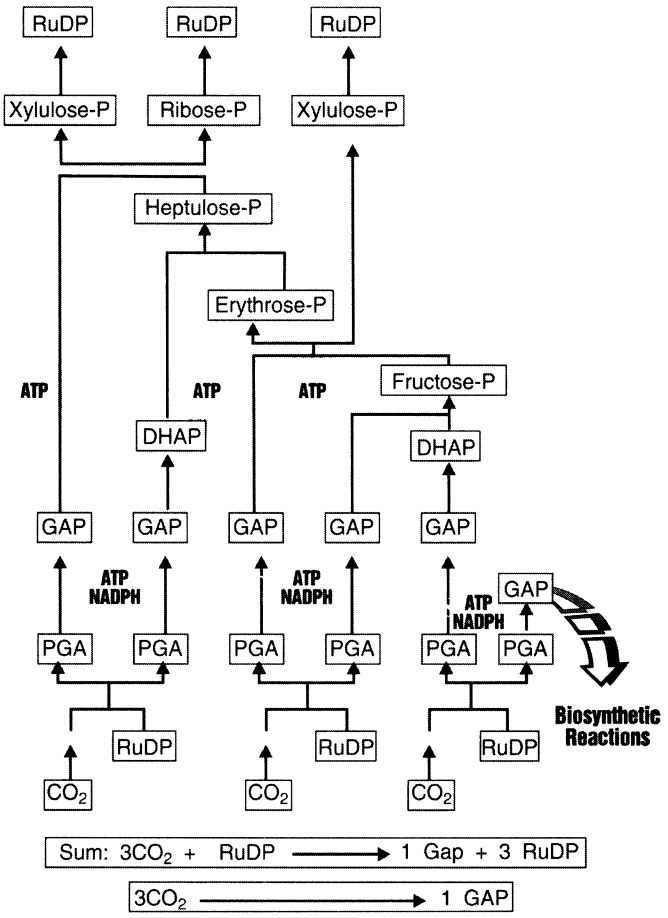


Figure 4.19. The Calvin cycle.

4.9 METABOLIC CONTROL OF GEOCHEMICAL PROCESSES

We have seen that living cells extract energy from reduced compounds, such as organic matter, by oxidation; transfer electrons from these oxidation processes to various electron acceptors; and store the energy produced in a form that can be tapped as needed. In order to carry out these functions, energetically favorable reactions ($\Delta G < 0$) are coupled with energetically unfavorable reactions ($\Delta G > 0$). The oxidation of glucose in glycolysis, for example, requires that the cell to first spend 2 molecules of ATP initiate the reaction sequence and thereby produce a net gain of 2 ATP molecules. These metabolic pathways are regulated by enzymes that catalyze the reactions in a particular sequence and at an appropriate rate.

In studying geochemically important processes that are carried out by microorganisms, it is necessary for researchers to consider limitations imposed by these metabolic processes. Living cells must conform, not only to thermodynamic constraints but also to biochemical constraints. The mere fact that a particular reaction

is energetically favorable is often not sufficient for that reaction to occur. In addition, the cell must have the enzymatic mechanisms to carry the reaction to completion. Because many important geochemical processes are mediated by microorganisms in ground-water systems, it follows that there are biochemical constraints on many geochemical processes as well.

There are many examples of this. Most geochemically important redox reactions—oxygen reduction, nitrate reduction, Fe(III) reduction, sulfate reduction, and methanogenesis—are mediated by microbial biochemical pathways and electron transport systems. In order to understand the occurrence and distribution of these processes in subsurface environments, therefore, it is necessary to understand the biochemical constraints imposed by these mechanisms. Perhaps the best example of enzymatic control of a geochemically important reaction is that of Fe(III) reduction.

High concentrations of dissolved iron (Fe(II)) are very common in anaerobic ground-water systems. Traditionally, the occurrence of Fe(II) in ground water was explained in terms of equilibrium thermodynamics and Eh-pH diagrams (Hem, 1985). Because Fe(III) oxyhydroxides are thermodynamically unstable in the presence of organic compounds under anaerobic conditions, Fe(III) reduction was considered to occur spontaneously and reversibly according to the reaction

$$Fe(OH)_3 + CH_3COOH \rightarrow Fe^{2+} + 2CO_2 + H_2O + 6H^+ \qquad (9)$$

It had long been known that particular microorganisms were able to take advantage of this exergonic process in order to obtain some energy for growth. However, with the isolation of *Geobacter metallireducens,* it was apparent that this was a major metabolic strategy of some microorganisms (Lovley and Phillips, 1988). It quickly became apparent that not only could microorganisms obtain energy by iron reduction, but that this reaction required microbial enzymatic catalyzation to proceed at all. In a series of experiments, Lovley et al., (1991) showed that none of the most common organic compounds found in natural systems could reduce Fe(III) oxyhydroxides at circumneutral pH (5–9) and low temperatures (25°C), even if the "eH" (redox potential) of the experiment was low. In addition, this reaction did not occur in the presence of a microoganism such as *E. coli* that lacked the essential enzyme system, iron reductase, that mediated the reaction. However, in the presence of *Geobacter metallireducens*, which did contain iron reductase, these reactions proceeded quickly.

Thus far, this story has proceeded fairly predictably. An important geochemical process, Fe(III) reduction, turns out to require the electron transport system of specialized microorganisms. In this manner, Fe(III) reduction is the same as, say, sulfate reduction. There is, however, a twist. Studies with *Geobacter metallireducens* showed that the iron reductase enzyme was membrane-bound; that is, it was located in the membrane of the cell. Because Fe(III) oxyhydroxides are solids, Fe(III) cannot diffuse into the cell as an oxyhydroxide. It follows that, for the cell to be able to reduce Fe(III), the cell membrane has to be in direct contact with a solid Fe(III) oxyhydroxide particle. This is, in fact, what is observed for *Geobacter metallireducens*. If the microorganisms were separated from the solid Fe(III) oxyhydroxides by a semipermeable membrane, no Fe(III) reduction would occur. Thus,

iron reduction is subject to several constraints, such as the requirement for physical contact between the Fe(III) and the cell membrane, in addition to the more obvious thermodynamic ones.

It is tempting, sometimes, to think of microoganisms as magic boxes that use fancy biochemistry to perform novel and unlikely processes. Indeed, the ability to couple thermodynamically favorable and unfavorable processes into metabolic pathways enables microbes to do some very unlikely things. This flexibility, however, is obtained at a price. This price is that microbially mediated processes must conform not only to thermodynamic constraints but also to constraints imposed by the enzymatic mechanism.

The Fe(III)-reduction example illustrates this point. For Fe(III) reduction to occur in natural systems, microorganisms with the appropriate enzymatic capabilities obviously must be present. However, these microorganisms must also be able to come into physical contact with Fe(III). It follows that Fe(III) present in the crystal structure of silicate minerals, such as biotite, is more difficult for microorganisms to reduce than Fe(III) oxyhydroxides. This last constraint, which limits Fe(III) reduction in many subsurface environments, could never have been deduced in the absence of detailed microbiologic investigations. Similar constraints on other geochemically important microbial processes remain to be worked out. This general topic will be a productive field of research for years to come.

4.10 SUMMARY

The ability to utilize energy in an organized manner is the hallmark of living entities. Metabolism in microorganisms and in the higher plants and animals consists of catabolism, the breaking down of organic molecules to extract energy, and anabolism, using energy to assemble needed proteins and other complex molecules. Energy-producing (exergonic) and energy-consuming (endergonic) reactions are coupled by means of energy-storing intermediate reactants in order to synthesize complex molecules. The most important of these energy-storing intermediate reactants is ATP, but microorganisms use other intermediates, such as GTP, as well.

Many of the energetically favorable reactions used by cells, such as aerobic glucose oxidation, proceed much too slowly at low temperatures to sustain life. Cells solve this problem through the use of enzymes, proteins that catalyze chemical reactions. Enzymes work by combining chemically with a substrate or a combination of substrates, and by bringing them together in a configuration so that reaction can occur. Enzymes are classified according to the kinds of reactions that they catalyze. Oxidases and reductases catalyze oxidation-reduction reactions, lyases remove functional groups from organic compounds, and ligases combine compounds together.

The regulation of enzymatic activity is crucial to the cell, and this is accomplished by means of a number of mechanisms. The production of enzymes can be induced or repressed at the genetic level by the presence or absence of particular substrates. Enzyme systems are also regulated by feedback inhibition mechanisms, in which the product of an enzymatic reaction acts to inhibit the function of that particular enzyme.

The two basic metabolic strategies available to microorganisms are fermentation and respiration. The basis for both strategies is using electron transfers to perform

work. In fermentation, however, electrons are retained in organic carbon compounds or molecular hydrogen. In respiration, electrons are transferred to mineral terminal electron acceptors, such as oxygen, nitrate, ferric iron, sulfate, or carbon dioxide. Respiration is much more efficient than fermentation, producing a much greater yield of ATP per mole of substrate oxidized than fermentation.

Microorganisms use energy obtained from catabolic processes to build the proteins, polysaccharides, lipids, and nucleic acids needed to repair damaged cell parts and to grow new cellular material. The basic strategy of the cell in building these compounds is to use intermediate products of metabolism as building blocks. Pyruvate, for example, which may be produced by both fermentative and respirative processes, may be used to build new polysaccharides, proteins, and lipids.

Numerous geochemically important processes in subsurface environments, oxygen reduction, Fe(III) reduction, sulfate reduction, and methanogenesis are carried out exclusively by enzymatically controlled microbial processes. Because of this, these geochemical processes are subject to biochemical as well as thermodynamic constraints. For example, the enzymatic reduction of Fe(III) oxyhydroxides requires physical contact between Fe(III) and a cell membrane for the reaction to go to completion. Similar biochemical constraints on other geochemically important processes are less well understood but are probably equally important.

QUESTIONS TO CONSIDER

1. Name three compounds that cells use to temporarily store energy.
2. What mechanism does the cell use to capture the energy released by electron transport chains?
3. What chemical characteristic of ferredoxins allows microorganisms such as clostridia to produce molecular hydrogen in anoxic environments?
4. What are the two primary carbon substrates that support methanogenic respiration?
5. Acetogenic bacteria have the unusual capability of reducing carbon dioxide to what compound?
6. In dissimilatory nitrate reduction, what is nitrate most commonly transformed to? What does this imply about the ecology of nitrate reduction?
7. In Fe(III) reduction, as carried out by *Geobacter,* why is it necessary for the cell to be in physical contact with a mineral source of Fe(III)?
8. How does the fact that Fe(II)-oxidizing bacteria obtain relatively low amounts of energy from each mole of Fe(II) contribute to the clogging of wellscreens?

CHAPTER 5

BACTERIAL GENETICS

In Chapter 4 we saw how the ability to use energy in an organized fashion is the defining characteristic of living organisms. By coupling energy-yielding and energy-consuming chemical reactions, the cell is able to synthesize the macromolecules of which it is composed and maintain its inherently unstable structure. However, it takes more than just available energy for these complex biochemical reactions to occur. There must be a "master plan" that organizes the sequence of reactions and supervises the construction of cellular components. This plan must contain all of the information needed to build each protein, carbohydrate, lipid, and nucleic acid present in the cell. Furthermore, this plan must be self-regulating, in the sense that only those cellular components needed at a particular time will be actually made and used. Finally, this plan must allow the organism to reproduce itself. In all living organisms, the information for this "master plan" is coded into nucleic acids that are present in each cell.

The term "gene," which refers to a unit of heredity, has been in use for about a century. In 1900, Hugo De Vries, K. E. Correns, and Erich Tschermak-Seysenegg discovered the rules of heredity, only to learn that they had been worked out independently 40 years earlier by Gregor Mendel. These investigators discovered, as had Mendel, that separate genetic characteristics are inherited independently. Early geneticists rationalized these observations by postulating the presence of particles, or genes, that carried inherited characteristics from generation to generation. It is one of the curiosities of history, however, that the term "gene" was used for at least 50 years before anyone had any idea what they were made of.

In the first half of the twentieth century, there were two schools of thought as to what constituted genes. One school favored proteins as carrying genetic information, and the other school favored nucleic acids. It wasn't until the structure of deoxyribonucleic acid (DNA) was deduced in 1953 by Francis Crick and James Watson that it became clear that genes were, in fact, composed of nucleic acids.

5.1 DNA: ITS STRUCTURE AND ORGANIZATION

The function of DNA, deoxyribonucleic acid, in most microorganisms is to store the genetic information needed to synthesize cellular components. One exception to this rule is the case of some viruses (the HIV virus that causes AIDS is an example) that use RNA to store genetic information. The chief property of DNA that suits it to this information-storing role is its highly ordered structure. Figure 5.1 shows the double-helix structure of DNA. In this structure, sugar molecules (deoxyribose) are bound together by phosphate groups to form two long strands. These two strands are linked together by hydrogen bonds between nitrogenous bases, and the entire structure is twisted into a helix. The structure is analogous to a spiral staircase, with the sugars and phosphates forming the two rails and the bases forming the steps.

The order of the structure is inherent in the positioning of the nitrogenous bases. The sequence of bases along each strand of DNA is capable of storing information in a manner exactly analogous to a code. In the Morse code, for example, letters are coded as sequences of dots and dashes. The code for *S* is dot-dot-dot and the code for *O* is dash-dash-dash. Thus, the coded expression dot-dot-dot dash-dash-dash dot-dot-dot is immediately recognizable as SOS. In the genetic code, se-

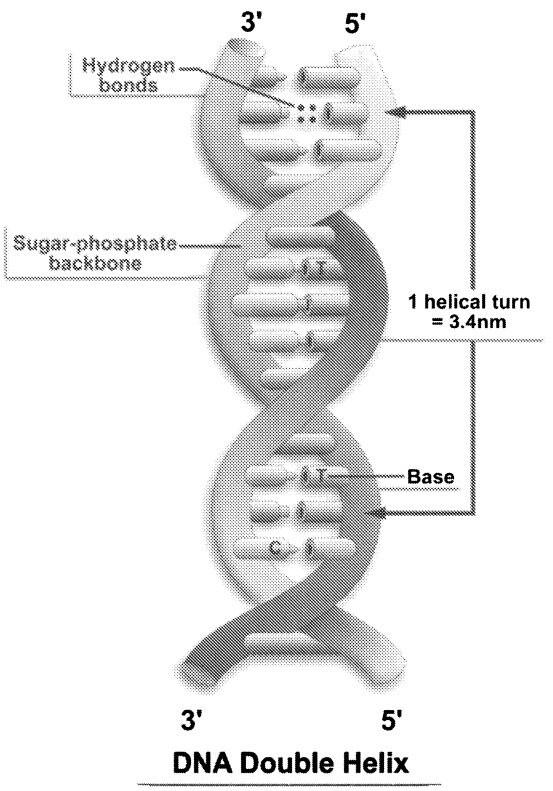


Figure 5.1. The structure of DNA.

quences of bases are used instead of dots and dashes. Specifically, sequences of bases on DNA code for the formation of particular protein molecules needed for cell maintenance and growth.

In living cells, however, simply the ability to store information is not sufficient. Living cells must also have the ability to reproduce, which means that copies of DNA must be made. The ability of DNA to make copies of itself, or replicate is inherent in its double helix structure and on the specificity of base pairs. Adenine on one strand of DNA binds only with thymine on the other strand. Similarly, guanine on one strand can bind only with cytosine on the other strand. Thus, if the DNA molecule is "unwound," each single strand is capable of exactly reproducing its complementary strand.

This process of replication is shown schematically in Figure 5.2. First, a double strand of DNA is unwound through the action of specialized enzymes. Each strand is then replicated by linking the complementary bases A-T and G-C with the mediation of DNA polymerase. As the new DNA is synthesized, sections of the new molecule are linked together by enzymes known as DNA ligases, enzymes that attach DNA at particular places. DNA ligases also have the important task of "editing" the new DNA—that is, checking to see that the new copy exactly matches the template from which it was made. If errors were made, DNA ligases are capable

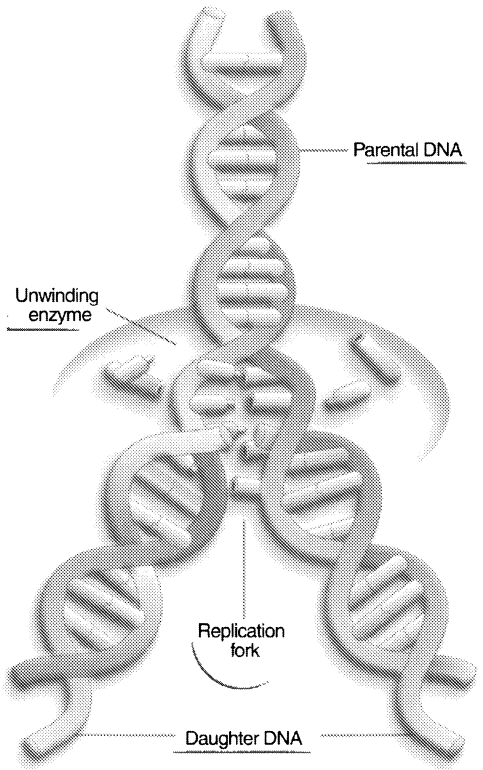


Figure 5.2. Replication of DNA (Modified from Norton, 1986, with permission courtesy of the Addison-Wesley Publishing Co.).

of making last-minute repairs. The net result of these complex replication processes are two exact copies of the original DNA molecule.

The nucleotide sequences present on a DNA molecule do not all code for specific proteins. Rather, it is now clear that DNA consists of functional areas, termed operons, that are separated by "start" and "stop" sequences (Fig. 5.3). The operons are often called structural regions, and the start, promoter, and stop sequences are called regulatory regions. Furthermore, only one strand of DNA, called the informational strand, is actively involved in protein synthesis. The other strand, called the complementary strand, is used only for DNA replication.

In bacteria, DNA that is involved in cell replication is called chromosomal DNA. Chromosomal DNA in the bacteria is different from that in eucaryotic organisms in that it is organized into closed loops. Chromosomal DNA is present in the nucleoid region of the bacterial cell and is seldom visible through a light microscope. Occasionally, the nucleoid region becomes visible after the cell has doubled its complement of DNA in preparation for cell division.

Bacterial cells often contain DNA that is not part of the chromosomes. This DNA, called plasmid DNA, is present as small loops embedded in the cell's cytoplasm. Plasmid DNA is copied much like chromosomal DNA but is not involved directly in cell replication. Rather, plasmid DNA codes for proteins that confer specific attributes to a cell. For example, many bacterial species contain plasmids that confer antibiotic resistance. Other plasmids may give the cell the ability to use specific organic compounds as substrates. Thus, plasmids are directly involved with the ability of an organism to deal with its environment. Plasmids have the important property of being able to transfer between cells. Thus, it is possible for a cell to obtain specific functions by plasmid transfer without modifying its chromosomal genome. This property, which is the basis of much genetic engineering of microorganisms, allows bacteria to respond to environmental stresses efficiently.

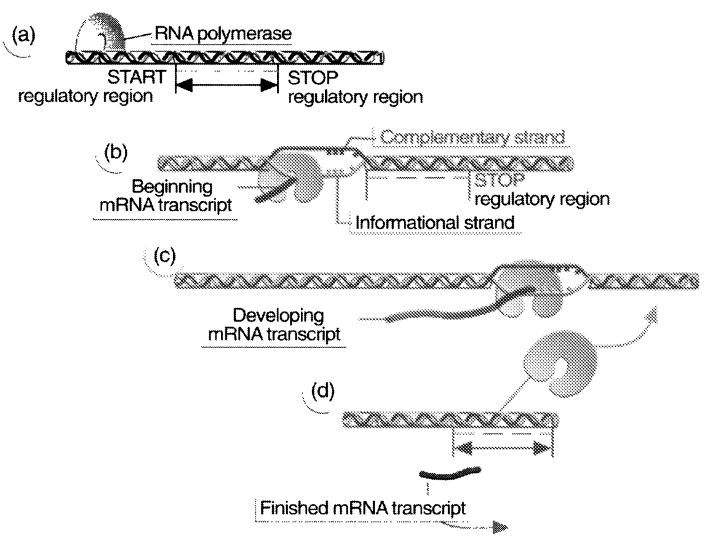


Figure 5.3. Transcription of mRNA from the DNA template (Modified from Norton, 1986, with permission courtesy of the Addison-Wesley Publishing Co.).

5.2 RNA: ITS STRUCTURE AND ORGANIZATION

DNA carries the blueprints for all of the components needed by a living cell. However, the translation of this information into specific proteins involves another class of nucleic acids, called ribonucleic acids, or RNA.

There are three major classes of RNA. Messenger RNA (mRNA) is transcribed directly from DNA and carries the genetic information from each gene to a place where the protein molecule can be assembled. Transfer RNA (tRNA) carries amino acids to the site of protein synthesis and delivers them to the appropriate locations on the growing protein molecule. Ribosomal RNA (rRNA) forms part of the ribosomes that are the surfaces upon which proteins are assembled.

5.2.1 Transcription

The process of transcription, or making mRNA, is shown schematically in Figure 5.3. First, an enzyme called RNA polymerase binds to a region on a DNA molecule that starts the coding of a particular genetic sequence. RNA polymerase unwinds a portion of the DNA molecule, and RNA nucleotides base-pair to the exposed DNA. Unlike DNA replication, however, RNA contains uracil in the place of adenine. Thus, RNA contains no thymine, which binds with adenine in DNA. The process of transcription continues until the RNA polymerase reaches a region on the DNA molecule that codes for the process to stop. The completed mRNA then moves to the ribosome to begin protein assembly.

5.2.2 Translation—Making Proteins

Molecules of tRNA and rRNA are transcribed from particular portions of the DNA molecule in an analogous manner. The function of tRNA is to carry amino acids and to deliver them to the appropriate location on mRNA. To this end, tRNA molecules have sites designed to bind only to specific sequences composed of three bases, called codons, on mRNA (Fig. 5.4). The portion of the tRNA molecule that binds to the mRNA is thus called an anticodon. The other end of the tRNA molecule has a binding site specific to a particular amino acid. Thus, when a tRNA molecule binds to a particular codon on mRNA, it always carries the amino acid specific for that codon. In this manner, amino acids can be delivered in the exact sequence in which the mRNA is coded.

Like any code, however, the genetic code requires a "key" in order to interpret messages. After the discovery of the structure of DNA in 1953, several groups of researchers worked on this problem. It was Francis Crick and his coworkers who first correctly proposed that three nitrogenous bases coded for a single amino acid, which was an important step in breaking the genetic code. The next step was to determine exactly which amino acids were coded for by each codon. Several research groups contributed to this work, and the 1968 Nobel Prize was jointly awarded to Robert Holley, H. G. Khorana, and Marshall Nirenberg for their work on breaking the genetic code.

Suppose, for example, a base sequence on mRNA is

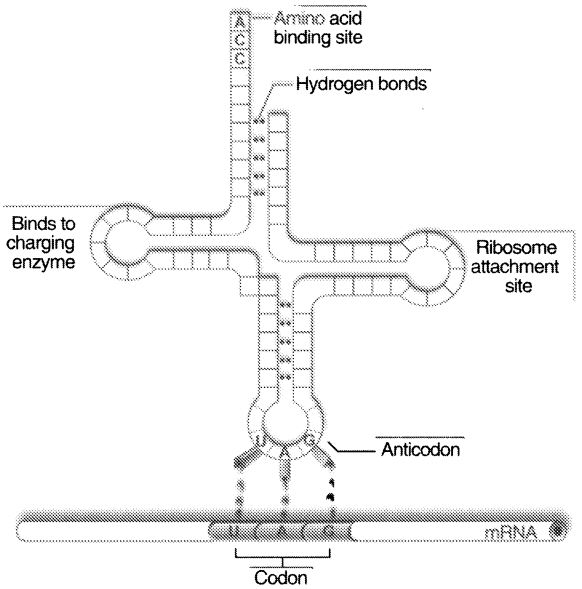


Figure 5.4. The structure of tRNA (Modified from Norton, 1986, with permission courtesy of the Addison-Wesley Publishing Co.).

CUU-AGA-AAA-UUU-AGU-GGG-ACU-UCU-UAA

The translation of this code into a protein chain would be

Leu-Arg-Lys-Phe-Gly-Thr-Ser

Protein synthesis, in addition to being able to translate the genetic code, requires an assembly area where amino acids can be physically linked together. These protein assembly areas are the ribosomes. Ribosomes are assembled from rRNA and associated proteins. The large subunit of a ribosome contains 2 strands of RNA and 32 proteins. The small subunit contains a single RNA strand and 21 proteins. Once the necessary RNA molecules have been synthesized, assembly of the protein can commence.

The process of assembling a protein molecule from the information coded in mRNA is called translation. The overall idea is to translate the sequence of nucleotides in mRNA to a sequence of amino acids in the protein. The process of translation is shown in Figure 5.5. First, the mRNA binds to the small subunit of the ribosome. Next, a tRNA molecule carrying the amino acid formylmethionine binds to the large ribosomal subunit. There are only two tRNA binding sites on a ribosome, and a tRNA molecule always initially binds to the first site where a peptide is attached to the amino acid. Once the peptide is attached, the tRNA molecule moves to the next site, providing space for another tRNA molecule to attach, bringing the next amino acid in the sequence. The two amino acids are then

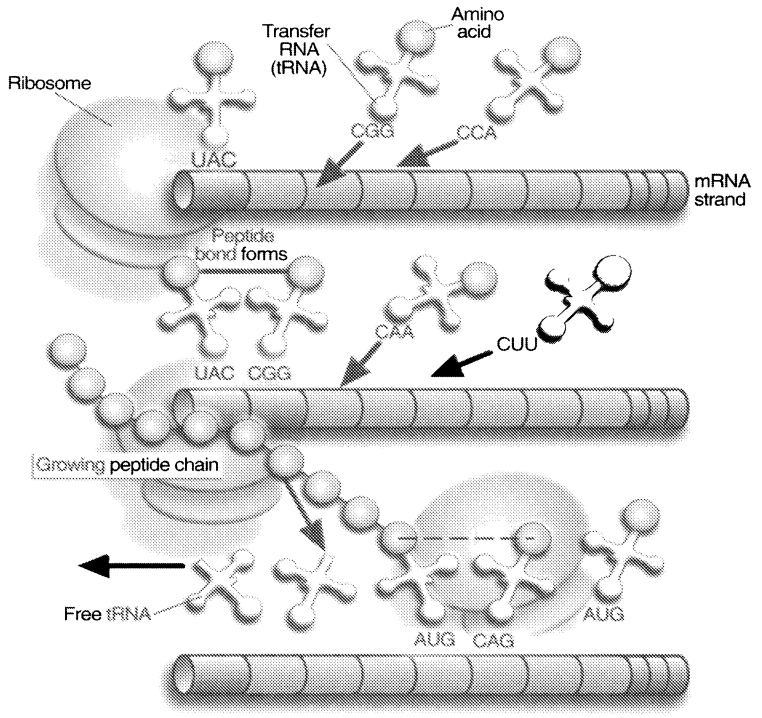


Figure 5.5. Translation of the genetic code from mRNA to the production of particular proteins (Modified from Norton, 1986, with permission courtesy of the Addison-Wesley Publishing Co.).

linked by the peptide bond. The first tRNA molecule is released, the second tRNA moves over, and another tRNA molecule bringing the next amino acid arrives.

The sequence in which the amino acids are linked is controlled by the interaction of tRNA with the template on the mRNA. Each codon on the mRNA codes for one, and only one, amino acid. However, the converse of this is not true, since several different codons code for the same amino acid. Thus, the codons UUU (uracil-uracil-uracil) and UUC (uracil-uracil-cytocine) both code for the amino acid L-phenylalanine. Similarly, the codons GCU and GCC code for L-alanine. The mRNA template is bound to the small subunit of the ribosome; as the tRNA molecules arrive, only those carrying the next amino acid required in the sequence are able to bind to the mRNA and hence the large subunit. Once the appropriate amino acid has been delivered and the peptide linkages made, the mRNA slides to the next codon, allowing the next amino acid to be delivered by tRNA. This process continues until a "stop" codon is encountered on the mRNA (UAA, UAG, or UGA), and the protein is complete. When the "stop" codon is reached, the protein is released from the ribosome. It may then be modified by forming or breaking disulfide bridges or be combined with lipids or polysaccharides before it takes on its biological role in the cell.

5.3 GENE EXPRESSION AND REGULATION

In addition to having the genetic information to build specific proteins and having mechanisms for transcribing this information and translating to the amino sequences required, it is necessary for the cell to regulate protein production. At one extreme, it is obvious that the cell need not make all the proteins that it is capable of making all the time. At the other extreme, the genetic information does little good if it cannot be "turned on" when required. Thus, the regulation of gene expression is a critical function of the cell.

5.3.1 Induction

There are numerous regulatory mechanisms used by bacterial cells. Perhaps the best studied is that of the lac-operon in *E. coli*. *E. coli* is capable of producing enzymes that allow it to metabolize the sugar lactose. However, because lactose is not always available as a substrate, it would be inefficient for *E. coli* to make these enzymes continually; rather, it is more efficient for the cell to be able to sense the presence of lactose when it becomes available, and subsequently to synthesize the lactose-degrading enzymes. After the lactose has been consumed and the enzymes are no longer needed, it would be advantageous for the cell to cease making the enzymes.

E. coli is, in fact, able to regulate production of these lactose-metabolizing enzymes by means of the lac-operon. The lac-operon, which is present in the chromosomal DNA of the cell, consists of three distinct regions. These regions are called the regulator, the promoter, and the operator regions. The regulator region codes for formation of a protein, called a repressor protein, that binds to the promoter region of the operon. This prevents RNA polymerase from binding to that portion of the DNA and prevents transcription of the operator that codes for the lactose-metabolizing enzymes. Thus, as long as the repressor protein is in place, *E. coli* will not be able to metabolize lactose.

The repressor protein is designed, however, to be removed from the DNA strand if an inducer binds to it. In the case of the lac-operon, the inducer is allolactose, a derivative of lactose. When allolactose binds to the repressor protein, the protein is removed. RNA polymerase can then bind to the promoter region of the operon and transcribe the operator region. Following transcription, the enzymes for metabolizing lactose are assembled, and lactose is then used as a substrate.

Once the available lactose has been consumed, allolactose is no longer available to bind the repressor protein. The repressor protein binds to the promoter region of the lac-operon, and production of lactose-metabolizing enzymes ceases. The enzymes that degrade lactose are referred to as inducible enzymes, since they can be induced and manufactured as required by the cell.

5.3.2 Repression

In addition to being able to induce production of enzymes in order to metabolize specific substrates, it is also frequently advantageous for the cell to be able to repress certain metabolic functions. For example, *E. coli* is able to obtain more

energy for growth by using glucose as a substrate than it can obtain from lactose. Thus, if both glucose and lactose are available, *E. coli* would obviously be better off using the glucose first. The cell accomplishes this by means of catabolite repression.

Catabolite repression acts by means of the promoter region of the DNA. The cell normally produces a number of specialized proteins, called catabolite activators. Catabolite activators bind to promoter regions of DNA, greatly increase the affinity for RNA polymerase, and promote the synthesis of particular enzymes. In *E. coli,* these promoter activators must be bound to cyclic AMP (adenosine monophosphate) and normally encourage production of enzymes for metabolizing sugars, such as lactose, arabinose, and galactose. However, when concentrations of glucose in the cell are high, the cell produces ATP via the glycolytic pathway, and concentrations of cyclic AMP are correspondingly low. Cyclic AMP is thus unable to bind to the catabolite activators and the transcription of the lactose-arabinose-galactose-metabolizing enzymes is depressed. This, in turn, allows the cell to concentrate on utilizing glucose via glycolysis. Once glucose becomes depleted, cyclic AMP levels increase, the activator proteins are again able to bind to promoter regions, and production of the lactose-arabinose-galactose enzymes may resume.

Bacterial cells exhibit a very complex interplay between induction and repression of particular metabolic patterns. The efficient interplay of these processes is greatly encouraged by competition between bacteria for scarce resources. Obviously, microorganisms with the most efficient combination of inducible enzyme systems, together with appropriate repression mechanisms, will have a substantial competitive advantage in nature. In ground-water systems, where there is fierce competition for limited resources, such mechanisms have undoubtedly selected for extremely efficient microorganisms. The nature of many of these specialized induction-repression mechanisms, however, is only understood at a rudimentary level. This is a topic of considerable interest for future research.

5.4 MUTATIONS

During the process of DNA replication, the bacterial cell employs several biochemical mechanisms to ensure that mistakes are not made. For example, DNA polymerase enzymes in procaryotes, the enzymes that attach complementary nitrogenous bases to the replicating DNA molecule, often exhibit exonuclease activity; that is, they have the ability to depolymerize a nucleic-acid chain. This allows the polymerase to correct errors in base sequences as needed. Similarly, procaryotic organisms have ligase enzymes whose sole function is to repair "nicks" in the newly synthesized DNA molecule.

One environmental factor that can damage DNA is radiation. Exposure to certain kinds of radiation may cause the formation of covalent bonds between adjacent pairs of thymine on the same strand of DNA. These thymine dimers are harmful, because they prevent the proper functioning of DNA polymerases. Many bacteria, particularly those commonly exposed to sunlight, have evolved mechanisms for repairing this kind of damage. In one mechanism, endonuclease enzymes remove the dimer, polymerase enzymes fill in the gap, and ligase enzymes attach the two ends of the DNA molecule.

These repair mechanisms, together with the inherent reliability of the base-pairing design of DNA, lead to an extremely low rate of errors during DNA synthesis. It is generally observed that copying errors occur at the rate of less than 1 per 100 million base pairs copied.

In spite of this accuracy, however, mistakes do occur. When a single base in the DNA strand is changed, it is referred to as a point mutation. There are several kinds of point mutations. A substitution mutation is when a particular base, say guanine (G), is incorrectly replaced by a different base such as thymine (T). This type of error has the potential for changing one codon. For example, if the sequence AAG on a DNA molecule is mistakenly changed to AAT, the resulting mRNA will no longer code for the amino acid phenylalanine but instead will code for leucine. This difference may be sufficient to inactivate the resulting protein. In addition, because several codons code for the same amino acid, all point mutations do not necessarily result in changes to proteins.

In addition to substitutions, it is possible for a particular base to be added or deleted from the sequence, called addition and deletion mutations, respectively. These kinds of mutations are potentially devastating to the microorganism, because they result in a frame shift in how the genetic code is read. Because the code is read in three-base units, the addition or deletion of one unit changes all of the codons in that particular sequence of DNA. This generally results in the formation of a completely inactive protein.

5.4.1 Mutagenic Agents

Mutations often occur as random events, due only to physical errors made by DNA polymerases. However, certain chemicals and radiation have the potential to interfere with the normal functioning of DNA replication and can cause mutations. For example, under ordinary circumstances, cytosine pairs only with guanine in a DNA molecule. However, hydroxylamine chemically alters cytosine to uracil so that, instead of pairing with guanine, it pairs with adenine. This results in the GC pair being replaced with an AT pair after the DNA molecule reproduces. Hydroxylamine, therefore, is referred to as a chemical mutagen.

In addition to chemically altering nitrogenous bases, several chemicals have configurations similar to nucleotides and can replace them in the DNA structure. For example, 5-bromo-uracil can replace thymine in the DNA structure and pair with adenine. This kind of chemical reaction results in substitution mutations and greatly increases rates of mutation.

The ability of particular chemicals to produce mutations is often evaluated by means of the Ames test. In this procedure, a strain of the bacterium *Salmonella typhimurium* is exposed to the chemical being tested in a range of concentrations and is grown on a medium that lacks histidine. Under normal conditions, *S. typhimurium* cannot synthesize histidine and will not grow. However, at particular concentrations of mutagenic agents, a mutation occurs that allows the bacterium to synthesize histidine and grow on the medium and to produce visible colonies. The Ames test is widely used to test chemicals for mutagenic activity. Because mutations in the human genome may result in cancer, the Ames test is also widely used to determine if a chemical is a potential carcinogen.

Chemicals are not the only source of mutations in bacteria. Radiation, particularly high-energy radiation such as X rays, can physically break DNA and cause

substitution mutations. In addition, ultraviolet light can create covalent linkages between bases on the same strand of DNA, producing thymine dimers. If not repaired, this damage can lead to catastrophic mutation and death of the microorganism. In fact, high doses of ultraviolet light are sometimes used as a sterilization procedure.

5.4.2 Transposable Genetic Material

The locations of particular nucleotide sequences on a chromosome were once thought to be fixed. However, it is now known that particular segments of the bacterial chromosome are capable of moving from place to place. One kind of transposable genetic agent is known as an insertion sequence. These genetic agents have what are termed inverted repeated terminal sequences; that is, they have an identical nucleotide sequence on both ends, such as

G G G T C T G . . . . . C A G A C C C

C C C A G A C . . . . . G T C T G G G

This configuration, for reasons not well-understood at the molecular level, allows the sequence to move around the chromosome inserting itself at different locations. There appear to be particular enzymes involved in translocating insertion sequences, so that base pairing is not the only factor involved in translocation.

Insertion sequences have several possible effects on the expression of an organism's genome. For example, if an insertion sequence is placed in the middle of an operative gene, the protein coded for by that gene will be altered. Similarly, if the insertion sequence is placed at the promoter region of a gene, expression of that gene will be altered.

Insertion sequences are fairly small genetic elements and do not appear to code for particular proteins. Their function in the biology of cells is not always clear, but they may be involved in specifying sites at which DNA recombination may occur. Larger transposable genetic elements that do code for specific proteins are called transposons. Many of the transposons that have been studied code for antibiotic resistance. Furthermore, their ability to detach and to insert themselves in another portion of the DNA molecule has led to their use in genetic engineering applications.

The evolutionary significance of transposable genetic agents in cell biology remains controversial. It is not clear whether these kinds of genetic agents confer a biochemical advantage to individual cells or whether they are simply a mechanism for introducing variability into the expression of genes. It is interesting to note, however, that a new copy of the transposable agent is generated during transposition. This, in some cases, leads to multiple copies of the insertion sequence throughout the genome. This has led some biologists to postulate the existence of subcellular competition between different parts of the genome. If this is true, it may be that transposons are in some cases the biological equivalent of a computer virus, which exists only for self-replication and which may actually be detrimental to the organism as a whole.

5.5 NATURAL GENETIC EXCHANGES

Most procaryotic cells reproduce by binary fission, in which the two daughter cells formed are genetically identical to the parent. However, genetic uniformity is often not in an organism's best interest. Natural selection, the mechanism that allows populations to adapt to changing conditions, requires a source of genetic variability to act upon. Sexual reproduction, which is practiced by some eucaryotic cells, is one method for introducing genetic variability. However, this mechanism is not available to procaryotic microorganisms. As we saw earlier, some genetic variability is conferred by mutations. However, more common means for introducing genetic variability to bacterial populations involves natural genetic exchanges.

5.5.1 Recombination

The term recombination refers to any process by which DNA from two separate cells is combined into a single cell. When recombination occurs, the resulting microorganism retains some of the characteristic of each donor cell. Thus, recombination serves to introduce genetic variability into microbial populations.

When bacterial cells undergo recombination, genetic material from a donor cell is transferred to a recipient cell, and the DNA is physically combined. The process by which the combination of DNA from different organisms is carried out is called integration. The actual mechanisms involved with integration are very complex and remain the subject of active research. However, there are basic steps involved. First, the delivery of DNA is made from the donor to the recipient cell. Second, the two sets of DNA line up to match sites where the DNA can be cleaved with endonucleases. For integration to occur, the strands of DNA must be roughly homologous; that is, the complementary base sequences in the two strands must be similar, although they need not be identical. Third, endonucleases break the two DNA strands at roughly compatible sites. Finally, the donor DNA is joined with the recipient DNA by DNA ligases. Once the DNA has been integrated, it becomes part of the genome of the recipient microorganism and is replicated during cell division. The daughter cells therefore will possess features of both the donor and the recipient cells.

Transformation There are several ways in which DNA is physically transferred among microorganisms, initiating recombination. Transformation is the simplest mechanism for DNA transfer. In transformation, DNA is released from the donor cell when it dies and is then taken up by a living cell and integrated into its genome. Transformation has been observed in a number of different genera of bacteria under laboratory conditions and certainly occurs in the environment. Although transformation appears to be most common between closely related strains, it is possible that interspecies transformation also occurs in nature.

Conjugation Plasmids, extrachromosomal loops of DNA, are capable of conferring the ability for some bacteria to "mate," or exchange DNA, with adjacent cells. The process by which bacteria cells exchange DNA by this means is called conjugation.

A closely studied example of this novel behavior is the *F* (fertility) plasmid in some strains of *E. coli.* The F plasmid codes for the formation of proteinaceous pili, often called sex pili, that serve to connect two cells. Strains of *E. coli* that have the F plasmid (F^+) are able to form a sex pilus and attach to cells that lack the F plasmid (F^-). Once the connection is made, DNA from the F^+ cell is transferred to the F^- cell and either incorporated into the chromosomal DNA of the recipient cell or maintained as an independent plasmid. When the F plasmid is incorporated into the chromosomal DNA, the resulting strain retains the capability for forming sex pili and is referred to as an Hfr (high frequency of recombination) strain.

Hfr strains maintain the ability to form sex pili and hence the ability to transfer DNA. In this case, however, a portion of the chromosomal DNA, rather than just the F plasmid portion, is transferred. In fact, the portion of DNA transferred often lacks the F plasmid so that the resulting cell usually remains F^-. However, the recipient cell has obtained a large portion of the donor cell's DNA, and daughter cells exhibit a corresponding share of the donor's characteristics.

Transduction In addition to genetic transfers between bacteria by means of transformation and conjugation, genetic material may also be transferred between bacteria by viruses. Many kinds of viruses, termed bacteriophages, are parasites of bacteria. In the process of parasitizing bacteria, viruses may act to combine genetic material from different cells.

When viruses are involved in genetic transfers between bacteria, the process is referred to as transduction. When a virus infects a bacterial cell, the viral DNA often integrates with the chromosomal DNA of the bacterium. When the virus has reproduced itself and leaves the cell, it occasionally carries portions of the host's chromosomal DNA. When the virus infects a second host cell, these fragments of DNA are integrated into the new host.

Transduction by bacteriophage lambda, a virus that infects *E. coli* cells, has been extensively studied. Lambda is capable of entering the cell and incorporating its DNA into the genome of its host. When lambda enters a cell, it binds to the host DNA at one particular place, between the genes coding for galactose and biotin production. Normally, when the virus leaves the host DNA, it detaches from the chromosome, leaving it unchanged. Occasionally, however, the genes for galactose or biotin are detached with lambda and can be carried to a new *E. coli* cell. Once in the new cell, the lambda virus carrying, say, the galactose genes is defective and cannot attach to its normal binding site. However, if a normal lambda phage attaches to the binding site, the galactose end of the defective lambda can bind with the normal phage and the normal end of the defective lambda can bind with the chromosomal DNA. This produces an *E. coli* variant that contains two sets of genes coding for the production of galactose.

5.6 DNA TECHNOLOGY

Humans have been manipulating DNA for thousands of years. It's impossible to know just when people first realized that certain characteristics of plants and animals could be selectively bred for. However, we do know that domestic dogs di-

verged from the gray wolf between 15,000 and 10,000 years ago. We also know that the domestication of wheat occurred between 12,000 and 10,000 years ago. Thus, it is evident that selective breeding—or, in other words, DNA manipulation—has been practiced by humans at least for 10,000 years.

But selective breeding is a difficult way to manipulate genes. In the 1970s, it became possible, for the first time, to manipulate DNA directly in vitro. This, in turn, made it possible to synthesize new DNA molecules and introduce them into living organisms. These methodologies, which are variously termed "genetic engineering," "gene splicing," or "recombinant DNA," make it possible to select for given genetic characteristics at the molecular level.

In this section, an overview of the methods and techniques for manipulating DNA is given. These methods are becoming important tools in microbial ecology, and have greatly expanded our ability to examine microbial communities in subsurface environments. In addition, they have expanded our ability to probe these communities for specific metabolic capabilities. As such, they are and will continue to be important tools for studying subsurface microbiology.

5.6.1 Analyzing DNA

When the structure of DNA was first deduced by Watson and Crick in 1953, scientists immediately began looking for ways to study and manipulate nucleotide sequences. Genes, after all, are discrete nucleotide sequences. If it was possible to determine exact nucleotide sequences, it might be possible to understand how genes worked. In the 1950s and 1960s, this seemed to be an impossible task. After all, even in bacterial chromosomes, DNA molecules are enormously long. The chromosome of *E. coli,* for example, contains 4.6 million nucleotides. These DNA molecules could be physically broken, of course, but separating and identifying individual genes did not seem possible.

Restriction Nucleases This began to change in the 1970s with the discovery of restriction nucleases in bacteria. A nuclease refers generically to any enzyme that hydrolyzes a phosphodiester bond in a nucleic acid. But restriction nucleases have the additional property that they cut DNA only at particular sites characterized by particular sequences of nucleotides. These enzymes were discovered when it was noticed that genetic exchanges were resisted by certain bacteria. Specifically, when it was attempted to introduce DNA into some bacteria, the DNA was always degraded. A search for the cause of this phenomenon revealed the presence of nucleases that catalyzed DNA degradation. They were named restriction nucleases because they restricted the transfer of DNA between certain strains of bacteria.

The fact that restriction nucleases cut DNA only at specific nucleotide sequences makes it possible to directly probe DNA for particular sequences. For example, if DNA from a particular microorganism is digested with one restriction nuclease, the distribution of fragments obtained will be specific to that microorganism. The way this is done is shown schematically in Figure 5.6. First, DNA is extracted from a microorganism. Second, the DNA is treated with a particular restriction nuclease such as Eco RI. Next, the fragments of DNA are separated using gel electrophoresis. Gels, usually made of argose or polyacrylamide, contain a microscopic network of pores that allows smaller DNA fragments to pass easier than do larger

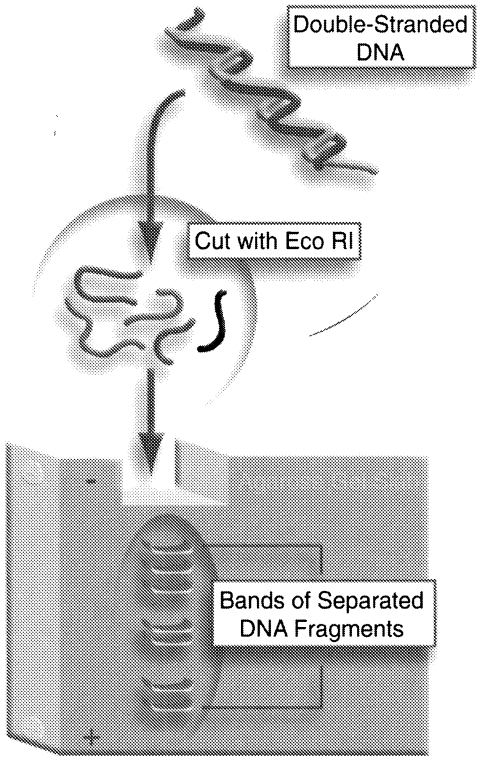


Figure 5.6. Characterization of DNA using a restriction enzyme (Eco Ri) and gel electrophoresis.

fragments. When a voltage is applied to the gel, negatively charged DNA migrates toward the positive pole of the electrical field and the resistance of the gel separates the DNA fragments by size. After a certain amount of time, the "bands," or DNA fragments of a given size, can be viewed either by staining methods or by radiolabeling the DNA with ^{32}P and using autoradiography. The pattern of bands that results is characteristic of that specific DNA molecule. This, in turn, made it possible for scientists to begin comparing differences in DNA composition between found in different microorganisms.

Sequencing DNA The use of restriction enzymes provided scientists with a way to "map" the location of restriction enzyme cleavage points in DNA, which provided much information. Later, techniques were developed for deducing the actual sequence of nucleotides present in fragments of DNA (Sanger et al., 1977). There are now several methods now available to sequence DNA. One of the most common current methodologies is illustrated in Figure 5.7. This method uses the fact that a derivative of deoxyribonucleoside triphosphates—called dideoxyribonucleoside triphosphate—lacks a hydroxide group. The lack of this hydroxide group stops the DNA polymerization process. When dideoxyribonucleoside triphosphate is linked to a given nucleotide, the resulting molecule will stop polymerization of the molecule at that nucleotide. Thus, if small amounts of the four bases GCT and A are

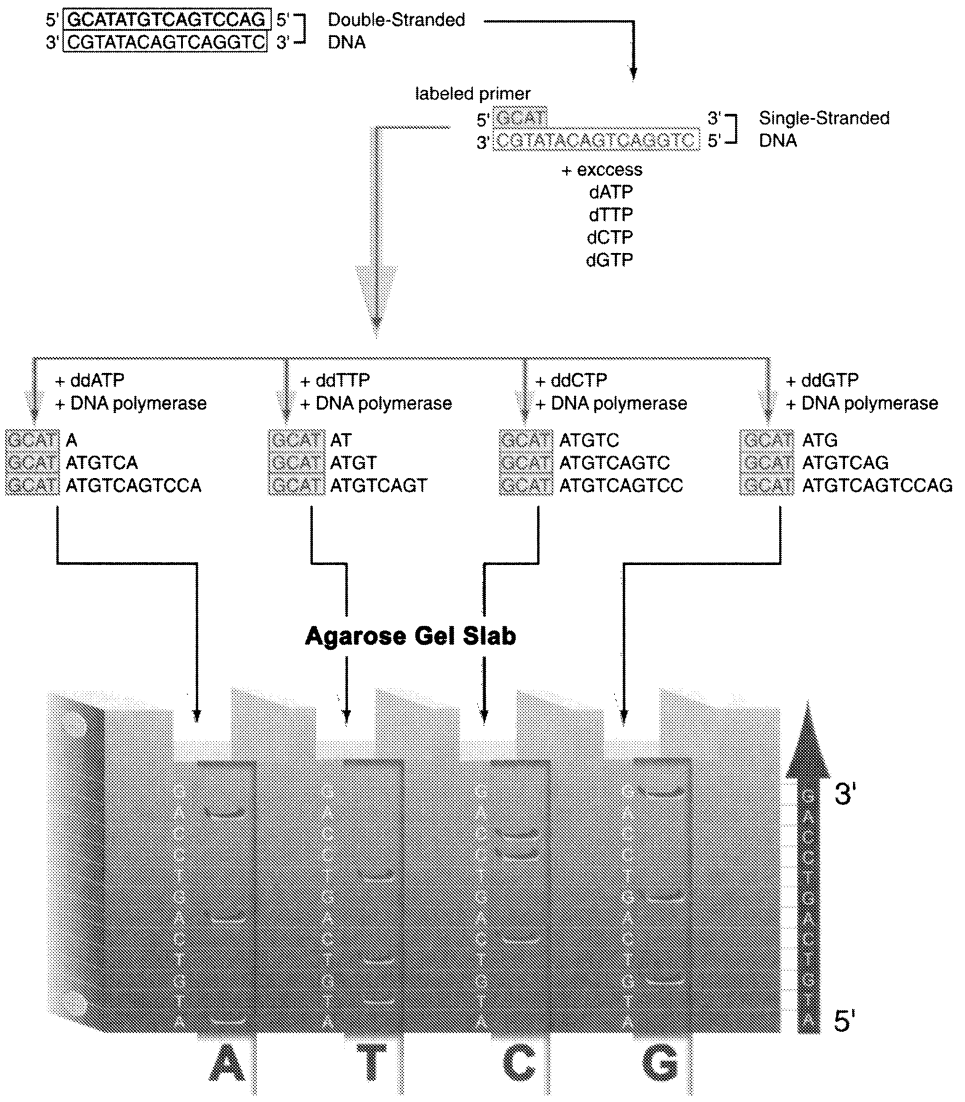


Figure 5.7. Methodology for sequencing DNA.

linked with dideoxyribonucleoside (ddG, ddC, ddT, and ddA) and mixed with normal deoxyribonucleoside linked to the four bases (dG, dC, dT, and dA), mixed with many copies of an unknown single strand of DNA, a ^{32}P-labled primer, and DNA polymerase, the polymerization for each four treatments will stop at known points. By lining up the resulting gel bands, the sequence of the DNA can be deduced (Fig. 5.7).

Hybridization Once a gene of particular interest has been isolated from a bacterial genome—for example, the genes coding for 16S RNA—it may be of interest to look for those genes in other microorganisms. This can be done by taking ad-

vantage of the double helical nature of DNA. The two strands of the DNA double helix are linked by relatively weak hydrogen bonds. These bonds can be broken using heat, or by raising or lowering the pH. This results in the DNA being denatured, or, in other words, the two complementary strands split apart. This is a reversible process, and if the temperature is lowered or the pH returned to neutral, the two strands will bind together again, or hybridize. Because DNA will hybridize only with its exact complement, this provides a powerful tool for identifying specific DNA sequences.

In order to use hybridization to search for specific DNA sequences, it is first necessary to have a length of nucleic acid to search with. These short lengths of single-stranded DNA, often called DNA probes, or oligonucleotides, are typically between 10 and 1000 nucleotides long. These probes can be obtained from natural sources, such as directly from microorganisms, or they may be artificially synthesized. One common way that probes are used, called Southern blotting, is illustrated in Figure 5.8. DNA from the target organism is first treated with restriction enzymes to cut it into fragments. These fragments are then separated by electrophoresis onto a gel. An alkali buffer is then drawn through the gel, which denatures the DNA into single strands and adheres it to nitrocellulose paper. This paper is then treated with a ^{32}P labeled probe. If the probe sequence is present in the DNA, the probe hybridizes with its complement, and the labeled bands can be visualized using autoradiography.

Hybridization is a powerful tool for analyzing DNA for specific sequences, and thus analyzing bacteria for specific functions. This technique has a wide range of applications in environmental microbiology, microbial taxonomy, and microbial ecology.

5.6.3 DNA Cloning

We have seen that DNA can be cut into fragments with restriction enzymes, that these fragments can then be separated and concentrated by gel electrophoresis, that the sequence of nucleotides can be determined, and that DNA probes for particular nucleotide sequences constructed. Now we will consider how these procedures can be used to make clones, or copies, of particular DNA sequences.

We have also seen how restriction enzymes cut DNA. DNA ligase performs the opposite task—it acts to join strands of DNA together. The combined use of restriction enzymes and DNA ligase makes it possible to insert pieces of DNA into microorganisms. When these microorganisms replicate, they make millions of copies of the DNA fragment.

The way this is usually done is shown schematically in Figure 5.9. First, a closed circular strand of plasmid DNA, called a cloning vector, is selected. The cloning vector is then cut using a restriction nuclease to open the circle. Next, the fragment of DNA to be cloned is inserted into the plasmid, and the ends joined together and sealed with DNA ligase. The plasmid is then inserted into bacterial cells, and the cells allowed to replicate. This typically produces millions of copies of the original DNA fragment. This DNA can then be recovered and purified by lysing the cells, centrifuging to separate the plasmid DNA from chromosomal DNA, and the clones of the original DNA fragment recovered using appropriate restriction enzymes.

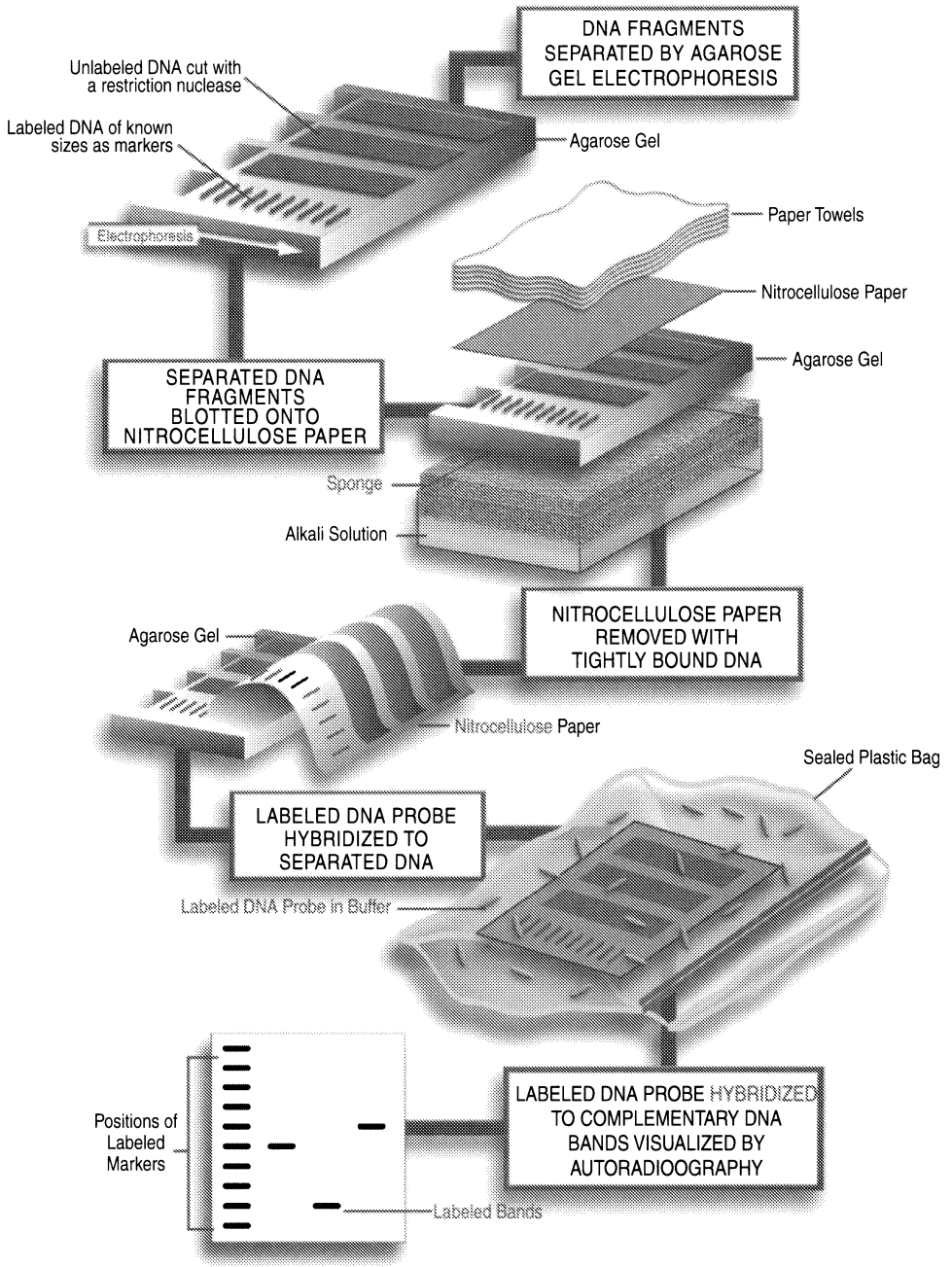


Figure 5.8. The Southern blot method of probing DNA for particular sequences.

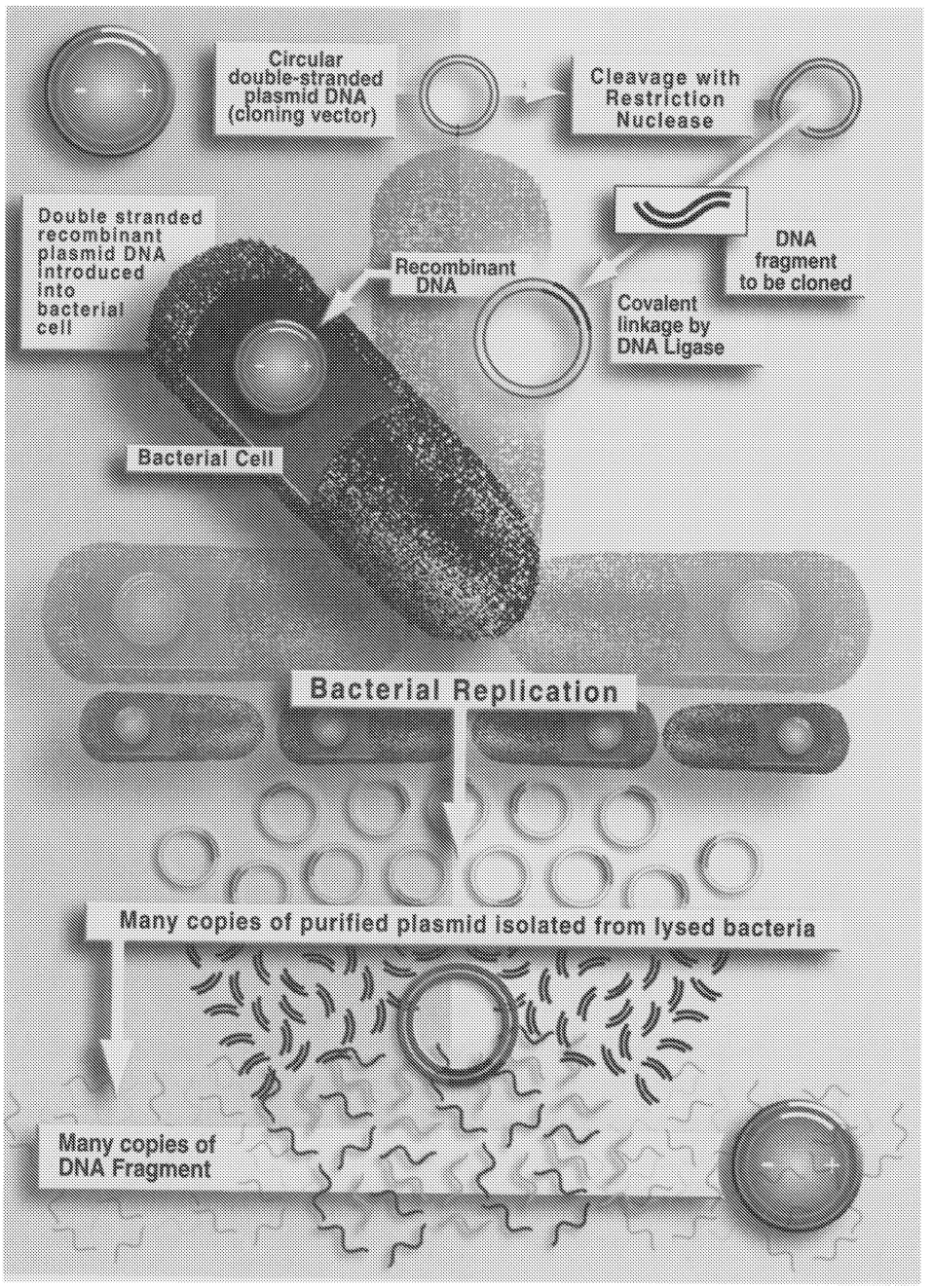


Figure 5.9. An in vitro method for cloning fragments of DNA.

The Polymerase Chain Reaction Prior to about 1988, cloning genes by inserting them into bacterial plasmids was the only efficient method of coping fragments of DNA. However, the polymerase chain reaction (PCR) is a method for replicating DNA entirely in vitro without the use of plasmids or bacterial cells. This process is conceptually very simple, and is illustrated in Figure 5.10. First, a fragment of double-stranded DNA is heated to about 100°C, which separates the DNA strands. Next, the temperature is lowered to about 70°C, and a short oligonucleotide, called a primer, is attached to each strand of DNA. Finally, a DNA polymerase that can withstand the elevated temperatures is added to the mixture. This high-temperature polymerase was first isolated from a microorganism, *Thermus aquaticus,* that was isolated from a hot spring in Yellowstone National Park. Thus the polymerase derived from this microorganism is named Taq. The primer guides the Taq polymerase to the place where replication is desired and initiates, or "primes," the reaction. The polymerase completes the synthesis of DNA, giving two double-stranded fragments of DNA where once there was one. Repeated cycles of this replication process can be used to make as many as a million copies of one original DNA fragment.

Denaturing Gradient Gel Electrophoresis (DGGE) Standard gel electrophoresis is a powerful tool for analyzing the relative sizes of DNA fragments generated by treatment with restriction nucleases. An important variation of this technique, which has wide application in analyzing the products of PCR, is called denaturing gradient gel electrophoresis (DGGE).

The theory behind DGGE is simple. The complementary strands of a DNA molecule, which are held together by hydrogen bonds, can be separated if those hydrogen bonds are subjected to sufficient force. For example, if DNA is simply heated, the increased kinetic energy will separate, or "melt," the strands. Chemicals such as urea or formamide can also separate, or "denature," DNA molecules. DGGE uses the fact that DNA molecules, which may differ only by a few nucleotide sequences, will separate with different efficiency. Those DNA molecules that separate more easily will become entangled in the gel matrix and become immobilized earlier in the gel. With DGGE, a gradient of DNA-separating mixture of urea and formamide is prepared within the gel. This allows easily separated strands of DNA to be efficiently differentiated from strands more resistant to separation.

Complete strand separation during DGGE is usually prevented by the presence of a "GC clamp" attached to one end of the DNA molecule. Because the hydrogen bonds between G and C are relatively strong, a relatively long sequence of G-C bonds will prevent complete strand separation. This clamp can be added during PCR amplification using a PCR primer that has a sequence of about 40 GC on it's 5′ end. After being reconstituted during PCR, the clamp prevents complete separation of the DNA during DGGE and facilitates differentiation of DNA strands that may differ only by one or two nucleotide sequences.

One important application of DGGE is to select particular DNA strands for reamplification during PCR. After separation on a DGGE gel, particular bands of DNA can be excised using a knife or razor blade. This particular DNA can then be PCR-amplified in order to provide purified samples for rDNA sequence determination. Thus, DGGE followed by PCR provides a way of bypassing cloning as a way of generating sufficient DNA for sequencing analysis (Fig. 5.10).

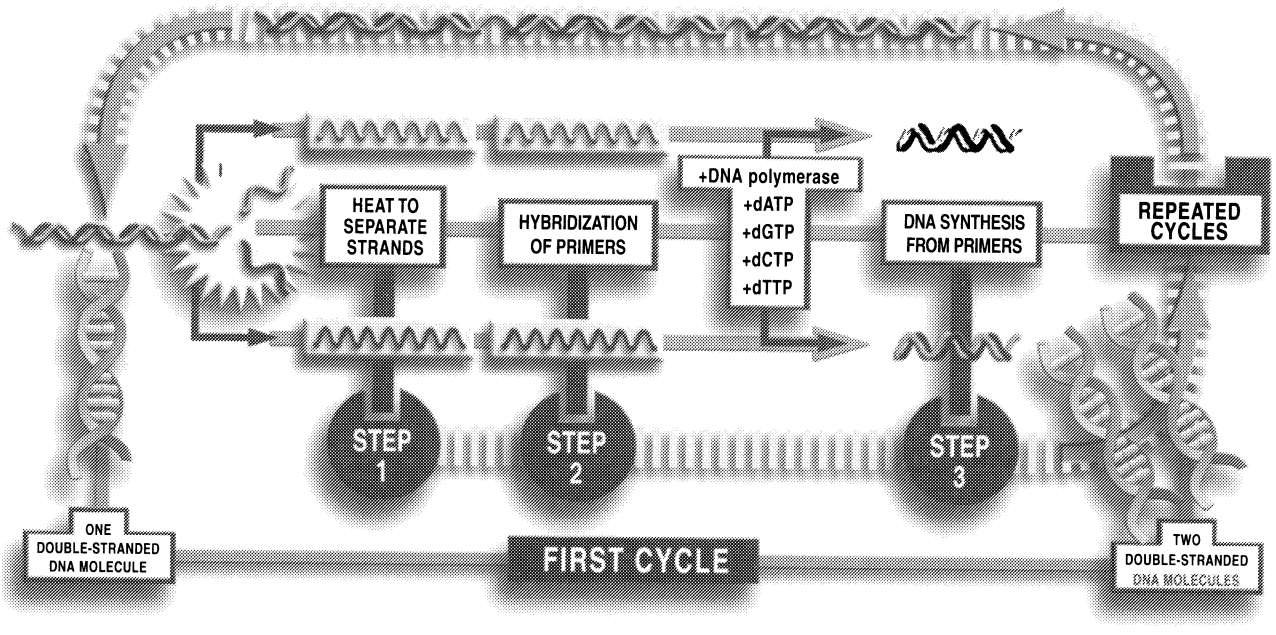


Figure 5.10. Using the polymerase chain reaction to copy DNA.

5.7 GENETIC ENGINEERING

In preceding sections, we have seen that microorganisms can transfer genetic information among themselves in the absence of sexual reproduction. These genetic transfers are advantageous to individual species, as they increase genetic variability and provide leeway for species to evolve in the face of changing environmental conditions. Since the 1970s, however, microbiologists have learned how to manipulate these natural DNA-transferring mechanisms in the laboratory, and they have learned to manipulate DNA in vitro. These techniques have enabled scientists to insert specific genetic capabilities into specific microorganisms. This, in turn, enables microbiologists to "program" cells to produce particular products or to carry out useful functions. The technology that enables humans to manipulate the genomes of bacterial cells in this manner is called genetic engineering.

To many people, the term "genetic engineering" brings to mind images of Frankenstein and of scientists creating other horrible monsters. The truth is more mundane. Genetic engineering simply redirects natural processes of transformation, conjugation, and transduction, or uses naturally occurring restriction nucleases, ligases, and polymerases in order to place certain genes in the genome of certain bacteria. This process serves exactly the same function as selective breeding does in plants or animals. The difference is that, instead of focusing on the macroscopic exchange of genetic information (breeding), the focus is on exchanging genetic information at the molecular level. It is important to remember that all of the techniques for engineering microorganisms mimic naturally occurring gene-transferring processes.

5.7.1 Plasmids

The occurrence of plasmid DNA in bacterial cells has been recognized since about 1960. The first studies of plasmids were motivated by the observation of antibiotic resistance in strains of pathogenic bacteria. These "R factors" present in the bacteria could be traced by epidemiological (epidemiology is the study of disease patterns) techniques, implying that some sort of infectious agent conferred the antibiotic resistance. It was quickly realized that these agents were very similar to viruses, since they were capable of replicating themselves within bacteria and were capable of moving between bacterial cells. In fact, plasmids capable of transferring between cells by means of conjugation have been termed "epiviruses." Many microbiologists do not make a logical distinction between plasmids, bacteriophages, transposons, or insertion sequences, preferring, rather, to consider them as one family of unnamed parasitic organisms. In this view, the only difference between viruses and plasmids is that viruses may produce many copies of themselves within bacterial cells, whereas plasmids (as well as transposons and insertion sequences) replicate as the bacterial cell replicates.

Putting philosophical questions aside, the fact is that many genetic-engineering techniques are based on manipulating plasmid DNA in bacteria. Plasmid DNA has the advantage of being easy to isolate from cells, easy to manipulate as desired, and easy to reintroduce into other cells. The first step is to lyse the cells and concentrate the plasmid DNA. In the second step, the plasmid DNA is cut at particular points by restriction endonucleases. These restriction enzymes bind to spe-

cific nucleotide sequences and cut the DNA molecule at that point. An important feature of restriction enzymes is that they break the DNA molecule unevenly, leaving "sticky ends," or ends that have the ability to base-pair. Because restriction enzymes act only on specific sequences, different strands of DNA can be cut so that they have complementary sticky ends. If different DNA strands that have been treated with the same restriction enzyme are mixed, the different fragments will bind together at the sticky ends. These fragments can then be covalently bonded together by treating the mixture with DNA ligase.

This procedure, termed in vitro recombination, is shown schematically in Figure 5.11. First, a plasmid and a segment of foreign DNA are treated with the same restriction endonuclease. Ideally, the plasmid is cleaved at one site, whereas the foreign DNA is cleaved at two locations. This allows the foreign DNA to be inserted into the plasmid. The foreign genes are then covalently bonded into the new plasmid by means of DNA ligase.

5.7.2 Vectors

Once the new molecule of DNA has been synthesized, the next step is to insert it into a cell. As before, these insertion procedures are based on processes carried out naturally by the cell. DNA can sometimes be introduced into cells by transformation. The most common technique for transformation is to treat the recipient bacteria with calcium chloride to make the cell more permeable to DNA. The mechanism of this transformation is neither well understood nor particularly efficient. However, transformation of *E. coli* cells with some small plasmids yields a success rate of only about 1 in 10,000 to 1 in 100,000 DNA molecules. For some applications, this low success rate will suffice. For other applications, however, the new plasmid is carried directly into the target cell by active transport systems called vectors. This greatly increases the efficiency of genetic transfer.

Plasmid Vectors Recall that some plasmids, such as the F plasmid in *E. coli,* carry genetic instructions for conjugation and can move from cell to cell by means of pili. These plasmids can be engineered to carry foreign DNA into cells directly. These plasmids, which are very useful in genetic-engineering applications, are called plasmid vectors.

The strategy in using plasmids for carrying foreign genetic material into a new cell involves several steps. First, the plasmid is cleaved at an appropriate place and the genes to be transferred are inserted. Next, the plasmid is inserted into a host cell by means of transformation. Once transformed, the plasmid-containing cells are capable of conjugation with adjacent cells and subsequent DNA transfer. Most plasmid vectors are specific for *E. coli.* However, a few plasmids, called promiscuous plasmids, are capable of existing in other gram-negative bacteria. Promiscuous plasmids are important, because they extend cloning technology beyond *E. coli* to other genera, such as *Pseudomonas.*

Once inserted, the first step is to identify those cells that contain the new genetic material. A common technique for such identification is to place antibiotic resistance genes on the plasmid vector. After conjugation, the resulting hybrids are plated onto media containing antibiotic. Only cells containing the desired plasmid can grow on the antibiotic-laced media, and they can be readily purified. Once the

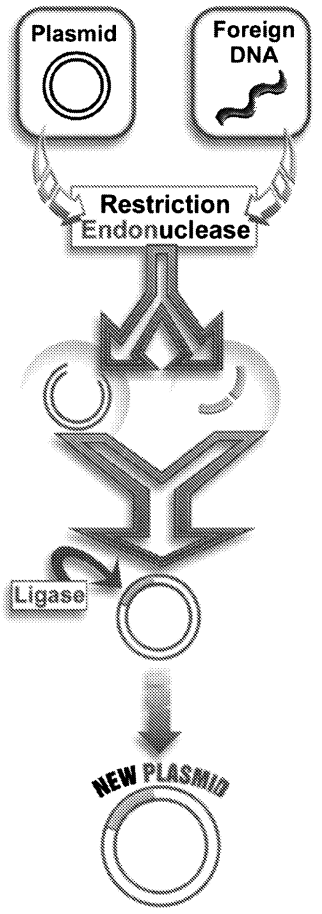


Figure 5.11. An example of in vitro recombination.

transformed cells are purified, they are allowed to reproduce, which creates numerous copies of the new plasmid. The process of mass-producing copies of the plasmid is termed cloning, as the idea is to obtain identical copies of the DNA. The cloned DNA can then be concentrated and inserted into additional cells as needed.

Phage Vectors There are many different known bacteriophages. However, only lambda has been extensively used as a vector in genetic engineering technologies. A major drawback of using lambda as a vector is that it limits the size of the foreign DNA insert. Two major types of lambda are used in genetic engineering. The first has a single restriction enzyme site within a known gene. This allows insertion of foreign DNA into a particular site. When DNA is cloned into this site, the gene is inactivated, allowing the new phenotype to be identified. The second type of phage vector has two targets for the restriction enzyme. This allows replacement of part of the phage's DNA with foreign DNA. If the foreign DNA has

a readily recognizable phenotype or, alternatively, if the deleted DNA sequence is recognizable, the transduced cells can be readily identified.

5.8 APPLICATIONS OF DNA TECHNOLOGY

Genetic-engineering technology has created a whole new industry since the mid-1970s. Prior to these technologies, often the only way to mass-produce certain biomolecules was by means of cumbersome chemical techniques, followed by extensive purification procedures. With the advent of recombinant DNA technology, however, it became possible to direct living cells to produce the biochemicals. This made it possible to produce useful chemicals in commercially feasible quantities. Furthermore, these chemicals could be produced in unprecedented purity.

5.8.1 Insulin Production

Perhaps the best-known example of how recombinant DNA technology has been used for practical benefit is the production of human insulin. Prior to 1983, all insulin used by human diabetics was manufactured from the pancreases of slaughtered cows or sheep. Unfortunately, cow insulin is slightly different from human insulin, and many diabetics became allergic to it. The genes for making human insulin were recovered from pancreatic cells, recombined with plasmids, and inserted into *E. coli* cells. The plasmids enabled the *E. coli* cells to mass-produce human insulin, which could be recovered by harvesting the cells. Presently, much of the insulin used by diabetics in the United States is produced by this technology.

5.8.2 Enhanced Biodegradation

There has been much interest in producing genetically engineered cells for environmental applications. One of the first efforts in this direction was the work of Ananda Chakrabarty and his associates in engineering microorganisms to contain hydrocarbon pollution. More recent efforts have been focused on engineering microorganisms to degrade a wide range of xenobiotic halogenated hydrocarbons.

Aliphatic Petroleum Hydrocarbons The steps taken by Chakrabarty in the early 1970s to engineer an aliphatic hydrocarbon-degrading bacterium are a good example of how recombinant DNA techniques have been used in environmental applications. The investigators knew that certain strains of *Pseudomonas* had the capability for degrading particular aliphatic hydrocarbons. Specifically, they isolated a strain of *Pseudomonas aeruginosa,* designated strain AC59, that rapidly degraded C_6, C_8, and C_{10} aliphatic hydrocarbons but that was not capable of degrading C_{12} or C_{14} hydrocarbons. Strain AC59 harbored a plasmid, termed the "OCT plasmid" (for "octane"), that conferred this ability. Next, they isolated another *P. aeruginosa* strain, designated strain AC63, that grew rapidly on C_{12} and C_{14} aliphatic hydrocarbons but that could not grow on C_6, C_8, or C_{10} hydrocarbons. The ability to utilize these shorter molecules was conferred by the DOD plasmid.

Unfortunately, both of these plasmids were nontransmissible. However, it was possible to splice the OCT plasmid with a transmissible CAM plasmid. The

CAM-OCT plasmid, when transferred to strain AC63, resulted in a strain capable of metabolizing aliphatic hydrocarbons from C6 through C18.

Chakrabarty's research is notable for historical and legal reasons, as well as for technical reasons. Chakrabarty planned to use his engineered microorganism to help remediate oil spills. However, once introduced into the environment, the microorganism could conceivably be reisolated by other individuals and used at other spills without Chakrabarty's permission. Therefore, Chakrabarty applied for patent protection for his engineered microorganism. This patent application was challenged, and the issue was placed before the U.S. Supreme Court. In 1980, in *Diamond v. Chakrabarty,* the Supreme Court ruled that the microorganism constituted a "manufactured" product and therefore was subject to patent protection. This ruling gave a tremendous boost to applying recombinant DNA techniques to environmental problems, because it affirmed that those investing in engineering useful microorganisms could expect to have their investments protected.

Aromatic Hydrocarbons Since the early 1970s, an astonishing amount of information has been learned about the manner in which plasmids encode for particular catabolic processes. One particular plasmid that has been extensively studied is the TOL plasmid, which codes for the degradation of toluene, xylene, and related aromatic hydrocarbons. Various configurations of this plasmid occur in species of the genus *Pseudomonas*.

There are at least 11 separate steps in the overall process, each of which are catalyzed by different enzymes. The "upper" pathway enzymes, which are involved in oxidizing the methyl group of toluene to form benzoate, are coded for in one operon. The "lower" pathway enzymes, which are involved in cleaving the ring, are coded in another operon. The pathway is therefore organized into two functional parts: (1) the conversion of the hydrocarbons to aromatic carboxylic acids, and (2) the conversion of these carboxylic acids to aliphatic metabolites. The production of the enzymes involved in this pathway is induced by toluene, xylene, and related compounds.

In addition to illustrating the complexity of plasmid-mediated degradation pathways, the TOL plasmid is a good example of how plasmids can be manipulated in order to confer desired traits on bacterial strains. One good example of these kinds of manipulations was worked out in K. N. Timmis's laboratory in the early 1980s. These investigators described how they "recruited" genes from the TOL plasmid for the lower pathway enzymes, and by a series of transfers succeeded in building a new plasmid (Fig. 5.12). They started with a plasmid capable of degrading 3-chlorobenzoate (i.e., 3CB+) and attempted to add genes from the TOL plasmid that would confer the ability to degrade 4-chlorobenzoate (4CB). The problem was to be able to transfer both the degradative genes, D and L, with the promoter gene, S. Without the promoter gene S, the degradative genes would not be expressed and therefore would be of no use. Unfortunately, cleaving the TOL plasmid with the s endonuclease separated the degradative genes from promoter genes.

In order to solve this problem, the S gene was isolated by cleavage with the x endonuclease and inserted into an intermediate plasmid that already coded for the degradation of 3-chlorobenzoate. The D L E G genes were cleaved separately and inserted into a different intermediate plasmid. These genes were then cloned, copied by allowing the *Pseudomonad* strains to reproduce, in preparation for the final step.

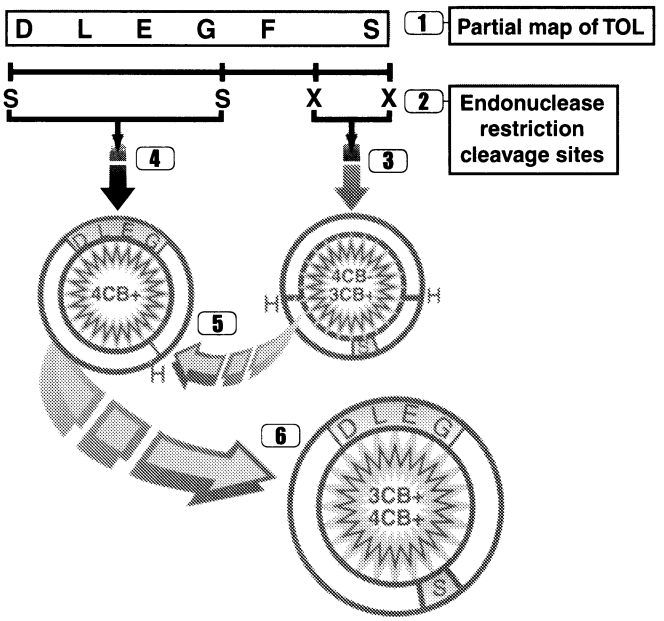


Figure 5.12. Construction of a degradative plasmid by genetic engineering.

In the final step, the S gene, together with the genes for degrading 3CB, were removed from one intermediate plasmid with the H endonuclease and inserted into the other plasmid at a single H endonuclease site. The resulting plasmid could catabolize 4-chlorobenzoate in addition to 3-chlorobenzoate. The net result, therefore, was an increase in the degradative capacity for the plasmid.

5.9 DNA TECHNOLOGY IN SUBSURFACE MICROBIOLOGY

DNA technology has several actual and potential applications to subsurface microbiology. These include phylogenetic analysis of subsurface isolate, probing isolates for contaminant-degrading capabilities, and describing ecological diversity (Chapter 6). In this section, examples of these applications to subsurface microbiology is given

5.9.1 Phylogenetic Analysis of Fe(III)-Reducing Bacteria

Microorganisms that couple the oxidation of organic matter with the reduction of Fe(III) are important components of the flora present in surface and subsurface sedimentary environments. The first microorganism, *Geobacter metallireducens,* shown to grow with this metabolism was isolated from the bed sediments of a river (Lovley and Phillips, 1988). Subsequent to the isolation of *G. metallireducens,* strains of Fe(III)-reducing microorganisms were also isolated from a Cretaceous-age deep subsurface environment (Lovley et al., 1990), and from a petroleum hydrocarbon-contaminated aquifer. In addition, several strains other microorganisms

capable of reducing Fe(III) were found in existing culture collections. Interestingly, it was found that several species of microorganisms formerly thought to grow only with the reduction of sulfur (S^0) could also grow with the reduction of Fe(III). Given this variety of Fe(III)-reducing microorganisms, the question naturally arises as to how closely related they are. For example, is Fe(III) reduction characteristic of only closely related species, or is Fe(III) reduction more widely spread and found in distantly related species?

To answer these questions, Lonergan et al. (1996) undertook a phylogenetic analysis of known Fe(III)-reducing microorganisms. Their procedure was to isolate DNA from each microorganism, treat the DNA with a restriction enzyme (Rnase) that cuts out the 16S RNA genes, amplify these genes using PCR techniques, and sequence them (Fig. 5.13). The sequences (about 1,200 base pairs) were then aligned, and the differences between species determined. Finally, statistical analysis was used to evaluate the relative relatedness of each strain.

The results of Lonergan's analysis are shown in Figure 5.14, and show that the members of the genera *Geobacter, Desulfuromonas, Pelobacter,* and *Desulfuromusa,* all of which can use Fe(III) and/or S^0 as terminal electron acceptors, are linked phylogenetically and can be grouped into a single family, Geobacteraceae. However, the analysis also shows that the capacity to reduce Fe(III) is widely distributed through the bacteria, and not specific to the Geobacteraceae. The wide phylogenetic dispersion of the Fe(III) reducers suggests that Fe(III) reduction evolved relatively early in earth's history, and that it is possible that Fe(III) and/or S^0 reduction was one of the first mechanisms of respiration that evolved. Also,

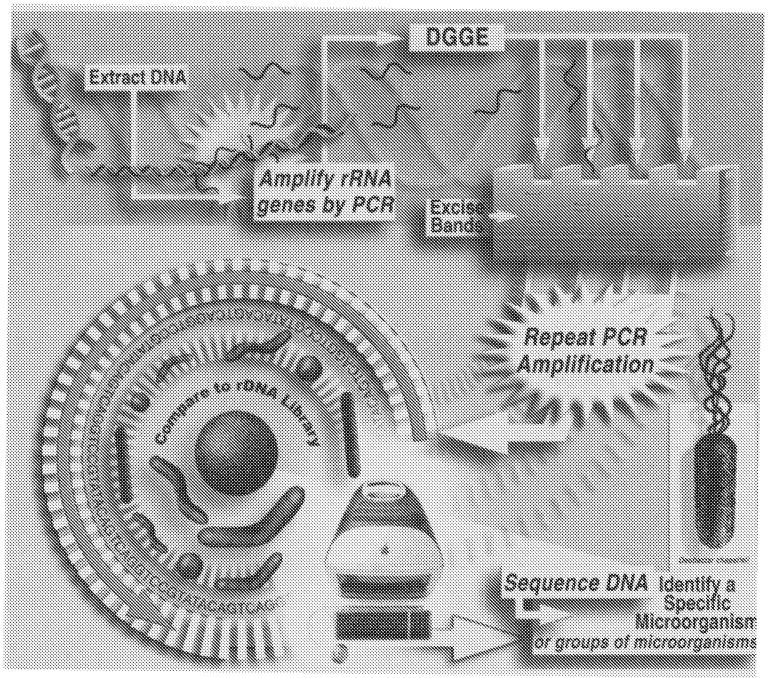


Figure 5.13. Identifying specific microorganisms present in the environment using a molecular biology approach.

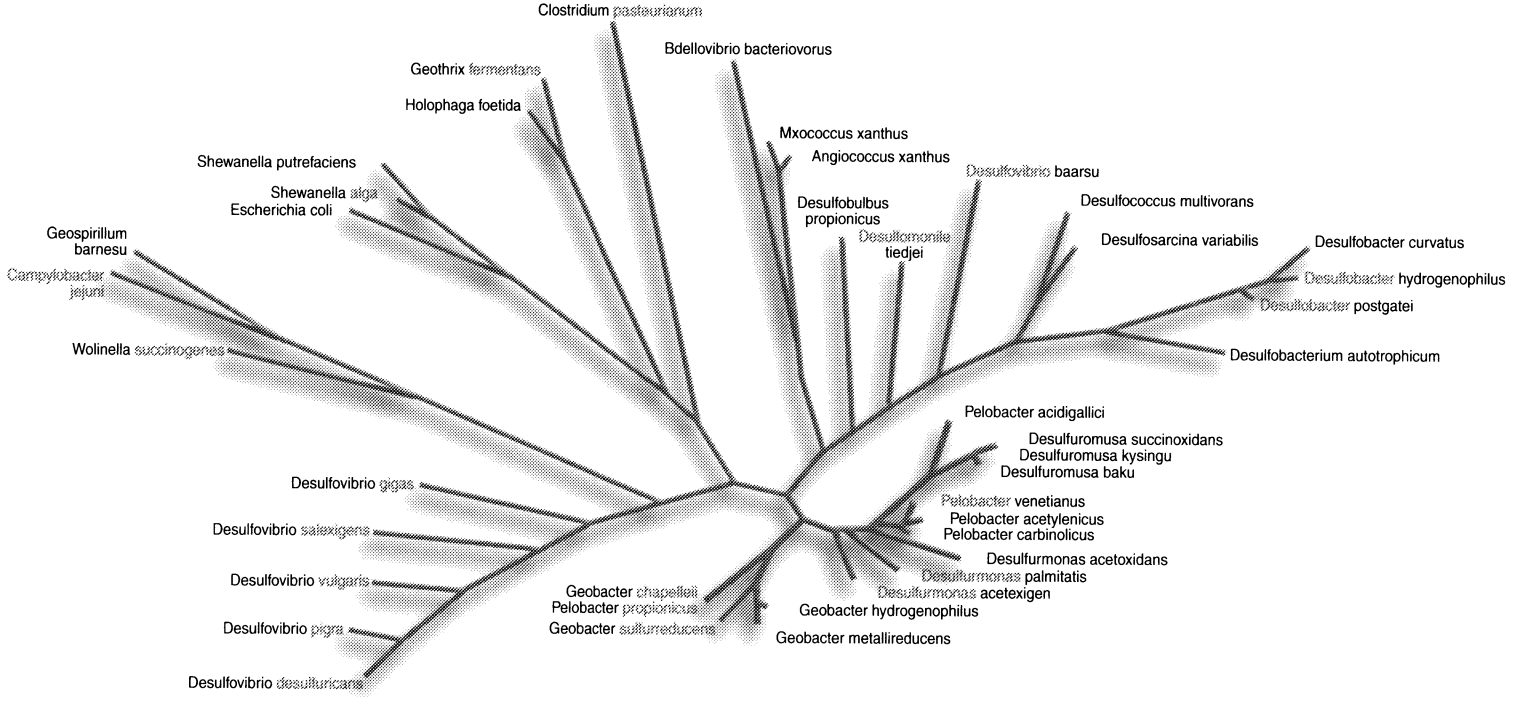


Figure 5.14. Phylogenetic analysis of bacteria capable of Fe(III)and/or S^0 reduction.

the results suggest that recovering DNA directly from sediments in the environment, and probing for specific 16S rRNA sequences characteristic of the Geobacteracea, may be a useful tool for identifying Fe(III)-reducing bacteria without using culture methods.

5.9.2 Using DNA Probes to Find Contaminant-Degrading Microorganisms

The ability of plasmids to reproduce is intimately tied to the reproductive success of their host microorganisms. Those plasmids that confer functions that are useful in particular environments will tend to enhance the survival of the microorganism—and thus the plasmid—in those environments. Conversely, plasmids that confer functions not needed for a particular environment may actually be a hindrance to microorganisms if energy resources are spent on unproductive functions. Thus, the kind of plasmids inhabiting microorganisms from a particular environment may give some information about the kinds of stresses encountered.

The best example of this general principle comes, not from natural systems, but from hospitals. Hospitals are an environment in which microorganisms are much more likely to encounter antibiotics than, say, pristine aquatic sediments. Not surprisingly, since the advent of antibiotics in 1945 the incidence of plasmid-mediated antibiotic resistance has increased enormously in hospitals. Those plasmids that conferred antibiotic resistance gave their host microorganisms a competitive advantage over strains that lacked the plasmid. Consequently, both the plasmid-bearing microorganism and the plasmid itself were able to thrive where other bacteria were simply killed. This resistance does carry a price, however. In some cases, it has been documented that some hospital microorganisms are so loaded down with resistance-encoding plasmids and so used to not having to compete with other microorganisms that they are unable to survive outside of the hospital environment.

Plasmids are commonly present in soil, aquatic sediment, and aquatic bacteria. For example, about 50% of the marine bacteria living at the air–water interface exhibit the presence of plasmids. In shallow aquatic sediments, generally about 10% of isolates contain plasmid DNA. Since most plasmids present in environmental strains of bacteria have not been characterized, it's very difficult to say what useful functions, if any, these plasmids perform. Some studies have shown that plasmids are more common in polluted environments than in pristine ones. For example, it has been found that hydrocarbon-polluted waters in the Gulf of Mexico contain a higher incidence of plasmids than do pristine waters. However, this is not always observed. Burton et al. (1982) found that there was no difference in plasmid abundance in polluted river sediments versus pristine sediments. Clearly, the success of microorganisms in different environments reflects many factors in addition to plasmid occurrence.

There have been relatively few studies of plasmid occurrence and function on subsurface bacteria. Ogunseitan et al. (1987) found that up to 8% of isolates from shallow pristine aquifers contained plasmids. Furthermore, these investigators found some evidence that plasmid occurrence increased (up to 35%) in ground water that was contaminated with aromatic hydrocarbons.

The first comprehensive study of plasmid occurrence in deep subsurface sediments was conducted by Fredrickson et al. (1988). Interestingly, these investigators

found that the frequency of plasmid occurrence tended to increase with depth. In the relatively shallow Upland and Tobacco Road Sand units, the percentages of stains containing plasmids were 14% and 16%, respectively. In the deeper sediments of the Black Creek and Middendorf aquifers, the percentage of plasmid-bearing strains increased to 39% and 38%, respectively.

Documenting the presence of plasmids in these subsurface bacteria is one thing, but documenting the functions (if any) of the plasmids is quite a different, and extremely difficult, question to address. The first obvious question is whether the plasmids conferred antibiotic resistance. The resistance of the bacteria to various antibiotics was measured by the disc plate method. This involves plating the bacteria onto media and placing discs containing certain concentrations of antibiotics on the plates. Those strains resistant to the antibiotics can grow next to the discs. Conversely, those strains sensitive to the antibiotics cannot grow next to the discs.

A high percentage (70%) of the strains tested by Fredrickson et al. (1988) showed some level of resistance to penicillin, whereas a low percentage (5%) showed resistance to tetracycline. Significantly, 100% of the penicillin-resistant strains showed beta-lactamase activity. Beta-lactamase is an enzyme that inactivates penicillin, and high levels of beta-lactamase activity are normally associated with plasmids. It is possible, therefore, that some of the antibiotic resistance found in the subsurface isolates was plasmid conferred.

Fredrickson et al. (1988) were particularly interested in whether the observed plasmids conferred unusual catabolic properties on the bacteria. This, as one might imagine, is a particularly difficult question to address. Their approach was to probe the plasmid DNA for homology with a well-characterized TOL plasmid (pWWO). First, the TOL plasmid DNA was concentrated and treated with chemicals to "unwind" the DNA structure. The DNA was then allowed to hybridize with nucleic acids that had been labeled with radioactive phosphate (32P). The radiolabeled, single strands of DNA were then used as "probes"; that is, they were added to single-stranded plasmid DNA and examined for homology, indicating that the plasmid DNA was similar to the TOL plasmid.

A number of the subsurface strains assayed by Fredrickson et al. (1988) exhibited significant homology with the TOL plasmid. While it was unlikely that this indicated that the bacteria had been exposed to toluene or xylene in the recent past, it did suggest a more intriguing hypothesis: The subsurface bacteria were adapted to metabolizing aromatic hydrocarbons that are present naturally in these sediments. As the enzymes required to mineralize these aromatics could be similar to those required to mineralize toluene, this could indicate similar pathways used by the subsurface bacteria.

The study by Fredrickson et al. (1988) is a good example of how DNA probes are potentially useful for studies of subsurface microbiology. DNA probes can be prepared to assay for the presence or absence of many metabolic processes. This technique, in turn, could prove useful for a variety of applications in subsurface microbiology.

5.9.3 Release of Genetically Engineered Microorganisms to the Environment

There has been considerable debate among microbiologists concerning the possible release of genetically engineered microorganisms (GEMs) into the environment.

The principal fear, more often expressed by nonmicrobiologists, is that GEMs, since they would be new to the immune systems of plants, animals, and humans, could produce catastrophic epidemics. This fear has been greatly compounded by the fact that most of genetic-engineering research and technology involves *E. coli,* a normal component of the human digestive tract. Genetically engineered strains, so goes the argument, might not be recognized and attacked by the human immune system. This could lead, in turn, to massive infection and death.

Fears surrounding the release of GEMs to the environment have, for the time being anyway, prevented the widespread use of this technology for cleaning up environmental contamination in ground-water systems (Forsyth et al., 1995). While several experimental studies have been undertaken since the late 1980s, most of these studies are confined to microcosms in the laboratory, or to carefully controlled lysimeters. For example, Holmes et al. (1999), reported on the effects of a genetically engineered strain of *Klebsiella planticola* on the growth of wheat plants in the laboratory. The results of this study indicated that the GEM persisted long enough to increase the number of nematodes present in the soil. Many other studies have focused on developing mechanisms for tracing GEMs in the environment. For example, Leff and Leff (1996) reported inserting a green fluorescence protein gene into *E. coli,* which makes it much easier to trace their fate in aquatic systems using fluorescence microscopy.

The non-use of GEM technology has not had a large impact on the bioremediation of petroleum hydrocarbons. When Chakrabarty began his research on engineering microorganisms to degrade aliphatic petroleum hydrocarbons (the 1970s), the extent to which such microorganisms are present in ground-water systems was not known. Since that time, it has been observed that petroleum hydrocarbon–degrading bacteria are ubiquitous in shallow ground-water systems. Thus, engineering bacteria to perform this function has not been necessary. However, this does not preclude the possibility that more recalcitrant contaminants, such as PCBs, might be effectively remediated using GEMs. However, until there is a political consensus that the benefits of this technology (degrading contaminants) outweighs the potential risks (new pathogens), it is unlikely that GEMs will be commonly utilized to remediate ground-water systems.

5.10 SUMMARY

The information required for the metabolism, growth, and reproduction of bacterial cells is stored in chromosomal and plasmid DNA present in each cell. DNA is capable of reproducing exact copies of itself—a property that allows genetic information to be efficiently passed to each succeeding generation. Genetic information is coded into DNA by the particular sequence of nitrogenous bases. This information is then translated to produce specific proteins by means of several different kinds of RNA. Messenger RNA is transcribed directly from the DNA template and carries the information to a protein assembly site. Transfer RNA physically carries the specific amino acids to the mRNA template, where they are assembled in the required order with the mediation of ribosomal RNA.

The expression of genes is carefully regulated in the cell by a number of mechanisms. The production of certain enzymes can be induced if the substrate for that enzyme becomes available from the environment. Alternatively, if a substrate is not

present, production of degradative enzymes can be repressed. It is common for the production and repression of enzymes to be regulated by feedback inhibition loops, in which the substrate for a particular reaction induces enzyme production and where the product of that reaction acts as a repressor. In this manner, cells are able to balance enzyme production with their changing needs.

Genetic variability is introduced to bacterial cells by a number of processes, including mutations and transposable genetic material. Genetic exchanges between microorganisms is effected by a number of processes, including transformation (DNA from a dead cell being taken up by a live cell), conjugation (DNA transferred by means of quasisexual joining of cells), and transduction (DNA transferred by viruses). These natural processes, when manipulated in the laboratory, form the basis for genetic-engineering technologies.

Plasmid DNA is often the focus of genetic-engineering technologies. These relatively short DNA molecules often confer useful biochemical functions, such as the capacity to degrade xenobiotic chemicals. Furthermore, because their DNA can be cut up and spliced into new combinations, it is possible to "engineer" microorganisms with useful characteristics. In one of the first applications of this technology during the 1970s, Chakrabarty engineered microorganisms with the ability to degrade the relatively long hydrocarbons associated with hydrocarbon spills. This research, in turn, led to the extension of patent protection to genetically engineered microorganisms. However, fears of releasing genetically engineered microorganisms into the environment have largely precluded the use of this technology on a routine basis.

DNA-manipulating technologies are based on the properties of nuclease restriction enzymes, which cut DNA at particular base sequences, the DNA-denaturing properties of heat and high pH, ligases that anneal DNA, and polymerase that enables single-stranded DNA to form their base complements. These procedures have revolutionized microbial taxonomy. It is now possible to evaluate the phylogeny of microorganisms by sequencing the genes coding for 16SrRNA. Significantly, these methods do not require the microorganism to be present in pure culture, or even that the microorganism can be cultured. Because of this, these methods have great potential for evaluating the microbial ecology of natural environments.

QUESTIONS TO CONSIDER

1. What physical property distinguishes the DNA of procaryotic cells from that of eucaryotic cells?
2. What is the difference between transcription and translation?
3. What functional regions of a DNA strand contribute to the process of induction in bacterial cells?
4. What property of DNA makes genetic exchanges between different microbial species not only possible but virtually certain to occur?
5. Give two properties of plasmids that make them useful in genetic engineering.
6. Explain how heat, and how DNA responds to heat, makes it possible to exploit the polymerase chain reaction in the laboratory?

7. How do molecular approaches to identifying microorganisms, as shown in the *Geobacter* example, improve our ability to evaluate the microbial ecology of subsurface environments?
8. Has genetic engineering improved our ability to bioremediate petroleum hydrocarbons in ground-water systems? Why or why not?

CHAPTER 6

MICROBIAL ECOLOGY OF GROUND-WATER SYSTEMS

Ecology is that branch of biology dealing with relationships between organisms, and relationships between organisms and their physical surroundings. An ecological community consists of all the organisms—microbes, plants, and animals—that live in an area. A community, together with the physical environment to which it is tied, is called an ecosystem. The major focus of ecological research is to determine how communities are structured, how species in the community interact with each other, and how communities interact with their physical environment.

In order to simplify the investigation of communities, ecologists usually study some subset of the organisms present in a given environment. For example, an ecologist might be mainly concerned with a particular taxonomic group such as birds. Thus, one might read about "bird communities." Similarly, microbial ecologists are mainly concerned with relationships between microorganisms and focus on "microbial communities." Given this considerable simplification, determining how organisms in particular communities interact with each other and their environment might appear to be perfectly straightforward. In practice, however, there are often considerable technical and interpretive problems to be overcome.

Consider, for example, a particularly simple ecological question—one concerning blackbirds in marshy environments of the northwestern United States. It is observed that during the mating season, part of a particular marsh is populated exclusively by yellow-headed blackbirds and another part of the marsh populated only by red-winged blackbirds. The ecological question is, What factor or combination of factors lead to the observed segregation of the two populations?

In order to answer this question, the ecologist would begin by making observations on how the two species interact. In this case, the ecologist would observe that in the spring, male red-winged blackbirds arrive in the marsh several weeks before its cousin, the yellow-headed blackbird. The redwing males promptly stake out all of the best nesting sites, which consist of cattail clumps surrounded by deep water. This ensures a constant supply of insects to feed upon while the nestlings are growing. However, when the larger male yellowheads arrive in a couple of

weeks, they forcibly evict the redwings from the best sites. This forces the redwings to retreat to less desirable sites. By the time the females arrive and mating starts, the yellowheads inhabit one part of the marsh—the part with the most food—totally excluding redwings. For their part, the redheads inhabit an entirely different, less productive, part of the marsh. The two populations do not overlap.

Given these observations, it's perfectly clear how the yellowheads exclude redwings—they kick them out. Less clear is why yellowheads do not also inhabit the poorer nesting sites as well and thus totally exclude the redwings. To answer this question, the ecologist would have to observe the rate at which yellowheads successfully raise their chicks as a function of the productivity of the nesting site. It turns out that the physical size of the yellowheads, in addition to providing the advantage in one-on-one encounters with redwings, also carries a price. This price is that they are not able to successfully raise their young in nesting sites that do not produce sufficient food. The young yellowheads simply require too much food. The redhead, however, with their smaller size can, successfully support offspring in the poorer nesting sites. The redwings exploit an environment closed to the yellowheads, proving that competition can cut two ways. The yellowheads have a competitive advantage in direct confrontation between the two species. The redheads, on the other hand, have a competitive advantage in exploiting available habitat.

Having made these observations, the next challenge to the ecologist is to determine the evolutionary factors that led to the observed relationship between the two species. The yellowheads are bigger, perhaps a result of natural selection favoring the ability to oust redwings from high-quality areas. On the other hand, the size of the yellowheads might reflect a completely different factor, such as a relatively low temperature in its winter range, or perhaps their size makes them less vulnerable to predators. In this case the yellowhead's ability to outcompete redwings for productive nesting sites is unrelated to the actual competition between the species. For their part, are the redwings smaller due to natural selection for inhabiting poorer nesting sites? Alternatively, does the smaller size of the redwings reflect something like migration habits or food availability in their winter range? These kinds of questions, central to the study of ecology, are very difficult to answer with confidence and require careful study. The relationship between the two blackbird species used here as an example, for instance, is still a matter for investigation and debate.

Given the inherent difficulties associated with unraveling relationships between species such as birds—species that can be directly observed—the formidable difficulties faced by the microbial ecologist studying subsurface environments can be more readily appreciated. The microbial ecologist faces the same difficult questions concerning relationships between microorganisms and their environments, but in this case it is very difficult to directly observe either the microorganisms or the environments that they inhabit. Furthermore, the sheer number of microbial species inhabiting many subsurface environments can be daunting. It has been only recently that microbial ecologists have begun to examine relationships between microorganisms in ground-water systems (Ghiorse and Wilson, 1988). Nevertheless, the special problems that arise in studying these unique ecosystems place subsurface microbial ecology as an important subdiscipline of both microbiology and ecology. This chapter surveys our present understanding of microbial ecology of ground-water systems.

6.1 SCOPE OF SUBSURFACE MICROBIAL ECOLOGY

The microbial ecology of many natural environments has been carefully investigated over the years. Examples of environments that have received particular attention include soils and aquatic sediments. It is no accident that these environments have been selected for intensive study. The microbial ecology of soils is directly related to the agricultural productivity of farmland. Understanding the microbial ecology of soils, and understanding how microbial populations adjust to different agricultural practices has helped boost agricultural production in the twentieth century. Thus, the microbial ecology of soils has been closely studied because it is a problem with major economic applications.

Similarly, the microbial ecology of aquatic sediments has received considerable attention because of practical reasons. In the nineteenth and early twentieth centuries, rivers and streams in the United States and Europe were routinely used for direct waste disposal. It was observed, however, that if raw sewage, to use one example, was placed in a river, the resulting pollution decreased rapidly downstream. Furthermore, it was observed that microbial processes in the fluvial sediments were largely responsible for consuming the dissolved organic carbon and nutrients in the sewage, thus renovating the river. Understanding the nature of these pollution-attenuating processes as well as understanding their rates and capacities was therefore an important practical problem. Also, much of the worldwide cycling of carbon and nutrients occurs in aquatic sediments. In order to understand global carbon balances, for example, it is necessary to understand carbon cycling in aquatic sediments.

The microbial ecology of subsurface environments has emerged as an important topic because of similar practical considerations. In the 1970s it became apparent that there was widespread contamination of shallow water-table aquifers due to a variety of human activities, including waste disposal, agricultural practices, and fuel storage. Gradually, it became clear that subsurface microorganisms played an important role in the mobility and fate of many contaminants (McNabb and Dunlap, 1975; Freeze and Cherry, 1979; Matthess, 1982). As such, understanding the ecological interactions between microorganisms in the subsurface assumed much more importance than had been placed on it earlier in the twentieth century (Ghiorse and Wilson, 1988).

The scope of microbial ecology as applied to the terrestrial subsurface is closely tied to the occurrence of ground water. While ground water by definition can be produced only from aquifers, confining beds (also called aquicludes) play an important role in shaping ground-water flow patterns and have important affects on ground-water chemistry (Back, 1986). Thus, the microbial ecology of subsurface environments has come to include both water-bearing aquifer sediments and water-retarding aquiclude sediments. In addition, unsaturated sediments overlying water-table aquifers are generally included in the scope of subsurface microbial ecology. To some degree, this introduces some overlap with traditional soil microbial ecology. However, subsurface microbial ecologists tend to focus on unsaturated sediments below the root zone, whereas soil microbial ecologists focus on the root zone.

The questions considered in the microbial ecology of the terrestrial subsurface are similar to those considered by aquatic sediment microbial ecologists. Energy,

most of which comes originally from the sun, is trapped by photosynthetic plants in organic carbon compounds and flows through an ecosystem. The subsurface microbial ecologist seeks to elucidate the pathways through which the flow of carbon and energy passes, and the processes that control the flow. Closely linked to the flow of carbon are the flow of nutrients, such as phosphorus and nitrogen, and the flow of electron acceptors that are coupled to carbon oxidation.

One important regulating mechanism of carbon flow in subsurface environments are microbial food chains. It is impossible for any single microbe to have the enzymatic capability for degrading all of the organic carbon compounds present in sediments. Thus, it is common for particular microbes to extract energy from organic carbon by performing specialized metabolic processes and transforming the carbon. The transformed carbon compounds may then be used by other microbes as primary substrates.

A simple example of how microbial food chains may regulate carbon and energy flow in a water-table aquifer is shown in Figure 6.1. In this example, the chain begins with the fermentation of lignin under anaerobic conditions, perhaps by *Clostridia,* to produce molecular hydrogen and carbon dioxide. Some of the hydrogen and carbon dioxide produced may diffuse upward to an oxygenated zone near the water table, and be utilized by hydrogen-oxidizing bacteria. Under anaerobic conditions in which mineral electron acceptors such as nitrate, Fe(III), or sulfate are absent, methanogenic bacteria take up a portion of the hydrogen and carbon di-

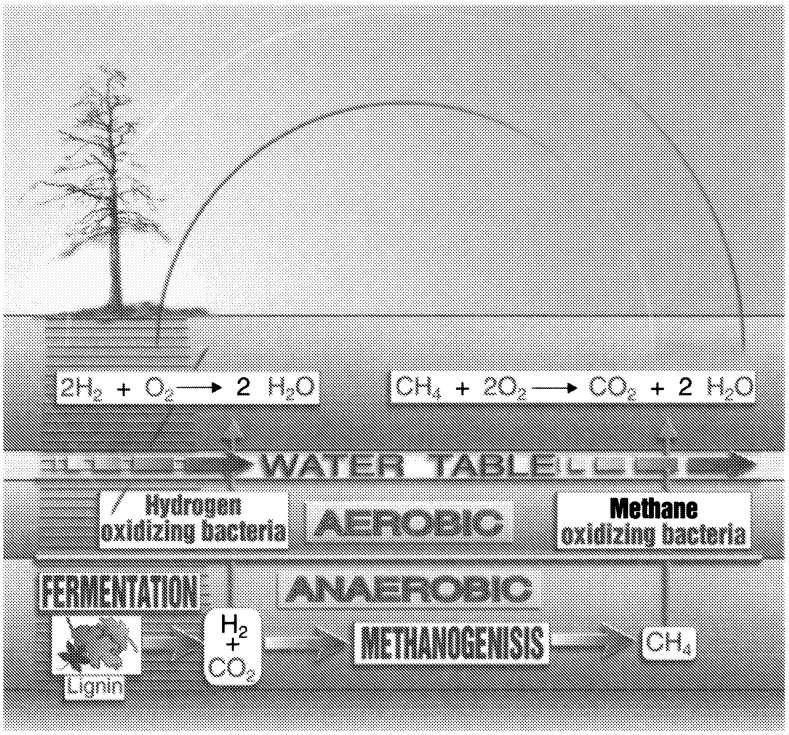


Figure 6.1. Schematic diagram showing how carbon and energy flow in a shallow water-table aquifer proceeds via food chains.

oxide, and produce methane. Some of the methane produced in this manner may then diffuse upward to oxygenated water near the water table and is oxidized to carbon dioxide.

Because sediments typically contain many different kinds of organic compounds that are potential substrates for microbes, it is commonly observed that numerous food chains operate parallel to each other. For example, the lignin present in sediments typically consists of many classes of complex organic compounds, including aliphatic side chains and carbohydrates linked to aromatic molecules. Metabolizing these different compounds requires specific enzymatic capabilities, not all of which are present in particular fermentative microorganisms. Thus, a number of different species may be present, which not only attack certain classes of compounds but also produce different fermentation products. These different fermentation products, in turn, form different food chains.

The summation of these ongoing, parallel food chains give rise to the community structure of a subsurface environment. This community structure may be fairly simple, with only a few species actively metabolizing in a given system. Alternatively, the community structure may be very complex, with hundreds of strains of microbes involved in organic matter degradation. Determining the community structure of aquifer systems is a major goal of the subsurface microbial ecologist.

Also of primary importance to the microbial ecologist is determining the trophic levels associated with subsurface environments. In classical ecology, the concept of a trophic pyramid is widely used (Fig. 6.2a). In this model, the base of the pyramid is made up of photosynthetic primary producers such as plankton or plants. The next level of the pyramid contains the primary consumers, principally plant-eating herbivores. At the same trophic level are the decomposers, microorganisms (fungi, bacteria) that oxidize the organic matter. The next trophic level is filled by secondary consumers (carnivores) that feed on the primary consumers and decomposers. There may be additional trophic levels filled by progressively higher animals.

Subsurface environments are fundamentally different from many ecosystems in that the number of trophic levels present is greatly restricted. For example, there

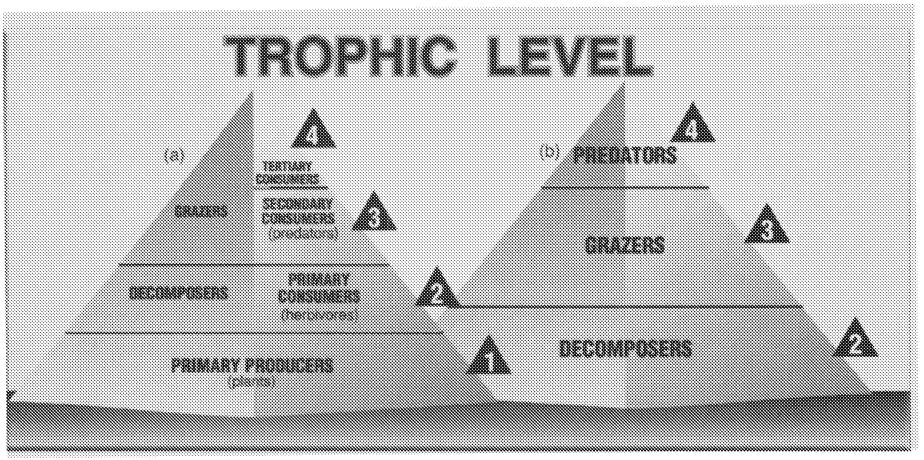


Figure 6.2. The trophic pyramid model (a) as typically used in classical ecology, and (b) those trophic levels present in ground-water systems.

can be no photosynthetic primary production in subsurface environments isolated from sunlight. It is possible for primary production to be based on chemolithotrophy, and this occurs in some subsurface environments. However, that is the exception rather than the rule. In most subsurface systems, there is a greatly attenuated trophic structure (Fig. 6.2b). Because there is no primary production and no primary consumers, decomposers, largely bacteria but also including fungi, form the base of the trophic pyramid. Superimposed on the decomposer level is a secondary consumer level. This trophic level is largely filled by protozoa that may actively graze on the decomposers, although it is possible that some predatory bacteria such as *Bdellovibrio* are present in some systems. There may be, in some systems, higher trophic levels present. An example of these higher trophic levels in ground-water systems would be the blind fish that inhabit some carbonate aquifers. These fish feed on protozoa that in turn feed upon the decomposers.

Understanding the energy flow, food chains, community structure, and trophic levels of different subsurface environments are important goals in subsurface microbial ecology. The motivation for achieving these goals is varied and depends upon the systems under consideration. For example, in a contaminated shallow water table aquifer, the effects of protozoan predation on decomposer populations—and therefore rates of contaminant decomposition—might be an important consideration. For a clastic aquifer system consisting of alternating beds of sand and clay, the important ecological question might be how community structure differs between the two sediment types and how these differences impact carbon and energy flow. The focus of an ecological study for any subsurface environment therefore depends largely on the questions being asked. The nature of the relevant questions is then a major factor in selecting appropriate methods of study.

6.2 METHODS IN SUBSURFACE MICROBIAL ECOLOGY

Microorganisms can only be directly observed using a microscope. In order to achieve the resolution required to observe bacteria using either light or electron microscopy, only a very small portion of the microbial habitat can be observed at the same time. Aquifer systems that compose the habitat of subsurface microorganisms are often hundreds of square miles in area and may be hundreds of feet thick. Clearly, if the decision is made to directly observe bacteria in an aquifer, it will not be possible to observe the aquifer itself in a meaningful way. This difference in scale between the microorganisms and their habitat is a basic conflict in studying the microbial ecology of subsurface environments. In practice, this conflict can be partially resolved by adjusting the scale of the observation technique used to the scale of the feature of interest. To use an example familiar to geoscientists, one cannot observe cross-bedding by looking at thin sections of sandstone with a petrographic microscope. Such features can be observed only at a hand-specimen or outcrop scale. Conversely, one cannot observe individual sand grains in hand specimen; one must use a petrographic microscope. In general, therefore, the scale of observation must match the scale of the feature that is of primary interest.

This scale conflict is especially important in subsurface microbial ecology. In selecting a scale suitable for observing microorganisms, little information concerning the microbial environment (i.e., the aquifer system) can be gleaned. Alternatively, if a scale suitable for observing a large aquifer system is selected, little

direct information concerning individual microorganisms can be gleaned. This scale conflict is a fundamental consideration in choosing the most appropriate method for studying a given environment.

The most commonly used methods to study the microbial ecology of subsurface environments include:

1. Culture techniques (small-scale)
2. Direct observation (micro-scale)
3. Biochemical marker techniques (small-scale)
4. Activity measurements in microcosms (small-scale)
5. Ground-water geochemistry (large-scale)
6. Molecular techniques (small-scale)

Most of these techniques make observations at a small scale (i.e., samples from 1 to 1,000 grams of aquifer material) or micro scale (i.e., samples less than 1 gram). The only large-scale observations (volumes of aquifer material greater than 10 square meters) that can be made are those using ground-water geochemistry. As we shall see, each of these approaches and their corresponding scales have particular advantages and disadvantages that must be carefully evaluated by the investigator. As a practical matter, the selection of a method or combination of methods should reflect the questions being asked. If the presence of microorganisms capable of particular metabolic functions (such as degradation of organic chemicals, for example) are of primary interest, culture techniques or activity measurements (small scale) are appropriate. On the other hand, some questions, such as determining overall rates of microbial metabolism in regional aquifer systems require the larger scale of geochemical techniques.

6.2.1 Culture Methods

Procedures for obtaining microorganisms in pure culture and then analyzing pure cultures for their biochemical properties are deeply embedded in the practice of microbiology. Many of the most important advances in medical and agricultural microbiology were made by pure culture methods. These methods can, in some instances, be usefully applied to the microbial ecology of subsurface environments. The basic idea is to isolate as many strains of bacteria as possible from sediments recovered aseptically from the subsurface, and characterize them on the basis of colony morphology, cell morphology, cell arrangement, gram reaction, and physiological/biochemical characteristics. This data can then be used qualitatively and quantitatively to characterize populations of microorganisms and to compare populations from different environments.

The first comprehensive use of culture techniques for evaluating the microbiology of a subsurface environment was described by Kolbel-Boelke et al. (1988). These investigators isolated 2,700 strains of bacteria from a shallow Pleistocene aquifer in the Lower Rhine region of Germany near the Netherlands. Samples were obtained from aseptically cored sediments as well as from water samples from wells screened in the aquifer. Sediments were sampled by placing about 25 g of aquifer material in 100 mls of sterile water, shaking them with glass beads for 30

minutes, and spread-plating 0.1 ml samples of the resulting suspension onto a peptone-glucose media. Ground water was filtered through sterile cellulose nitrate filters and the filters placed on plates of the media. After 21 days of incubation, individual colonies were picked and streak-plated onto fresh media in order to purify the strains. Each of these strains recovered was then categorized numerically by means of 155 characteristics, which were scored as positive (1), negative (0), or not tested (2). These characteristics included colony morphology (1–40), cell morphology (41–77), and physiological/biochemical properties (78–155). For example, if the isolate formed a circular colony (character 2), then character 2 would be scored positive and the other colony-shape characters (characters 1,3,4) would be scored negative. Thus, for the character "colony form" (characters 1–4), the isolate would be scored 0100. This process is then repeated for all 155 characters considered in the analysis. These morphological characteristics can be compiled in tabular form so that different populations can be compared.

Kolbel-Boelke et al. (1988) compared bacteria isolated from ground water pumped from a shallow aquifer (i.e., a ground-water community) to bacteria isolated from sediments cored (i.e., a sediment-bound community) from the same aquifer using these culture methods. The ecological question was, How similar are the two communities? In the ground-water samples, 23.5% of the isolates were gram positive gram variable and 76.5% gram negative. In the sediments, the percentage was roughly similar; 31.9% being gram positive/variable and 68.1% being gram negative. Based just on gram reaction, therefore, the two communities seem fairly similar. Interestingly, however, 61.2% of the ground-water isolates showed flagellar movement, whereas only 46.6% of the sediment isolates showed this feature. On the basis of this difference, one might speculate that there is a greater adaptation toward motility in the free-living ground-water community, and less adaptation for motility in the sediment-bound community. This speculation, which seems intuitively reasonable, could then form the basis for studies designed specifically to test that hypothesis.

Of particular interest is the ability of isolates to metabolize different organic compounds (characters 78–135) under oxidative (O) or fermentative (F) conditions. Thus, if the isolate could oxidize glucose (GLU-O), but could not ferment glucose (GLU-F), could not oxidize arabinose (ARA) or xylose (XYL), could oxidize galactose (GAL), mannose (MNE), fructose (FRU), lactose (LAC-O), but could not ferment lactose (LAC-F), the strain would be scored 100011110 for characters 78–87. Kolbel-Boelke et al. (1988) described the potential "physiological activity" of a particular strain as

$$pA = 100\,[P/(P + N)]$$

where pA is the potential physiological activity, P is the number of substrates for which a strain was "positive" (i.e., could utilize it under the given conditions), and N is the number of substrates for which the strain was negative. If a strain could oxidize all 58 substrates, its pA would be 100%, if it oxidized 29 substrates, its pA would be 50%, and so forth.

Similarly, Kolbel-Boelke et al. (1988) described the potential activity of an entire community recovered from a sampled zone using the equation

$$pCA = 100 \{P(a)/[P(a) + N(a)]\}$$

where pCA is the potential community activity, $P(a)$ is the number of strains positive for the a^{th} substrate, and $N(a)$ is the number of strains negative for the a^{th} substrate.

The distribution of potential community activity in both ground water and sediments as determined by Kolbel-Boelke et al. (1988) showed several notable features. First, there appeared to be considerable diversity in all of the communities studied. In other words, in each community there were strains present that degraded a very narrow range (less than 10%) of the substrates and strains that degraded a broad range (more than 50%). Somewhat surprisingly, there were no clear similarities between each of the ground-water communities or between the sediment communities.

When the kinds of substrates degraded by each community are considered, some differences become apparent. For example, a high percentage of isolates in each community exhibited the ability to oxidize glucose (GLU-O). However, only isolates from the sediment communities consistently exhibited the ability to ferment glucose (GLU-F). As ground water is aerobic in this aquifer, it stands to reason that free-living bacteria would gain little advantage by retaining the ability to ferment. However, because microenvironments in sediments may become anaerobic, sediment-bound microorganisms may gain some advantage by retaining this ability.

Another difference was in the ability of the communities to reduce nitrate (NO_3N_2). This characteristic was more pronounced in ground-water communities than in sediment communities. At first glance this might seem inconsistent with the aerobic-anaerobic hypothesis for the distribution of the ability to ferment glucose given above. However, because nitrate is a negatively charged ion, it would tend to be repelled from negatively charged mineral surfaces and be concentrated in the pore water. Microbial communities in pore water could thus have more access to nitrate as a substrate and have more use for a nitrate-reducing ability.

The basic approach of Kolbel-Boelke et al. (1988) has been widely used in documenting diversity of subsurface microbial communities. Balkwill et al. (1989) reported isolating 626 physiologically distinct types of bacteria from deep sediments of the Atlantic Coastal Plain. Like the studies of Kolbel-Boelke, the physiological capabilities of individual strains were investigated. Balkwill's group, however, employed commercially available API Rapid NFT test kits (Analytab Products, Plainview, N.Y.) for this purpose. The use of such rapid-identification test kits greatly reduces the amount of time and effort required to characterize the metabolic potential of isolated microorganisms.

Culture methods for investigating the microbial ecology of subsurface sediments have both advantages and disadvantages. The chief advantage is that culture methods can demonstrate the diversity of microbial communities. The principal conclusion of Kolbel-Boelke et al. (1988) was that the communities, both water-borne and sediment-bound, were fairly diverse. This result could not have been predicted a priori. This method also has the advantage of being able to compare microbial diversity between different horizons. For example, the community of sediment probe 2-04 was characterized by a very high percentage of gram-negative bacteria, which set it apart from other sediment communities. Thus, the culture method is

also effective in documenting heterogeneity within aquifers. Culture methods have been extensively applied in other ground-water systems to document microbial diversity and heterogeneity (Balkwill, 1989).

Culture methods also have significant disadvantages, the most serious of which is the selectivity introduced by using culture media. Many of the bacteria present may not grow on the media and culture conditions offered, or grow so slowly as to be underrepresented. This was observed to be the case in studies of a shallow water table aquifer in Fort Polk, Louisiana (Ghiorse and Balkwill, 1983). These authors noted that when particles of aquifer material were placed on culture media, small colonies would begin to grow. However, when these cultures were transferred to fresh media, they could not grow. Ghiorse and Balkwill (1983) concluded that a significant number of microorganisms present were not culturable with the methods being used. The net result of such problems in culturing subsurface bacteria is that not only may the information on diversity and community structure be skewed, but there is no way to know how much this has affected the data.

In addition to the selectivity problem, there is the problem of strain duplication. How does one determine whether two strains of isolates are closely related or whether they simply came from two cells of the same strain? If all isolates are treated as different strains, then isolating a large number of cells of the same strain would create the appearance that many strains have similar properties. On the other hand, if one were to take the position that isolates with similar properties belong to the same strain, one takes the risk of obscuring the presence of closely related but different organisms.

Another problem with culture methods has to do with the "activity" of bacteria in culture versus under in situ conditions. Just because a particular strain exhibits the ability to reduce nitrate in culture, for example, does not mean that particular strain is involved in nitrate reduction in situ. In general, it is not feasible to evaluate which processes are important in situ from culture data alone. For these important ecological questions, other techniques must be utilized.

6.2.2 Direct Observation

An important limitation of culture methods in subsurface microbial ecology is that they give little information as to processes that are active in situ. Other limitations are that culture methods give no information as to the niches inhabited by microbes or to their nutritional status. In order to deal with these problems, microbial ecologists have employed a number of direct observational techniques to address questions of community biomass, diversity, and nutritional status.

An example of how microbes present in aquifer sediments can be directly observed was given by Ghiorse and Balkwill (1983). These investigators employed a two-step approach. First, they prepared smears of aquifer sediment on microscope slides and stained them with acridine orange. Acridine orange binds with nucleic acids present in cells forming a fluorescent dye. When excited by ultraviolet light, the nucleic acid–acridine orange complex emits visible light that can be observed microscopically. In theory, the only light visible should be stained cells. In practice, however, acridine orange binds with mineral and organic particles present in the sediment, and these AO-mineral complexes may also fluoresce. Distinguishing be-

tween bacterial cells and sediment particles is often straightforward due to size and shape considerations. However, a measure of experience and skill is involved in such determinations.

The second step used by Ghiorse and Balkwill (1983) for directly observing aquifer microorganisms was to separate the microbes from sediment particles, embed the microbes into epoxy, and observe them with transmission electron microscopy (TEM). Microorganisms were released by placing sediment in a Waring blender with a solution of 0.1% pyrophosphate and 3.0% glutaraldehyde. The purpose of the pyrophosphate was to help dislodge the microorganisms. The glutaraldehyde was added to fix the microorganisms and prevent growth during the mounting procedures.

Using the sediment smear–acridine orange method, it was found that microorganisms were not uniformly distributed in the sediment. Rather, bacteria tended to occur in microcolonies consisting of anywhere from three or four to hundreds of cells. Several other features of interest, such as bacteria present in filaments, were also observed. Both rod-shaped and coccoidal cells were observed as well as cells that exhibited features of eucaryotes. Additionally, cells that appeared to be in the process of dividing were observed. This indicated that the cells were actively growing in situ immediately before or during the sampling procedures.

These observations are important from an ecological standpoint because they provide direct evidence as to how microbes interact with their environment, evidence that cannot be gleaned from culturing microorganisms. For example, culture studies could not have demonstrated that microcolonies were the predominant mode of occurrence of subsurface microorganisms. Furthermore, while culture studies can show that microorganisms are capable of growth and replication, they cannot show that this occurs in situ. The direct observations of Ghiorse and Balkwill (1983), as well as direct observations made in other studies (Wilson et al., 1983; Harvey and George, 1987), show that such growth does occur in situ. Harvey and George (1987) went further and determined by direct observation the percentage of cells that were in the process of dividing. This data was then used to estimate growth rates of subsurface bacteria.

At an even smaller scale, direct observation of cells with transmission electron microscopy (TEM) can also provide evidence of ecological importance. For example, Ghiorse and Balkwill (1983) showed that many cells contained inclusions of a substance similar to poly-beta-hydroxybutyrate (PHB). PHB is used by bacterial cells as an energy-storing compound. This, in turn, indicates that the microorganisms at the Fort Polk site were subjected to, and probably adapted for, starvation stress.

TEM observation also showed that about two-thirds of the bacterial cells had gram-positive cell walls. Initially, this was thought to be a surprising result since culturing microorganisms from subsurface environments often revealed a preponderance of gram-negative cells (Kolbel-Boelke et al., 1983). Interestingly, this observation was repeated at the Lula, Oklahoma site where direct observation of microorganisms showed a preponderance of gram-positive cells (Wilson, et al., 1983), but cultures of the same sediments indicated that gram-negative cells predominated. This raises the possibility that the methods used to culture the microorganisms systematically discriminated against gram-positive cells. Exclusive use of culture methods may therefore present a false impression of the relative abun-

dance of gram-positive and gram-negative cells in a system. As pointed out by Ghiorse and Balkwill (1983), however, such determinations can be made only by supplementing culture methods by direct observation of subsurface sediments, or by employing biochemical techniques that directly reflect in situ conditions.

6.2.3 Phospolipid Fatty Acid Analysis

Direct observational techniques for observing microorganisms in deep subsurface sediments suffer primarily from two limitations. First, only a very small volume of aquifer material can be accurately screened due to the high magnification required to resolve microbes. Second, direct observations of microorganism give only limited information as to the phylogenetic types present. It is feasible, as we have seen, to differentiate rod versus coccoid forms and to discriminate between gram-positive and gram-negative microorganisms. However, in order to accurately evaluate the community structure present, it is desirable to have better resolution of different microorganisms present.

One very powerful tool for providing this better resolution has been introduced by David C. White and his collaborators (White et al., 1983; Balkwill et al., 1988). The basis of this technique is that different microorganisms exhibit significant biochemical differences in the phospholipids and fatty acids (PLFAs) that comprise cell walls and internal storage granules. By directly extracting and characterizing PLFAs, information concerning total biomass, cell wall type, presence or absence of eucaryotic organisms, and nutritional status of the microorganism present may be derived.

Phospholipids are part of every bacterial membrane (section 1.2) and maintain a relatively constant proportion of the cell mass. Furthermore, phospholipids are turned over fairly rapidly after cellular death. Because of these two features, the amount of phospholipid present in sediments is a potential measure of the living biomass present. Moreover, the fatty acid composition of the phospholipids can give informations as to the kinds of microorganisms present. Eucaryotic microorganisms, for example, are characterized by long-chain polyenoic fatty acids. The presence of such fatty acids in sediment is therefore a direct indicator of the presence of eucaryotic microorganisms. Similarly, anaerobic microorganisms form cis-vaccenic acids and therefore indicate the relative importance of anaerobic and aerobic metabolism in a given sediment.

As with direct microscopic observation, biochemical techniques also indicate the relative abundance of gram-negative and gram-positive microorganisms. Gram-negative bacteria average about 14 umol muramic acid/g dry wt and gram-positive bacteria average 38 μmol/g muramic acid, a difference that reflects the relative proportion of peptideoglycan in cell walls. Also, gram-positive bacteria often have acidic macromolecules, called teichoic acids, associated with the cell wall (*teichoic* comes from the Greek work *teichos,* which means "wall"). These teichoic acids are composed of repeating glycerol or ribitol units connected by phosphate esters. Thus, the abundance of ribitol in particular is a rough measure of the relative abundance of gram-positive bacteria. The abundance of gram-negative bacteria can be estimated by the abundance of hydroxy fatty acids in the lipopolysaccharides.

The extraction procedure used to separate lipids from sediment samples was as follows. First, lipids were removed from 45–50 g of sediment using a chloroform-

methanol extraction procedure. Part of the recovered lipids was analyzed directly and part was hydrolyzed for analysis of various components, such as teichoic acids, lipopolysaccharides, exopolymers, and poly-beta-hydroxyalkanoic acids (PHA).

PLFA analysis was first applied to the microbial ecology of subsurface sediments by White et al. (1983). In this example, White and coworkers compared shallow sediments recovered from Fort Polk, Louisiana (discussed in the previous section), with deeper sediments (410 m) recovered from the Bucatunna Clay near Pensacola, Florida. Data from these subsurface environments were also compared to data from modern estuarine and marine aquatic sediments.

Because cells contain about 5.0×10^4 nanomoles phospholipid per cell, and because a cell weighs about 10^{-12} Grams, biomass (B_m; cells/sediment (dry wt)) may be estimated from the equation

$$B_m = [PL/5.0 \times 10^4]/10^{-12}$$

where PL is the mass of phospholipid in nanomoles/gram of sediment.

Similarly, biomass can be estimated from measued concentrations of muramic acid with the equation

$$B_m = [MA/C_{ma}]/10^{-12}$$

where MA is the mass of muramic acid in nanomoles/gram sediment and C_{ma} is a constant equal to the amount of muramic acid per gram of dry cell material. Because gram-negative cells average about 14 micromoles muramic acid/gram (dry wt), and gram-positive average about 38 micromoles muramic acid/gram, the data indicates biomass of between 6.4×10^7 (if all the cells were gram negative) and 1.6×10^7 (if all cells were gram positive) cells per gram of dry sediment.

Finally, biomass of gram-positive cells ($B_{m(gp)}$) that contain teichoic acids can be estimated from measured concentrations of ribitol according to the equation

$$B_{m(gp)} = [R/2.1 \times 10^5]/10^{-12}$$

where 2.1×10^5 is the amount of ribitol in *Staphylococcus aureus* (nanomoles per gram of dry cells). Using this equation gives an estimate of 1.3×10^7 cells per Gram.

It is notable that the estimates of biomass using total phospholipids, muramic acid, and teichoic acids were all in the range of 10^7 cells per gram dry weight for the Bucatunna Clay.

A more recent review of this technology, and how it can be used to answer questions about deep subsurface environments, was given by Ringelberg et al. (1997). It was shown that this technology can help verify the integrity of samples retrieved from the subsurface since the PLFA patterns of microorganisms in drilling fluid were distinct from those in uncontaminated cores. Also, PLFA analysis indicated that cores stored at 15°C exhibited a marked change in both biomass and community structure over time. Finally, it was shown that multivariant analysis of of PLFA profiles could readily distinguish microbial communities present in near-

surface soils, vadose zone sediments, and deep subsurface communities (Rigelberg et al., 1997).

6.2.4 Activity Measurements in Microcosms

Culture methods, direct observation, and biochemical marker techniques focus on the nature of organisms present and their physical and biochemical characteristics. Because the kinds of microorganisms present in any one environment are often so large, and the relationships between microbial species so complex, these methods are often quite cumbersome. An alternative to these methods, developed initially by soil microbiologists, is to focus on the net effects of microbial processes rather than on the kinds of organisms present. The guiding philosophy of this approach is that what the microorganisms do is important ecological information and can supplement the findings of culture studies.

An example of how activity measurements can supplement the use of culture methods in studying the microbial ecology of aquifer systems was given by Chapelle et al. (1988). The system being studied was the carbonate Floridan aquifer and its overlying confining bed, the Hawthorn formation. Ground water produced from the carbonate aquifer at this site contained up to 12 mmole/L of dissolved inorganic carbon—far more than could be accounted for by dissolution of carbonate minerals in a system closed to CO_2. The obvious hypothesis, therefore, was that CO_2 was being actively produced in the sediments of this system. The production of this CO_2 resulted in an open system with respect to CO_2 and vastly more carbonate material dissolution. In order to test this hypothesis, the first and most obvious question was ecological: Are there microorganisms present capable of producing CO_2?

To answer this initial question, culture methods similar to those employed by Kolbel-Boelke et al. (1988) were used to show the presence of a viable bacterial community. Not surprisingly, the results of these culture studies documented the presence of a wide variety of aerobic and facultatively anaerobic bacteria, including strains of *Bacillus, Pseudomonas,* and *Micrococcus*. However, the fact that those microorganisms could be coaxed into active growth on culture media in no way indicated that they actively produced CO_2 in situ, and that was the important question.

To address this question, samples of aquifer material were placed in sterile vials under aerobic and anaerobic conditions, and the production of CO_2 monitored with time (Fig. 6.3a). CO_2 was generated aerobically and somewhat slower anaerobically. However, sediment samples that had been sterilized by gamma radiation showed no CO_2 production.

While these data were consistent with the hypothesis that bacteria produced CO_2 in the sediments, the possibility that the sterilization technique had in some way interfered with an abiotic CO_2-producing process could not be ruled out. For this reason, a second experiment was performed. In this experiment (Fig. 6.3b), sediment samples were initially incubated anaerobically for seven days. CO_2 production was very low, ruling out the possibility that inorganic degassing of the sediments was producing significant amounts of CO_2 in the experiments. After seven days, oxygen was introduced into the vials and CO_2 production rapidly increased. How-

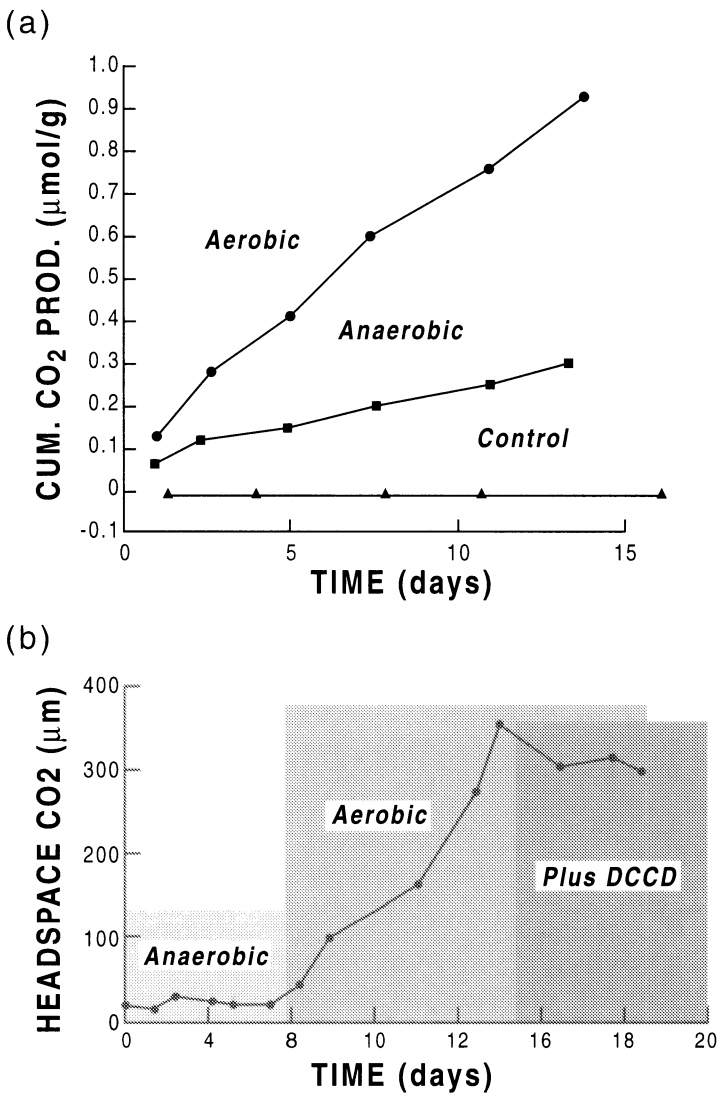


Figure 6.3. Production of CO_2 from (a) aerobic and anerobic incubations of aquifer sediments, and (b) inhibition of CO_2 production by DCCD. (Adapted, by permission, from Chapelle et al., 1988).

ever, that did not rule out the possibility that inorganic oxidation of organic material was producing CO_2. In order to test this possibility, dicyclohexylcarbodiimide (DCCD), an inhibitor that uncouples chemiosmosis from ATP synthesis in bacteria, was added to the sediment. This immediately shut off CO_2 production and showed unequivocally that bacteria in the sediments were capable of active CO_2 production. Thus, a combination of culture data and measurements of potential activity (Fig. 6.3) neatly answered an ecological question posed by observing the ground-water chemistry of this system.

Aquifer materials placed into laboratory vessels such as test tubes or septated serum vials for measurement of microbial activity are referred to as microcosms. *Microcosm* is defined in the dictionary as "a community or other unity that is representative of a larger unity." The reasoning for microcosms is that by understanding the activity of a small portion of an aquifer, much can be learned about the aquifer as a whole. Microcosms have been used extensively in studies of subsurface microbial ecology and are especially useful in assaying indigenous microbial communities for specific activities. In the example given above, the activity of interest was potential bacterial CO_2 production in a pristine aquifer. Often, however, the activity of interest deals with the potential of microbial communities to metabolize particular xenobiotic organic compounds.

One of the first uses of microcosm-activity studies to assay the potential of subsurface microbial communities to degrade xenobiotic compounds was described by Wilson et al. (1983). Studies by Wilson and his coworkers had previously documented the presence of a viable bacterial population in a shallow aquifer near Lula, Oklahoma. Now the question was whether, these bacteria were capable of degrading hydrocarbons such as toluene or trichloroethylene? Because the Lula site had not previously been exposed to such compounds, any degradation of them would reflect the activity of a nonacclimated microbial population. Furthermore, if such nonacclimated populations were could degrade these compounds, it would have important implications as to the potential fate of the xenobiotics in groundwater systems.

Because Wilson's group was particularly interested in volatile organic compounds—compounds that evaporate quickly in open vessels—the design of the microcosms had to allow sampling of pore water without exposure to the air. Aquifer material was aseptically transferred to a 50-ml test tube and organic compounds added in concentrations ranging from 85 to 666 μg/L. The tube was then sealed with a Teflon-lined screw cap (Teflon does not react with the organic compounds being studied), and the tube incubated. After incubation, it was necessary to sample the pore water in the microcosm while minimizing exposure to the air. This was accomplished by attaching a Teflon stopcock to the microcosm, attaching a separate tube filled with dilution water. The dilution water was then mixed with the pore water of the micocosm using a vortex mixer, and the tubes centrifuged until the water cleared. Aliquots of the diluted water were then analyzed.

The results of this study (Wilson et al., 1983) showed that toluene was rapidly degraded, with about 90% of the toluene disappearing within a week. Chlorobenzene was degraded by sediments cored from the unsaturated zone, but was not degraded significantly by deeper aquifer sediments. Other compounds, such as trichloroethylene and tetrochloroethylene, were not observed to undergo measurable degradation.

Measuring CO_2 production (Chapelle et al., 1988) or organic compound degradation (Wilson et al., 1983) activity in microcosms is suitable for answering specific questions concerning metabolic potential of microbial populations. They are not suitable for determining what part of a microbial community is involved in the degradation processes. It is common practice, therefore, to establish the presence of particular metabolic activity using microcosms and, if necessary, follow up with culture methods for isolating particular microorganisms capable of the activity.

6.2.5 Geochemical Methods

A feature shared by culture methods, direct observational methods, biochemical tracer methods, and microcosm methods is that the scale of observation is relatively small. Each of these methods is based on recovery of anywhere from one to a thousand grams of aquifer material. This scale is fixed largely by practical considerations of core recovery and sample handling in the laboratory. It is very difficult, however, to extrapolate meaningfully from such small-scale samples to the scale of regional aquifer systems, which may be hundreds of square kilometers in area. One way of enlarging the scale of subsurface microbiological observations is to use the chemistry of ground water as a tracer for particular microbial processes.

The basic premise of using ground-water geochemistry in this fashion is that particular microbial processes impact the chemistry of ground water in certain ways. For example, oxygen respiration in an aquifer closed to the atmosphere will result in the consumption of oxygen as ground water flows downgradient. Similarly, sulfate reduction will consume sulfate along aquifer flowpaths. In addition to consuming oxidants or substrates, some microbial processes will mobilize chemical species. Ferric iron reduction will result in the production of dissolved iron and methanogenesis will produce methane. Thus, the accumulation or depletion of particular chemical species in ground water can be used to indicate the presence or absence of certain microbial processes in aquifer systems.

An example of this approach was given by Lovley and Goodwin (1988). The system being studied was the Middendorf aquifer in South Carolina and the specific question was how predominant terminal-electron accepting processes are distributed. This kind of question is difficult to answer using culture techniques. The fact that sulfate-reducing bacteria may be cultured from aquifer sediments does not prove that such bacteria were actually active in situ. Also, because of the large area covered by this aquifer system, sampling sediments from the subsurface over a wide area was a practical and economic impossibility.

In view of these difficulties, Lovley and Goodwin (1988) took the approach of using ground-water chemistry as an indicator of microbial processes. First, they selected a series of wells oriented along a regional flowpath and sampled them for dissolved oxygen, nitrate, sulfate, and methane. Their data (Fig. 6.4) showed that oxygen and nitrate were present only near the outcrop-recharge areas of the aquifer. The presence of nitrate appeared to be related to agricultural practices near the outcrop area. As dissolved oxygen and nitrate was consumed along the aquifer flowpath, concentrations of dissolved iron increased. Farther downgradient, concentrations of dissolved iron decreased and sulfate concentrations also decreased. Even farther downgradient, concentrations of methane increased sharply.

This data suggested that the microbial ecology of this aquifer system was characterized by discrete zones, each dominated by particular microbial processes. Thus, in the part of the aquifer containing dissolved oxygen, oxygen reduction was the predominant process with ferric iron reduction, sulfate reduction, and methanogenesis largely being absent. Similarly, in the part of the aquifer where sulfate concentrations decreased, sulfate reduction was the predominant process and, where methane was being actively generated, methanogenesis was the predominant process. The ground-water chemistry data presented the picture of an aquifer segregated into discrete zones dominated by different physiologic microbial processes.

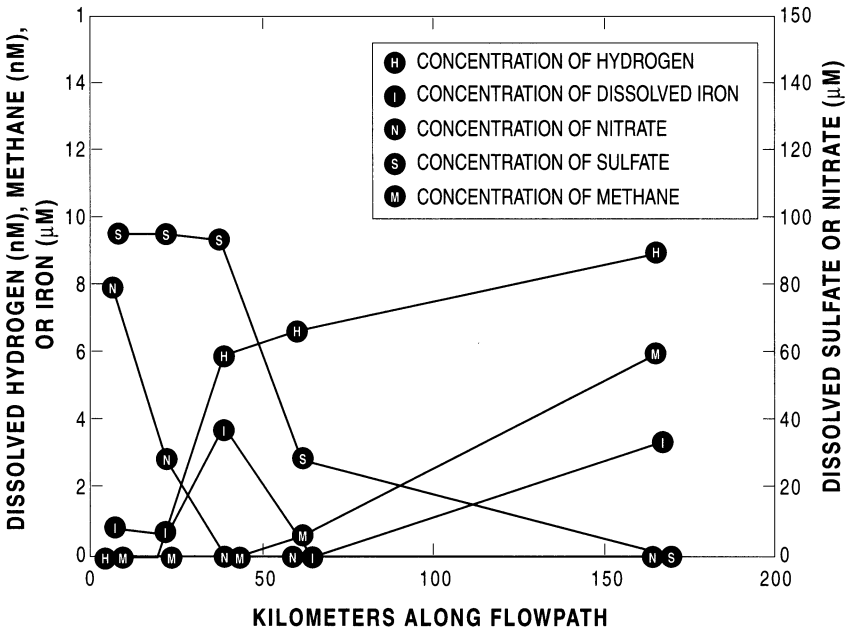


Figure 6.4. Concentration changes of oxygen (O), nitrate (N), iron (I), sulfate (S), methane (M), and hydrogen along a flowpath segment of the Middendorf aquifer. (Adapted from Lovley and Goodwin, 1988, with permission courtesy of Pergamon Press).

The drawback of using ground-water chemistry data in this fashion, however, is that direct evidence of the types and activities of microorganisms present is lacking. One method of providing this data would be to perform culture and activity studies of sediments cored from the different zones. In fact, extensive studies of the Middendorf aquifer have subsequently confirmed that in the aerobic zone, oxygen reduction is by far the predominant process with anaerobic processes such as sulfate reduction or methanogenesis being virtually absent (Hicks and Fredrickson, 1989; Jones et al., 1989). However, in much of the aquifer, culture studies were not feasible due to lack of cored material.

To deal with this difficulty, Lovley and Goodwin (1988) used a novel method dealing with concentrations of dissolved hydrogen gas in ground water. Hydrogen (H_2) is an intermediate product of fermentative bacteria and is consumed as an electron donor by ferric iron reducing, sulfate reducing, and methanogenic bacteria. In natural systems, therefore, hydrogen concentrations reach a steady state where production by fermentation is balanced by consumption by respiration. Significantly, the steady-state hydrogen concentration is dependent only upon the physiologic properties of the consuming bacteria and not on the rate of production or consumption. Thus, sediments dominated by ferric iron reduction have different steady-state hydrogen concentrations (0.1–0.8 nmolar) than sulfate-reducing (H_2 between 1 and 4 nmolar) or methanogenic (H_2 between 5 and 20 nmolar) sediments.

By showing that the parts of the aquifer characterized by sulfate consumption and methane production coincided with H_2 concentrations characteristic of sulfate reduction and methanogenesis respectively (Fig. 6.4), Lovley and Goodwin (1988)

provided independent evidence that these water-chemistry patterns resulted from microbial processes. This evidence, in turn, suggested that the microbial ecology of the aquifer system was characterized by a succession of predominant terminal electron-accepting processes. Such behavior had been postulated by Champ et al. (1979) by analogy with aquatic surface sediments (Frolich, 1979), but had not been previously demonstrated in ground-water systems.

As with other methods for studying the microbial ecology of subsurface aquifer systems, the use of ground-water geochemistry has advantages and disadvantages. The chief advantage is that ground-water chemistry provides a large-scale view of aquifers that cannot be obtained with other methods. Also, it is relatively inexpensive to sample water chemistry, whereas obtaining aseptic samples of deep sediments can be prohibitively expensive. However, ground-water chemistry can give no direct information as to the biomass, diversity, or biochemical potential of the organisms responsible for the processes under consideration. This can be a considerable disadvantage if, for example, the potential degradation of chemical contaminants is of primary interest. Furthermore, because ground-water chemistry integrates the effects of microbial processes, it is difficult to deduce the heterogeneity of microbial processes.

6.2.6 Molecular Microbial Ecology

One of the most frequent observations made in the microbial ecology of subsurface environments is that direct microscopic counts of bacteria commonly exceed plate counts by a factor of a hundred or more (Wilson et al., 1983; Balkwill, 1989). Because plate counts are dependent on culturing and bacterial growth, this has widely been attributed to the presence of large numbers of bacteria that cannot be cultured. Studies from other natural microbial habitats (lakes, oceans, aquatic sediments) show a similar pattern, and this has been termed "the great plate count anomaly" by Staley and Konopka (1985). The problems this presents to the microbial ecologist have long been recognized (Brock, 1987). In particular, it implies that there are many more bacteria in the subsurface then are evident by culture methods. This, in turn, suggests that microorganisms that perform important processes in the subsurface may not be recovered by culture methods.

The advent of molecular methods for studying and classifying bacteria (Chapter 5) has provided new methods for studying microbial ecology that are not dependent upon culture methods and, at least in principle, avoid culture bias (Amann et al., 1995; Head et al., 1998). A schematic diagram showing the general strategy for using molecular methods to study microbial ecology is shown in Figure 6.5. First, samples of sediment or water are obtained, the bacteria present are lysed, and the nucleic acids from the total microbial community are extracted. Second, the DNA coding for ribosomal RNA (rDNA), usually about 1,500 base pairs, is amplified using PCR with primers designed to isolate the 16S RNA genes. Alternatively, the rRNA present in the sample can be converted to DNA by means of reverse transcriptase, and the copied genes c rDNA amplified by PCR. Next, the amplified rDNA is either cloned using a plasmid cloning vector (Chapter 5) inserted into *E. coli,* or specific rDNA sequences obtained using denaturing gradient gel electrophoresis (Chapter 5) and reamplified with PCR. After sufficient DNA has been obtained, either by cloning or by DCCE-PCR, the rDNA is sequenced and the

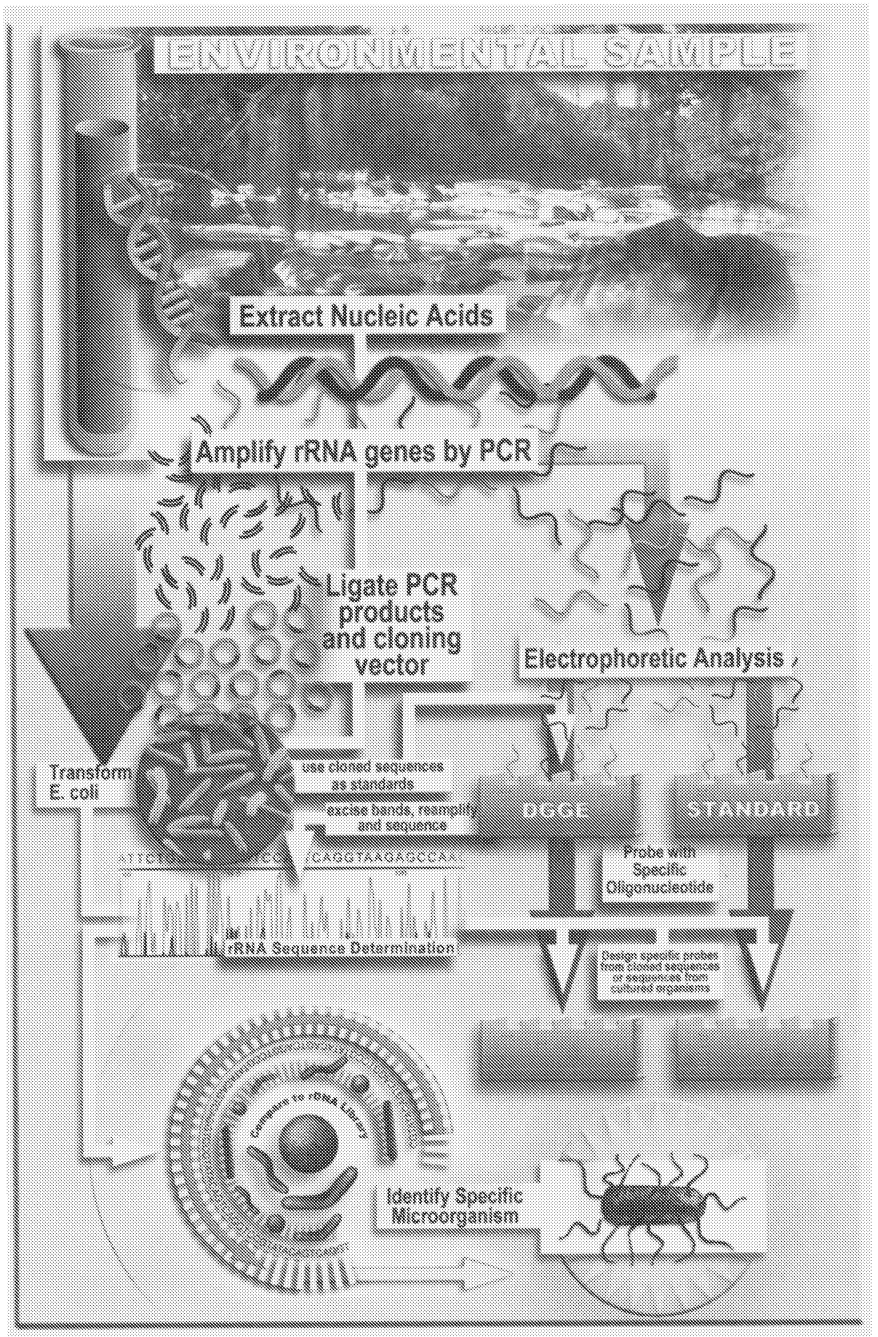


Figure 6.5. Schematic diagram showing the general strategy for using various molecular methods to study the microbial ecology of environmental samples.

results compared to sequences in an rDNA database. From this it is possible to determine genera, and sometimes species, of microorganisms present in an environment without having to use culture methods. Because of the lack of culture bias, it is possible to identify microorganisms that would otherwise be missed.

An example of how this technology has been applied to the microbial ecology of an aquifer system was given by Rooney-Varga et al. (1999). The aquifer being studied was a shallow glacial outwash aquifer located near Bemidji, Minnesota, that had been contaminated by a crude oil spill. Much of the risk associated with petroleum hydrocarbon–contaminated ground water comes from benzene, a known carcinogen. However, benzene is relatively recalcitrant under anaerobic conditions, and is often observed to migrate farther than other soluble contaminants. In accordance with this common observation, most sediments cored from Bemidji site do not contain microorganisms capable of oxidizing benzene. However, in some locations characterized by Fe(III)-reducing conditions, rapid oxidation of benzene was observed (Anderson and Anderson, 1998). The ecological question was, What microorganisms are present in the benzene-degrading zone that are not present in the zones where benzene was not degrading?

To address this question, Rooney-Varga et al. (1999) used polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGG), 16S rRNA sequence analysis, phospholipid fatty acid (PLFA), and most probable number polymerase chain reaction (MPN-PCR) methods to assess the ecological differences between benzene-degrading and benzene-nondegrading zones without the bias of culture methods. In MPN-PCR, the rDNA extracted from sediments is serially diluted and amplified by PCR. Those fragments that are present in greatest abundance appear in successively higher dilutions, and this can give a quantitative estimate of relative rDNA abundance (Anderson and Anderson, 1998).

PCR-DGGE patterns of DNA extracted from sediments differed markedly between the benzene-degrading and benzene-nondegrading zones. The diversity of bacteria appeared to be higher in the benzene-degrading zone, as indicated by the greater number of bands found in DGGE profiles. In addition, one distinct band present only in the benzene-degrading zone exhibited rDNA sequences characteristic of the Geobacteraceae family. This was consistent with the MPN-PCR analysis, which exhibited 10^7 copies of Geobacteraceae 16S rDNA in the benzene-degrading sediments as opposed to 10^4 copies in the benzene-nondegrading zone. Finally, PLFA analysis indicated greater numbers of bacteria in the benzene-degrading sediments, a higher degree of diversity, and higher concentrations of PLFA characteristic of known *Geobacter* species.

The obvious conclusion is that the benzene-degrading sediments were characterized by an abundance of bacteria associated with the family Geobacteraceae. Furthermore, because members of the Geobacteraceae are the only known microorganisms that have been shown to degrade aromatic hydrocarbons under anoxic conditions, it suggested that Geobacteraceae were responsible for the observed benzene biodegradation. Because attempts to isolate this microorganism were unsuccessful, however, this conclusion could not have been reached using traditional culture methods. While studies such as this using molecular techniques to answer ecological questions are in an early stage, it is likely that this approach will be increasingly used in future years.

6.3 MICROBIAL DIVERSITY AND NICHES IN AQUIFER SYSTEMS

A fundamental characteristic of aerobic aquifer systems is that they exhibit a high degree of morphologic and physiologic diversity in their bacterial flora. This was observed as early as the 1950s by German investigators (Wolters and Schwartz, 1956) using culture techniques. Subsequent studies, also using primarily culture techniques (Hirsch and Rades-Rohkohl, 1983; Balkwill and Ghiorse, 1985; Kolbel-Boelke et al., 1988; Balkwill et al., 1989), have shown that diversity among aerobic and facultatively anaerobic heterotrophic bacteria appears to be the rule rather than the exception. Similar culture studies of anaerobic aquifer systems have not been performed as extensively. However, the results of White et al. (1983) using biochemical marker techniques suggest that microbial diversity is also characteristic of anaerobic ground-water systems. In this section, the ecological implications of microbial diversity in ground-water systems is examined.

6.3.1 Measurement of Diversity

The term *diversity* describes heterogeneity within a system. As applied to ecology it refers to (1) the total number of species present in an ecosystem and (2) the number of individuals of each species present in that ecosystem. It is possible to define a "diversity index" based on these two quantities. The Shannon Index is the most widely used measure of diversity in ecology and consists of one term for describing total number of species (S) and a second term to describe the distribution of that species within the community (Dists). Total diversity (Divtot) may thus be defined as

$$\text{Divtot} = \text{S} + \text{Dists}$$

When dealing with macroscopic organisms, such as our earlier example of blackbirds, the information needed to quantify diversity may be obtained by direct observation. However, when dealing with microbes individuals can only be identified using culture techniques. The selectivity of culturing microorganisms in the laboratory automatically introduces bias into measuring diversity. Even with the limitations imposed by the selectivity bias, it is evident that determining the number of individuals of each species present is not a practical undertaking.

Faced with these difficulties for quantifying diversity, Balkwill et al. (1989) used a multitiered approach for expressing the horizontal and vertical diversity of microorganisms in a ground-water system. In this study, sediments from three continuous core holes in the Atlantic Coastal Plain were collected from land surface to a maximum depth of 265 meters. Sediment samples from various depths, spanning at least eight different geologic units, were used to isolate microorganisms on a peptone-tryptone-yeast extract-glucose media and a 1% dilution of this media. The physiologic characteristics of these isolates were then determined. These physiologic characteristics included the presence or absence of particular enzyme activities such as nitrate reductase, and the ability to assimilate different carbon sources such as D-glucose. Of the 1,112 isolates obtained, 626 were physiologically distinct. That is, they differed by at least one characteristic from all the other isolates.

This data was then used to investigate how the physiologic characteristics of the microorganisms varied spatially in the system. The first comparison made was between bacteria present in the soils (0 meters depth) with those present in deeper horizons (> 8 meters). In this case, the physiologic data were presented as the percent of isolates testing positive for a particular capability. By expressing the data in this manner it became evident that soil bacteria at the sites exhibited different diversity trends than bacteria isolated from deeper horizons. Specifically, the soil bacteria were able to metabolize a wider range of substrates than the subsurface bacteria. For example, about 69% and 85% of the soil isolates were able to oxidize L-arabinose and D-mantose, respectively, whereas only 46% and 53% of the subsurface isolates were able to oxidize these compounds. Balkwill et al. (1989) suggested that this difference in metabolic flexibility reflected the more limited kinds of organic matter present in the subsurface than in the soil.

It is important to note that there were considerable differences between the physiologic characteristics of isolates recovered on 1% PTYG agar and the characteristics of isolates recovered on PTYG agar. For the subsurface isolates there were significant statistical differences for 16 of the 20 physiologic tests between 1% PTYG and PTYG isolates. This implies, as expected, that culture methods have an important impact on the organisms recovered. Because of this impact, Balkwill et al. (1989) were careful to point out the media-specific nature of the diversity estimates. This, in turn, points out the need for methods that avoid the bias inherent in culture methods.

Another widely used technique for estimating microbial diversity in the subsurface is to recover microorganisms from pumped ground water (Hirsch and Rades-Rohkohl, 1983; Stetzenbach et al., 1986; Pedersen and Ekendahl, 1990), count them, and then identify them using physiologic criteria. For example, Pedersen and Ekendahl (1990) recovered four species of bacteria from a depth of 463 meters in a granitic aquifer in Sweden. The total plate count for this sample was 3.9×10^4 cells/ml. Of this total, 3.2×10^4 cell were identified as *Pseudomonas fluorescens*. These data show that most of the viable (i.e., culturable) cells present at that sampling depth were from a single species. That is to say, the observed diversity was fairly low.

In a similar study, Stetzenbach et al. (1986) identified bacteria isolated from ground water produced from deep wells in Tucson, Arizona. These data showed that more than 70% of the total viable microorganisms present were members of the genus *Acinetobacter*. Almost 10% of the microorganisms present were an unidentified pigmented organism. It is evident that, as in the example given by Pedersen and Ekendahl (1990), the diversity of the ground-water flora is fairly low. It appears, therefore, that microbial communities present in ground water are considerably less diverse than sediment-bound communities.

6.3.2 Niches and Sources of Microbial Diversity

In natural systems, habitats are seldom uniform. Rather, there is considerable variation in the availability of nutrients and the suitability of living space. A niche is defined as the physical space and immediate environmental conditions within which an organism survives and reproduces. A fundamental niche is determined by the organism's genotype and is all of the conditions under which it may survive. The

actual niche occupied by an organism, however, is generally limited by competition with other organisms and is referred to as the realized niche.

There are a wide variety of niches available in saturated subsurface environments. Some of the most readily apparent are shown in Figure 6.6. There are large pores ($> 2\ \mu$), small pores ($<.2\ \mu$), pores with open throats, pores with closed throats, grains composed of different mineralogy, and particles of organic matter that may also differ in composition. Just differences in mineralogy provide a wide range in potential niches. For example, a microorganism growing on a microcline grain would have a potential source of potassium, whereas a microorganism growing on a quartz grain would not. A microorganism growing on an organic matter particle would have a source of solid carbon, whereas one growing on a mineral grain would be limited to sources of dissolved or adsorbed organic carbon.

In general, one might expect a functional relationship between the number of exploitable niches in an ecosystem and the diversity of organisms. This depends, however, on how much each of the niches overlap and on the interaction between organisms. If there is significant overlap between niches and the organisms compete strongly, than a single organism may become dominant and the ecosystem exhibit low diversity. Alternatively, if competition is weak, there may be considerable diversity even if there is overlap of niches.

In ground-water systems, it would seem that some niches are largely isolated from others (i.e., a pore within a feldspar grain) and others with considerable overlap (a grain boundary between a quartz and feldspar grain). The observed high degree of diversity thus may represent the high number of available niches, the lack of competition, or both. It is likely that both of these factors enter in to the development of diversity in ground-water systems.

One possible explanation for the observed low microbial diversity found in ground water pumped from aquifers as opposed to the high diversity found in sediments is that ground-water samples represent a single niche. This niche would be the pore water present in the relatively large, open pores between sand-sized mineral grains. Water in these large pores is subject to less surface tension forces than water in small pores and is preferentially drawn into pumping wells. The large-pore niche, which would tend to be populated by free-living rather than attached

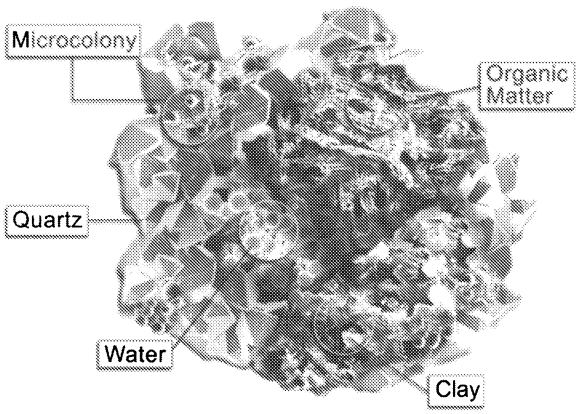


Figure 6.6. Some of the niches available to microorganisms in ground-water systems.

microorganisms, would be expected to exhibit much less diversity than the sediment as a whole. This, in turn, could explain the lower diversity in ground water relative to sediments.

6.3.3 Stress and Microbial Diversity

In addition to responding to the availability of nutrients and the abundance of niches, microbial diversity also responds to environmental stresses. The impact of environmental stress on microbial diversity has been extensively studied in surface water systems. These studies show a measurable decrease in microbial diversity as the load of pollutant increased. For example, Larrick et al. (1981) examined the diversity of heterotrophic bacteria as influenced by effluents from fossil fuel power plants. Total bacterial counts, as measured by number of colony-forming units (cfu), at his stations remained fairly constant with increasing stress from the effluents. However, the proportion of pigmented bacteria within the community correlated with physiochemical stresses. At an unpolluted reference site, the proportion of pigmented bacteria was about 13% of total cfu. At a heavily polluted ash basin, in contrast, pigmented bacteria made up almost 60% of the cfu.

Decrease in diversity due to pollution stress is often, but not always, observed. For example, in a study of sewage effluent discharge to Lake Huron, Stevenson and Stoermer (1982) found high diversity of diatoms in areas receiving effluent. They hypothesized that species diversity is low in algal populations that either grow very rapidly (low stress) or populations that grow very slowly (high stress). Intermediate growth rates, however, seem to encourage greater species diversity. Thus, species diversity could be greatest at a moderate level of pollution stress. This example points out that there is not always a simple relationship between species diversity and environmental stress.

Systematic studies of how diversity in subsurface microbial communities vary with stress are largely lacking. Based on analogy with surface-water systems, however, several effects would be anticipated. In pristine systems, highly oligotrophic aquifers (high stress) might be expected to have lower diversity than moderately oligotrophic aquifers. Similarly, the onset of pollution due to chemical spills or migration of landfill leachates (high stress) might decrease species diversity in a moderately oligotrophic (low stress) aquifer. This species-diversity decrease may coincide with an actual increase in cell numbers as a few well-adapted strains become dominant. Because changes in microbial diversity may either decrease or enhance the bioremediation potential of a system, this is a topic that deserves detailed study.

6.4 POPULATION INTERACTIONS

The ecology of microbial communities is strongly affected by interactions between populations that make up the community. The term "population" in this context refers to organisms that are phylogenically related to each other. In subsurface environments that lack a community of higher plants and animal, the most important interactions are those between microbial populations. Interactions between microbial populations are extremely varied. Positive interactions are those that

enhance the abilities of populations to survive within a community. In some cases, development of positive interactions allows the exploitation of resources that would otherwise not be available. This, in turn, allows some microbes to survive in environments that would otherwise be closed to them. Negative interactions are those that are in some way detrimental to certain populations. Negative interactions act as negative feedback mechanisms that limit population densities. These feedback mechanisms, in turn, adjust populations to the resources of the habitat. Negative interactions also tend to preclude the invasion of an established community by allochthonous populations and tend to maintain community stability.

6.4.1 Neutralism

The first kind of possible interaction is actually a lack of interaction. This is called neutralism. Neutralism occurs if, for some reason, populations are physically separated from each other. Furthermore, neutralism is more likely to occur under conditions of low population density that increase the chances for lack of contact. The most common example of neutralism would be the coexistence of a living bacterial population with endospores. Because the bacteria cannot use resources needed by endospores (which need none) and do not prey on or otherwise degrade endospores, bacteria have no effect on the endospore population. Similarly, because the endospores do not use resources or attack bacteria, the endospore population does not affect the bacterial population.

In subsurface environments characterized by large numbers of potential niches and low population densities, neutralism is probably very common. For example, populations growing exclusively on dispersed microcline grains would have negligible interaction with populations growing exclusively on equally dispersed biotite grains. Similarly, populations inhabiting closed pore spaces would have virtually no interaction with those in interconnected pore spaces. There is no direct evidence for the presence (or absence) of neutralism in subsurface environments. This almost certainly reflects the lack of techniques for documenting such interactions.

6.4.2 Commensalism

In a commensal relationship between populations, one population benefits from the interaction and the other population is unaffected. This type of interaction between bacterial populations is also probably very common in subsurface environments. The best, and probably most widespread, example of a commensal interaction in subsurface environments is consumption of oxygen. In and near outcrop areas of aquifers, oxygen is supplied to the system by vertically percolating recharge. As ground water flows downgradient, oxygen is systematically removed by aerobic and facultatively anaerobic bacteria. The removal of this oxygen by the aerobically respiring population creates the conditions required by obligately anaerobic bacteria. Thus, the anaerobic bacteria benefit by the presence of aerobes in aquifer systems. Since aerobes and anaerobes inhabit different niches, however, the aerobes are neither positively nor negatively affected by the anaerobic population.

In some commensal interactions, one microbial population physically alters the habitat in such a way as to allow a second population to exist. Another possible way this occurs in subsurface environments is the presence of boring bacteria.

These bacteria bore through solid grains, presumably to obtain substrates for growth. It is possible, however, for these borings to be opportunistically colonized by other bacteria as well. In this case, the opportunistic bacteria benefit with no corresponding benefit for the boring bacteria.

Another common commensal interaction is based on the production of specific growth factors. Some organisms produce specific compounds—vitamins or proteins, for example—and excrete them to the surrounding environment. These compound can then be taken up and used by other organisms to support growth. As long as these compounds are produced in excess and the producing organism derives no particular benefit in their removal, this interaction is commensal. It is common for this relationship to develop in communities of free-living bacteria and algae in aquatic environments. While there have been no reports of such relationships between subsurface microorganisms, this probably occurs quite commonly.

6.4.3 Synergism and Symbiosis

Synergism and symbiosis refer to relationships that benefit both populations. The difference is that two synergistic populations, while benefiting from interaction, are capable of living without each other. Symbiotic populations, on the other hand, require the interaction to sustain life. The distinction between synergism and symbiosis has not always been made. Symbiosis was originally used to describe any close relationship between populations. In present usage, however, the term symbiosis is restricted to interactions that are obligatory.

In microbial ecology, a classic example of synergism is the relationship between *Streptococcus faecalis* and *Lactobacillus arabionsus. L. arabinosus* requires phenylalanine, which is produced by *S. faecalis,* for growth. Similarly, *S. faecalis* requires folic acid for growth, and *L. arabinosus* happens to produce folic acid. These two microorganisms are quite capable of growing independently of each other, as long as these specific nutritional requirements are present in the media. However, if media are prepared that lack both folic acid and phenylalanine, *L. arabinosus* and *S. faecalis* can grow together in culture but they cannot grow separately.

Synergistic relationships are very important in the ecology of subsurface environments. The best example is the relationship between fermentative and respirative microorganisms in anaerobic ground-water systems. Fermentative bacteria are able to directly utilize complex organic matter, carbohydrates and lignins, present in subsurface environments to supply energy for growth. However, because fermentative metabolism cannot completely oxidize organic compounds (see section 3.6), fermentation products such as hydrogen and acetate still contain significant amounts of energy. Anaerobic respirative bacteria (Fe(III) reducers, sulfate reducers, methanogens) are incapable of directly metabolizing carbohydrates or lignins, but they are capable of metabolizing fermentation products. Thus, the presence of fermenting bacteria encourages the growth of respirative bacteria. Furthermore, since acetate and hydrogen are waste products, at least as far as the fermenters are concerned, their consumption by respirative bacteria encourages fermentation. Thus, both populations benefit.

The classic example of a symbiotic relationship between microorganisms is the growth of lichens. Lichens consist of photosynthetic algae or cyanobacteria and heterotrophic fungi. The algae fix atmospheric carbon dioxide and in some cases atmospheric nitrogen that provides the substrate for growth of the fungi. In return,

the fungi produce organic acids that help dissolve rock material allowing for further growth of the algae. These symbiotes inhabit extremely cold and dry environments of the Arctic desert. Individually, these species are not capable of surviving this extreme environment, whereas together they can.

Symbiosis has been closely studies by microbial ecologists for a number of reasons. One is that many ecologists feel that the formation of symbiotic relationships are a mechanism by which two populations unite to form new species. Many current theories of microbial evolution point to the structural similarities between organelles of eucaryotic cells (mitochondria, chloroplasts, for example) and procaryotic microbes. The consensus is that eucaryotic cells developed through the formation of symbiotic relationships between procaryotic microorganisms.

6.4.4 Competition

Different microbial populations in particular environments often strive to utilize the same resource. In this case, the relationship between the populations becomes competitive. The resource that is the object of competition can include nutrients, moisture, space, or any other commodity that is necessary for life functions and is in scarce supply.

Competitive interactions tend to separate populations in ecosystems. In microbial ecology there are numerous well-documented examples of population separation by means of competitive interaction. Perhaps the classic example is the separation of methanogenic and sulfate-reducing activity in aquatic sediments. It is ubiquitously observed in anaerobic marine sediments that a sulfate-reducing zone develops near the sediment–water interface and is underlain by a methanogenic zone (Froelich et al., 1979). For many years it was thought that this zonation reflected some direct inhibition of methanogenesis by sulfate-reducing bacteria. However, studies performed in M. J. Klug's laboratory at Michigan State University have shown that this separation reflects competition between sulfate reducers and methanogens for the same substrates (Lovley and Klug, 1983; Lovley and Klug, 1986).

In anaerobic sediments, both methanogenic and sulfate-reducing populations are supported by fermentation products such as acetate and hydrogen. These microorganisms thus compete directly for the same substrates. Because sulfate reducers have a higher affinity for acetate and hydrogen than methanogenic bacteria, sulfate reducers outcompete methanogens. Furthermore, because the sulfate reducers lower concentrations of these substrates below the threshold required by methanogens, methanogens are effectively excluded from the environment. This is termed competitive exclusion. Sulfate reducers continue to dominate the ecosystem until concentrations of dissolved sulfate are exhausted. Once sulfate reducers can no longer respire, concentrations of hydrogen and acetate increase to levels required by methanogens, and methanogenic bacteria become dominant.

It's interesting to note that methanogens have both a commensal and competitive relationship with sulfate reducers. While methanogens cannot successfully compete with sulfate reducers for fermentation products, methanogens depend on the sulfate reducers to deplete pore water with respect to sulfate, thus providing the sulfate-free environment they require.

Competitive exclusion is very important in the ecology of subsurface environments, and this is reflected in many commonly observed ground-water chemistry patterns. For example, one of the most frequently occurring ground-water quality

problems is the presence of high concentrations of dissolved iron. Furthermore, it is often observed that these high concentrations of dissolved iron occur in discrete zones. The Middendorf aquifer of South Carolina is characterized by a 40-mile-wide zone where concentrations of dissolved iron commonly exceed 0.3 mg/L (Fig. 6.7). Chapelle and Lovley (1992) have shown that this zonation of high-iron ground water reflects the competitive exclusion of sulfate-reducing and methanogenic bacteria by Fe(III)-reducing bacteria. Fe(III) reducers have a higher affinity for hydrogen, acetate, and formate than either sulfate reducers or methanogens. If suitable Fe(III) oxyhydroxides are present in the sediments, Fe(III) reducers lower concentrations of these substrates below thresholds required by sulfate reducers or methanogens, effectively excluding them.

Figure 6.8a shows that in the high-iron zone where sediments contain abundant Fe(III) oxyhydroxides, concentrations of acetate and formate are significantly higher than downgradient of the low-iron zone (Fig. 6.8b), where sediments contain little Fe(III) oxyhydroxides. This competition is reflected in the water chemistry as well. In the high-iron zone, hydrogen concentrations are in the 0.1–2.0 range characteristic of Fe(III) reduction. As Fe(III) oxyhydroxides become less abundant, concentrations of hydrogen increase to sulfate-reducing levels and dissolved iron concentrations decrease. This decrease reflects sulfide production by sulfate-reducing bacteria and subsequent precipitation of iron sulfides.

An interesting and, from a geoscientist's viewpoint, very important aspect of this example is that the presence or absence of solid Fe(III) oxyhydroxides is

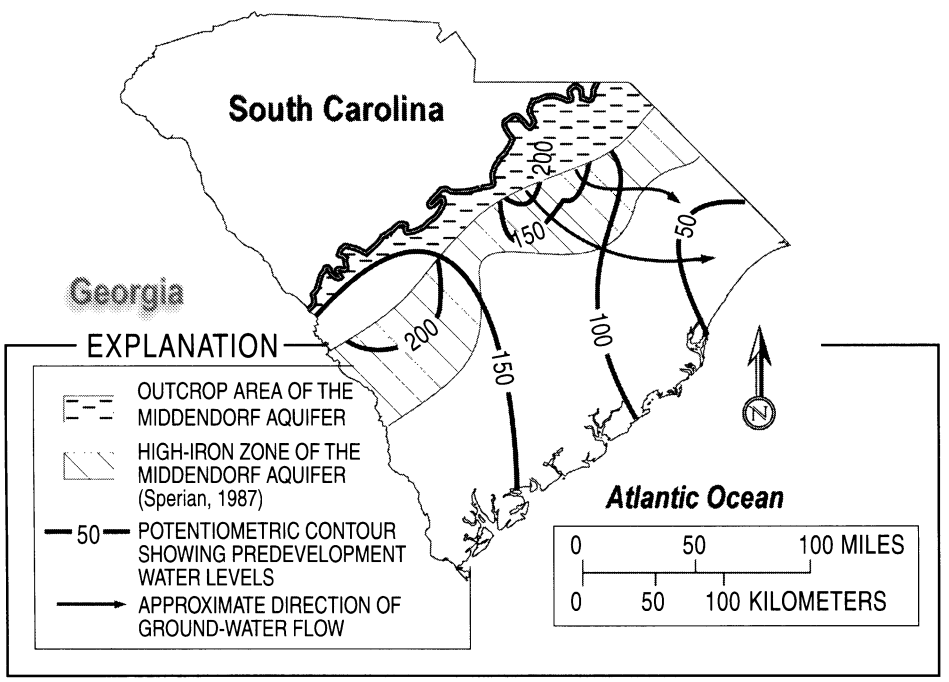


Figure 6.7. Zonation of high-iron ground water in the Middendorf aquifer due to the competative exclusion of sulfate reduction by Fe(III) reduction. (Modified from Chapelle and Lovley, 1992, with permission courtesy of the National Ground Water Association).

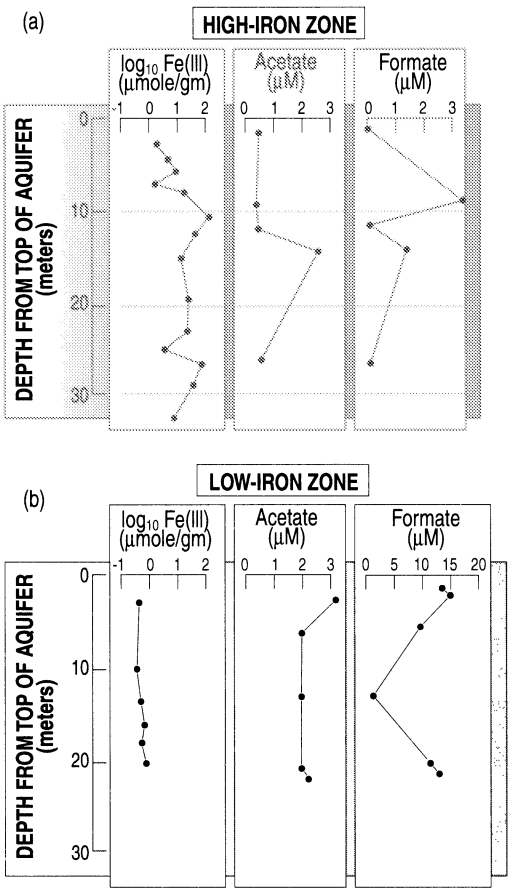


Figure 6.8. Concentrations of solid Fe(III) oxyhydroxides and dissolved acetate and formate in (a) the high-iron zone, and (b) the low-iron zone of the Middendorf aquifer. (Modified from Chapelle and Lovley, 1992, with permission courtesy of the National Ground Water Association).

largely controlled by geologic factors. In the updip portion of the Middendorf aquifer, the sediments are largely fluvial in origin, having been deposited in upper delta plain environments, and therefore contain significant amounts of Fe(III) oxyhydroxides that occur as grain coatings. The lack of Fe(III) oxyhydroxides in downdip sediments reflects a change in depositional environments from upper-delta plain (above tidal influence) to lower-delta plain (marginal marine) and delta front (open marine) sediments. Fe(III) oxyhydroxide-coated clays typically flocculate as salinity increases and therefore are not as common in sediments characterized by marine influence.

This example shows that the outcome of microbial competition in aquifer systems reflects both microbiologic and geologic factors. From a microbiologic viewpoint, Fe(III) reduction excludes sulfate reduction in the high-iron zone because of the presence of Fe(III) oxyhydroxides in the sediments. However, it is the geologic/sedimentology framework of the system that determines the distribution of Fe(III)

oxyhydroxides. Thus, understanding the outcome of microbial competition in aquifer systems requires both a microbiologic and geologic perspective.

Similar patterns of competitive exclusion have been reported from contaminated aquifers as well. Beeman and Suflita (1987) studied a shallow water table aquifer in Oklahoma that had been contaminated by municipal landfill leachate. Most of the contaminated zone was characterized by high organic carbon concentrations and low sulfate concentrations. In these areas, methanogenesis was the predominant terminal electron-accepting process. In one localized area, however, the ground water contained significant concentrations on sulfate and sulfate reduction was the predominant process. Significantly, while methanogenic bacteria were more numerous in the methanogenic zone than sulfate reducers, sulfate reducers were present. Similarly, while sulfate reducers were more numerous than methanogens in the sulfate-reducing zone, methanogens were also present. Thus, as was the case with Fe(III) reducers and sulfate reducers in the Middendorf aquifer, it is apparent that competition excludes the activity of microorganisms that are at a competitive disadvantage, but this does not lead to their total exclusion.

6.4.5 Antagonism, Parasitism, and Predation

When one population produces a substance that inhibits another population, the interaction is termed antagonistic. The population that produces the inhibitory substance thus gains a competitive advantage over the other. The production of antibiotics by some strains of bacteria and fungi are thought by some microbial ecologists to be an example of antagonism in nature. Curiously enough, however, it has not been unequivocally shown through ecological studies that antibiotic production actually confers a competitive advantage in natural systems. One reason for this may simply be that virtually all studies of antibiotic production are performed by medical microbiologists who are not specifically concerned with ecological interactions in nature.

Studies showing the presence or absence of antagonistic interactions between microorganisms in ground-water systems have not been performed. It is notable, however, that numerous strains of bacteria characterized by antibiotic resistance have been isolated from subsurface environments (Fredrickson et al., 1988). It is possible that this resistance reflects antibiotic production by some species in the subsurface, and thus may reflect antagonistic interactions. On the other hand, given the low population densities characteristic of subsurface environments, it's not clear that antibiotic resistance would confer much of a competitive advantage. As antibiotic production in different environments has been and will remain a topic of practical interest, studies on antagonistic relationships in subsurface environments are certainly warranted.

A parasitic relationship occurs when one population derives its nutritional requirements from hosts of another population. The parasitic population is clearly benefited by this relationship, generally to the detriment the host population. Host–parasite interactions require long-term contact between the populations, and members of the parasitic population are generally much smaller than members of the host population.

The most common host–parasite interaction in microbial communities is between viruses and bacteria. Viruses are obligate parasites and are able to reproduce

only inside other cells. Recent investigations have shown that a rather high percentage of planktonic marine bacteria harbor viruses. This shows, in turn, that parasitic interactions are rather common in that environment. Studies of viruses in aquifer systems have focused on the transport of human viruses rather than on viruses that parasitize indigenous bacteria. Thus, the extent that virus parasitism in ground water systems is largely unknown.

When one population actively consumes another population, the interaction is termed predation. The predatory population derives nutrients from this interaction and is clearly beneficial. Conversely, such interaction is clearly detrimental to the prey population.

The most common predator-prey relationship in microbial ecology is the interaction between protozoa and bacteria. Many protozoa actively feed, or graze as it is often termed, on bacterial populations. Early studies of the microbial ecology of subsurface environments noted a lack of protozoa in ground-water systems (Ghiorse and Balkwill, 1983), but subsequent studies have documented that protozoa are fairly common in aerobic aquifer systems (Sinclair and Ghiorse, 1989; Sinclair et al., 1990). Furthermore, there is evidence that protozoa actively graze on bacterial populations in some systems (Kinner et al., 1990). The observed higher numbers of protozoa in contaminated water table aquifers suggest that predation may act to limit bacterial degradation of contaminants in some systems. This important possibility needs to be explored in a variety of systems.

6.5 r AND K STRATEGIES IN MICROBIAL ECOSYSTEMS

Different populations in a microbial community employ different metabolic and reproductive strategies in their competition with each other. These different strategies allow different niches within a habitat to be successfully colonized. One scheme for classifying such strategies is to view them as falling along a gradient between pure r strategists and pure K strategists (Fig. 6.9). In this scheme, r strategists rely on high rates of reproduction to maintain themselves in the community. Similarly, r strategists tend to be characterized by catastrophic mortality events. The r strategists tend to have short life spans, high productivity–low efficiency utilization of resources, high migratory tendency, and variable population size. Conversely, K strategists are characterized by lower reproductive rates, longer life

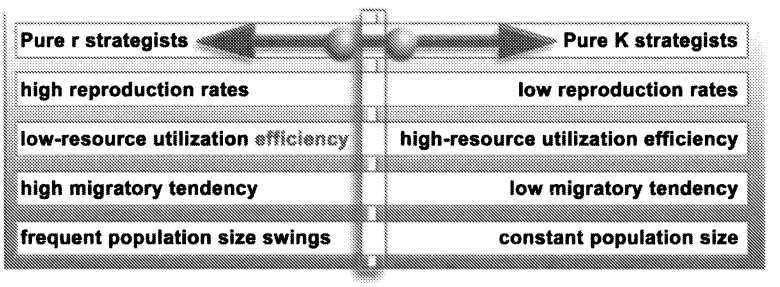


Figure 6.9. Some of the characteristics of pure r and K strategists.

spans, low productivity–high efficiency utilization of resources, low migratory tendency, and stable population size. Clearly, most microorganisms are neither pure r nor K strategists but fall somewhere between the two end members (Fig. 6.9).

The possible effects of r or K strategies on species survival can be illustrated by considering the emergence of a new volcanic island in the Pacific ocean. The creation of land obviously creates new habitat that can potentially be exploited by plants and animals. In colonizing this new habitat, r strategists have an immediate advantage. Plants that produce highly mobile seeds (palm trees, for example) reach the new habitat first. Once on the island, the plants that can reproduce the fastest are the ones that can claim the most space. Since resources (space) is not limiting, there is little initial advantage to use available resources efficiently. Clearly, r strategists have the advantage over K strategists when colonizing new habitat.

As time passes on the island, however, and more and more plants come and colonize, all of the available niches become crowded. Shortages of resources begin to appear and there might be mass mortality events among the r strategists. The ability to reproduce quickly becomes less and less important and the ability to utilize resources efficiently becomes more and more important. Plants with K strategy physiologic adaptations, adaptations that allow successful reproduction in space-limited conditions, have the advantage. Clearly, as time goes on, K strategists will gradually occupy most of the available niches.

The distinction between these two end-member strategies has been around a long time. Winogradsky, the great soil microbiologist of the early twentieth century, referred to some microbial species as being "zymogenous," or opportunistic. There are obvious similarities between the concept of zymogenous and r-selected microorganisms. Similarly, Winogradsky's concept of "autochthonous," or indigenous, microorganisms is analogous to that of K strategists.

6.5.1 r and K Strategies in the Aquifer Environment

At first glance, pristine subsurface environments would appear to be ideal for K-selected microorganisms, since there has been long-term stability in ambient conditions and nutrients are in limited supply. Indeed, available evidence suggests that K-selected microorganisms dominate in subsurface environments. For example, population densities appear to be fairly constant spatially (Balkwill et al., 1989), reproduction rates are very low (Harvey and George, 1987), and separation of populations by competitive exclusion mechanisms evident (Chapelle and Lovley, 1992). Furthermore, the presence of fairly diverse microbial communities (Balkwill, 1989) is consistent with the predominance of K strategies.

Interestingly, however, there is evidence that some microorganisms in pristine subsurface environments retain some r-strategy characteristics. For example, in an experimental investigation Hirsch and Rades-Rohkohl (1990) found that sterilized sediments exposed to ground water were rapidly (within 12 weeks) colonized by bacteria. Furthermore, the diversity of the colonizing microorganisms was considerably less than the microbial diversity in the surrounding aquifer sediments. This evidence suggests that r strategists do exist in subsurface environments that are able to rapidly take advantage of new habitat.

Ghiorse and Wilson (1988) suggest an intriguing hypothesis that may explain the long-term presence of r strategists in subsurface environments. Ghiorse and

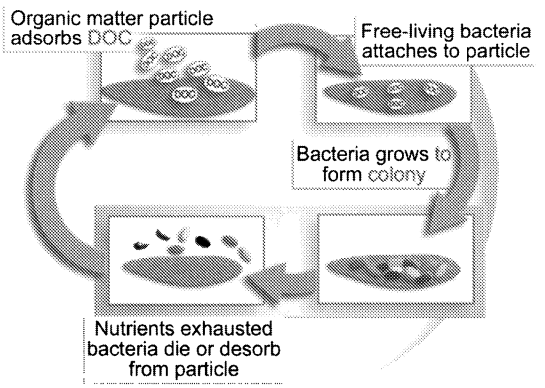


Figure 6.10. Cycle of microbial colonization and dispersal for r strategists in nutrient-poor ground-water systems.

Wilson were concerned with explaining why subsurface bacteria appear to divert considerable carbon resources to synthesize exopolysaccharides, a diversion that appears wasteful of scarce resources. They suggest that organic carbon particles in aquifers serve to absorb and concentrate dissolved organic carbon transported in from the overlying soil zone. As organic carbon absorption continues, the carbon particle becomes capable of supporting microbial growth. If a microorganism comes in contact with this enriched particle, it is crucial to bind strongly to it in order to fully exploit the resource. This neatly explains the presence of exopolysaccharides, which enable the microorganism to bind firmly to surfaces. After the organic resource is exhausted, the cells die or disperse, and the organic particle again begins to concentrate dissolved organic carbon. Ghiorse and Wilson (1988) thus envision a cycle of nutrient concentrations, colonization by microorganisms, exploitation, and finally dispersal (Fig. 6.10). Implicit in this cyclic model is the steady-state presence of r strategists capable of moving to and rapidly exploiting resources. Thus, the continued presence of r-selected microorganisms in subsurface environments may not be as surprising as it might appear at first glance.

6.6 CHAPTER SUMMARY

The ecology of microorganisms in subsurface environments may be studied using a number of different techniques, including culture methods, direct observations, biochemical marker methods, activity measurements, geochemical methods, and ecological modeling. Each of these methods has advantages and disadvantages. Ultimately, the methods used for a particular problem must reflect the nature of the questions being asked.

Subsurface environments present a number of different niches that may be exploited by subsurface microorganisms. Ground water in relatively large pores, for example, may support a free-living population of bacteria. Alternatively, bacteria

may attach themselves to a variety of aquifer materials, including mineral grains and organic matter. The large number of potentially exploitable niches in aquifers apparently leads to highly diverse microbial populations in many ground-water systems.

Populations interact in a number of different ways in ground-water systems. Neutralistic interactions are not either positive or negative for different populations. This is the case for bacteria that depend on different carbon sources. In a commensal relationship, one population benefits from interaction, whereas the other is unaffected. In synergistic relationships, such as between fermenting and respiring bacteria, both populations benefit. Symbiosis refers to a relationship in which interaction is a prerequisite for life. Relationships between populations can also be competitive, which is the case when each population exploits the same resource. Competitive relationships between different terminal electron-accepting microorganisms, each of which attempts to use the same carbon sources, are very common in subsurface environments. In particular, the competitive exclusion of sulfate reduction by Fe(III)-reducing bacteria leads to very distinctive zones of high-iron ground water in some systems.

Different microbial populations employ different metabolic and reproductive strategies in competing with each other. Pure r strategists rely on high rates of reproduction, with consequent less efficient utilization of resources, to maintain themselves. Pure K strategists, on the other hand, rely on the efficient utilization of resources while sacrificing a high reproduction rate to maintain themselves. The low-nutrient conditions characteristic of subsurface environments appears to favor efficient K strategists in many systems. However, there is evidence that fast-colonizing r strategists are also present in many systems. This may reflect the way carbon is cycled in subsurface environments and allows subsurface microorganisms to rapidly exploit niches as they become available. The term "population" in this context refers to organisms that are phylogenically related to each.

QUESTIONS TO CONSIDER

1. How do the large- or small-scale characteristics of available methods affect the study of microbial ecology in ground-water systems?
2. How reasonable is it to generalize results obtained from a 10-gram microcosm to an entire ground-water system? What steps can be taken to improve such generalizations?
3. Explain the assumptions used in applying phospholipid analysis for estimating the biomass of aquifer sediments.
4. Do hydrogen measurements in ground water produced from a single well give a small- or large-scale indication of predominant electron-accepting processes?
5. What criticism can always be made of using culture methods for studying the microbial ecology of ground-water systems?
6. What are the principal limitations in using PCR-DGGE methods for identifying contaminant-degrading activity in ground-water systems?
7. What properties of ground-water systems lead to different niches for microorganisms to inhabit?

8. How can competition between microbial populations lead to ground-water quality problems in ground-water systems? What property of aerobic bacteria tends to minimize such problems in oxygenated ground water?
9. How common do you think viral parasitism is in shallow ground-water aquifers? Do you think it becomes more or less common in deeper flow systems? Why?
10. Do you think microorganisms inhabiting deep petroleum reservoirs are r- or K-selected? Why?

PART II

MICROBIAL PROCESSES IN PRISTINE GROUND-WATER SYSTEMS

CHAPTER 7

ABUNDANCE AND DISTRIBUTION OF BACTERIA IN THE SUBSURFACE

The study of microbial processes and how they affect ground-water geochemistry begins with consideration of the abundance and distribution of bacteria. That bacteria inhabit virtually all subsurface environments is not currently a matter of dispute. However, the types of bacteria present, their abundance, and the kinds of physiologic processes that they carry out differ widely from environment to environment.

In order to deal with bacterial abundance and distribution in subsurface environments, it is first necessary to draw some fundamental distinctions between the types of environments most commonly encountered. This requires some basis on which to classify subsurface environments.

7.1 CLASSIFICATION OF SUBSURFACE ENVIRONMENTS

One possible basis for classifying subsurface environments, and one that is often used informally, is simply one of depth. It seems reasonable that deep subsurface environments are fundamentally different than shallow environments. Closer inspection of this as a classification strategy, however, immediately points out several difficulties. First, "deep" and "shallow" are relative terms that have different meanings in different geologic settings. "Deep" to a soil scientist would mean depths on the order of meters, whereas to a petroleum geologist it would conjure thoughts on the order of 10,000 meters. Aside from the semantic problems, the terms "deep" and "shallow" give no information concerning the hydrologic setting of the environment. A depth of 200 meters, for example, would be an aerobic water-table aquifer in the Tucson Basin of Arizona, whereas it might be an accumulation of petroleum in the Illinois Basin. Obviously, these two environments have little in common despite the fact that they occur at the same depth.

In describing microbial processes in subsurface environments, the hydrologic setting is the most important frame of reference. The hydrologic setting is what

determines such important factors as the amount of communication with the surface, rates of ground-water movement, and transport of dissolved substrates. Thus, classifying subsurface environments based on their hydrologic attributes is logical when considering microbial processes. Also, because the physics of ground-water flow does not vary between diverse hydrologic systems, such classification can be applied universally.

The most fundamental distinction between subsurface environments that may be drawn on the basis of hydrology is the difference between the saturated and unsaturated zones. The unsaturated zone is characterized by pore spaces that are incompletely filled with water. Pore space not filled with water is filled with gas. The significance of the unsaturated zone to hydrologists is that capillary forces between water and sediment particles prevent water from flowing to wells. Thus, wells completed in the unsaturated zone do not yield appreciable quantities of water. The amount of water present in the unsaturated zone varies widely and is highly sensitive to climatic factors.

Once the distinction between saturated and unsaturated hydrologic systems is made, the next step is to classify different types of saturated subsurface environments. Hydrologists have long recognized, on a qualitative basis, three categories of ground-water flow systems (Norvatov and Popov, 1961). These include an "upper zone" of active flow strongly influenced by local precipitation events, a "medium zone" of deeper flow only moderately affected by local precipitation events, and a "lower zone" of relatively stagnant water unaffected by local precipitation. This "zonation" was recognized entirely on an empirical basis and suffers from a vague notion of the importance of depth. As such, it offers little in terms of a classification scheme over just considering depth.

In the early 1960s J. Toth, a Canadian hydrologist, showed that the zonation of subsurface hydrologic systems follows logically from the physics of ground-water flow (Toth, 1963). By solving the steady-state ground-water flow equation

$$\frac{\partial^2 h}{\partial x^2} + \frac{\partial^2 h}{\partial z^2} = 0$$

for a hypothetical basin bounded on the sides by a ground-water divide and a valley bottom,

$$\partial h/\partial x = 0 \qquad \text{at } x = 0 \quad \text{and} \quad x = s$$

$$\text{for } 0 < z < z_0$$

bounded below by an impermeable layer,

$$\partial h/\partial z = 0 \qquad \text{at } z = 0$$

$$\text{for } 0 < x < s$$

and bounded above by a water-table surface that follows undulating topography,

$$h = g(z_0 + cx + \sin bx)$$

$$\text{for } 0 < x < s$$

Based on the governing equation and boundary conditions, Toth calculated lines of equal potential, termed equipotential lines, and from these deduced directions of ground water flow. In this context, the term "flow system" is defined rigorously as "a set of flow lines in which any two flow lines adjacent at one point of the flow region remain adjacent throughout the whole region." Figure 7.1 illustrates this concept. In the upper part of the system, the flow lines cluster into "cells" in which water recharges the aquifer at topographic highs and discharges at topographic lows. Because the flow lines in these shallow cells are parallel, they constitute one individual flow system. Deeper in the system, a set of flow lines passes underneath the shallow cells. Again, because these flow lines are parallel, they constitute one individual flow system. Finally, those flow lines that pass through the deepest part of the system constitute a third flow system.

This type of reasoning shows that three distinctly different types of flow systems develop for a wide variety of topographic configurations (Fig. 7.1). These different types of flow systems have the general characteristics:

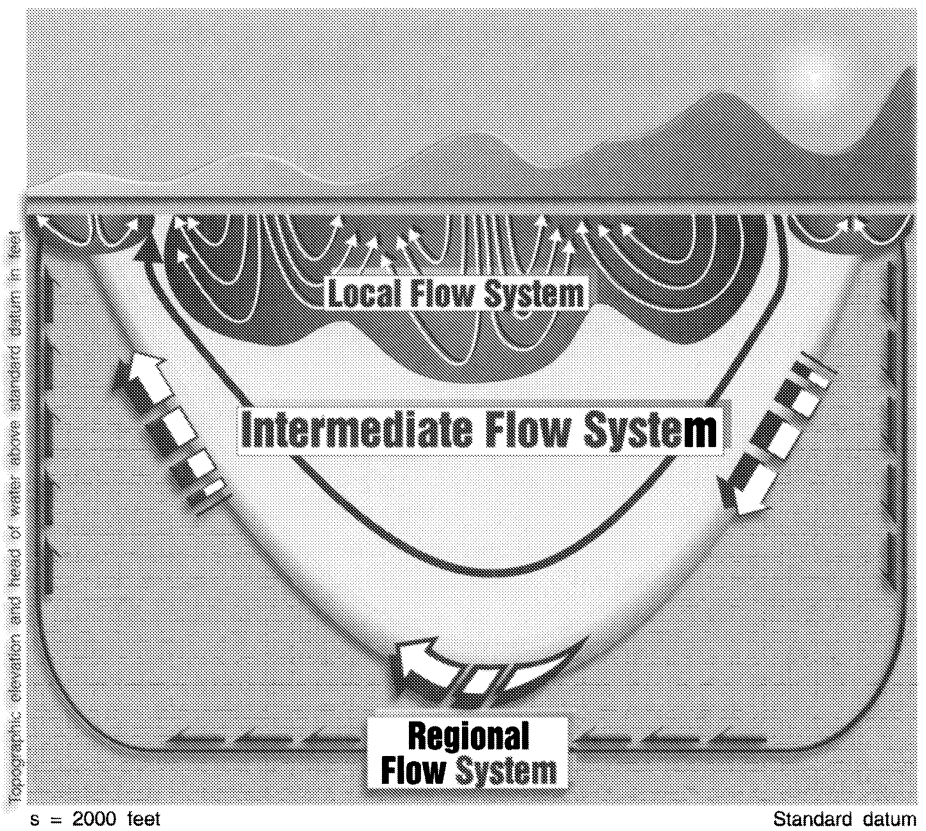


Figure 7.1. Delineation of local, intermediate, and regional ground-water flow systems.

1. A local system has its recharge area at a topographic high and its discharge area at a topographic low that are located adjacent to each other.
2. An intermediate system where recharge and discharge areas are separated by one or more topographic highs.
3. A regional system where the recharge area occupies the water divide and the discharge area occurs at the bottom of the basin.

These three different hydrologic settings are similar to the "zonation" that had been recognized empirically by Norvatov and Popov (1961). For example, recharge rates are greatest in the local (i.e., "upper") systems, and progressively less in the intermediate (i.e., "medium") systems and regional (i.e., "lower") systems. Thus, local systems will tend to respond quickly to local precipitation events, whereas intermediate and regional systems respond less or not at all. Also, rates of ground-water flow are much more sluggish in regional than local systems. Ground-water flow in regional (lower) systems may indeed appear to be "stagnant." There is, however, an important difference between Toth's classification and the empirical classification. This is that reference to depth is absent from the definition. In fact, Toth's definitions are independent of scale and depend only on the distribution of recharge and discharge to the hydrologic system. Thus, Toth's classification can be more universally applied than the empirical classification.

A major limitation of Toth's argument was that he assumed homogeneous and isotropic hydrologic properties within his hypothetical basins. This was necessary in the early 1960s because only analytical techniques were available for obtaining solutions to the ground-water flow equation. The advent of numerical methods for solving ground-water flow problems in the late 1960s, however, showed that the development of well-defined local, intermediate, and regional flow systems also occurs in nonhomogeneous and geometrically complex aquifer systems (Freeze and Witherspoon, 1967). This added complexity, however, tends to diffract flow lines so that flow in more permeable layers tends to become horizontal whereas flow in less permeable layers becomes largely vertical.

For example, given the fairly common case where material of low permeability is underlain by material of higher permeability, flow lines in local and intermediate regimes are mostly vertical and flow lines in the regional regime is almost exclusively horizontal. Similarly, if a zone of dipping high permeability material is overlain and underlain by lower permeability material, flow is concentrated in the high-permeability zone. While nonhomogeneity adds significant complexity to patterns of ground-water flow, this complexity occurs within the framework of the three hydrologic regimes identified by Toth (1963).

It is possible, therefore, to distinguish between four types of subsurface environments (Table 7.1). The first of these are unsaturated subsurface environments, which includes most soil zones, and are characterized by the lack of water flow to wells. For saturated environments, a rigorous distinction between local, intermediate, and regional flow systems can be drawn. If water from a recharge area discharges into an adjacent stream, that subsurface environment is defined as local. If water discharges into a river one or more basins removed is the recharge area, it is defined as an intermediate subsurface environment. Finally, if recharge discharges only at the center of a basin, it is defined as a regional subsurface environment.

The classification of subsurface environments shown in Table 7.1 emphasizes the different degrees of connection with the surface. Obviously, the unsaturated zone will be most closely connected with the surface, with water entering the system with every major precipitation event. Local flow systems also may be closely connected with the surface and may be strongly influenced by precipitation. Intermediate and regional systems, on the other hand, are much more isolated from events on the surface. The varying degrees of connection with the surface will obviously influence the kinds of microbial processes that may occur in different environments. For example, the unsaturated zone and local flow systems are often free to exchange gases with the atmosphere. Thus, a continuous source of oxygen is potentially available and aerobic bacteria may be able to continuously respire. In intermediate systems, however, the transport of dissolved oxygen is much more limited and anaerobic processes usually predominate. Regional systems are entirely isolated from atmospheric oxygen and anaerobic bacteria predominate.

7.2 THE UNSATURATED ZONE

An important hydrologic feature of the unsaturated zone is that it contains both water and gas in void spaces between sediment particles. More practically, water in the unsaturated zone is under a negative hydraulic pressure—that is, less than atmospheric pressure—and therefore water cannot flow into wellbores that penetrate it. The unsaturated zone is important from a hydrologic viewpoint because recharge to aquifers must first move through it. The rate that water moves through

TABLE 7.1 Classifications and Hydrologic Attributes of Subsurface Environments

Connection with Surface Recharge	Flow Rates	Oxygen Status	Potential for Microbial Transport from Surface	Example
		Unsaturated		
Extensive	Fast 0.5–5.0	Usually aerobic	High	Soil zones
		Saturated (Local)		
Extensive	Fast 0.5–5.0 ft/d	Often aerobic	High	Water table aquifer
		Saturated (Intermediate)		
Small	Slow 0.5–5.0 ft/yr	Generally anaerobic	Moderate	Confined aquifer
		Saturated (Regional)		
Virtually nonexistent	Almost stagnant	Anaerobic	Virtually nonexistent	Deep basin/ petroleum reservoirs

the unsaturated zone therefore directly controls the rate that an aquifer may be recharged. From a geochemical perspective, the unsaturated zone is important because gases are freely exchanged with water that ultimately recharges aquifers. Recharge water infiltrating through the unsaturated zone is readily charged with highly reactive gases such as oxygen and carbon dioxide due to the coexistence of the gas and aqueous phases. The carbon dioxide input is particularly important in studies of carbon isotopes in ground water (Rightmire and Hanshaw, 1973).

Microbial processes in the unsaturated zone are important for several reasons. First, bacterial processes such as oxygen consumption, carbon dioxide production, nitrification, and denitrification will directly impact the chemistry of infiltrating water reaching the water table. Secondly, anthropogenic compounds such as petroleum hydrocarbons, herbicides, pesticides, and a host of others must pass through the unsaturated zone in order to impact ground-water quality. Because many of these compounds are biologically active, bacterial processes in the unsaturated zone will affect their fate and transport.

The unsaturated zone is frequently divided into three components (Fig. 7.2). The first of these is the soil zone, generally a meter or two thick, which contains living roots and which supports plant growth. The porosity and permeability of the soil subzone is generally higher than that of underlying material. This underlying material, which varies in thickness from place to place, is often referred to as the intermediate zone (not to be confused with the "intermediate" zones of saturated flow systems) and consists of sediments or rocks that have not been exposed to extensive pedogenic (soil-forming) processes. The boundary between the unsaturated zone and the saturated zone is termed the capillary fringe.

Capillarity, the process that forms the capillary fringe, results from two forces: the mutual attraction (cohesion) between water molecules and the molecular attraction between water and solid mineral surfaces. Because of the forces, water will rise into small tubes to varying levels above water in a container. Pores in sediments are often of capillary size and therefore water is pulled from the saturated

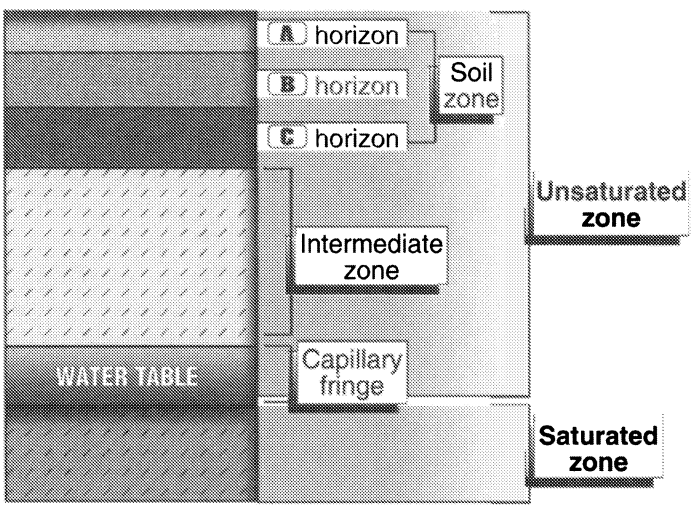


Figure 7.2. Three components of the unsaturated zone.

water table into the unsaturated zone. As a general rule, finer-grained sediments have thicker capillary fringes (up to 1 meter) than sandy sediments (generally 1 to 10 centimeters).

The abundance and distribution of bacteria in the unsaturated zone, particularly in soils, has been intensely studied over the last 150 years (Alexander, 1977). Much of the impetus for this research has come from the recognition that microbial processes are directly involved in soil fertility and therefore agricultural productivity. For example, it had been known for hundreds of years that composting manure under aerobic conditions leads to the accumulation of nitrate, an important component of plant fertilizers. It was the famous soil microbiologist S. N. Winogradsky who showed that this process is brought about by bacteria oxidizing the ammonia present in manure to nitrates. Winogradsky isolated in pure culture a bacterium that carried out this nitrification process in 1891. At about the same time, Winogradsky isolated an anaerobic bacterium of the genus *Clostridium* capable of nitrogen fixation: the process of converting atmospheric nitrogen into organic nitrogen. In 1888, the Dutch bacteriologist M. W. Beijerinck isolated a nitrogen-fixing bacteria from the roots of legumes, a microorganism later assigned to the genus *Rhizobium*. It's hard to overestimate the importance of these discoveries to modern agriculture, as they form the core of crop fertilization and crop rotation strategies.

The traditional association of soil microbiology with soil fertility and crop production has led to a curious lack of attention to microbial processes in sediments just below the root zone. In fact, the unsaturated zone between the root zone and the water table is probably the least studied of any subsurface environment. In many areas, there are only a few meters between the root zone and the water table, and this lack of attention is understandable. However, in some parts of the world, and particularly in arid climates, there may be hundreds of meters of unsaturated material below the root zone. Just from the standpoint of sheer volume, the deep unsaturated zone in these areas bears consideration.

7.2.1 The Unsaturated Zone As a Microbial Habitat

The unsaturated zone, by virtue of its proximity to photosynthetic organic carbon production at land surface, is the most biologically active and varied subsurface environment. By far the most biologically active part of the unsaturated zone is the soil.

There are five interactive factors that lead to the development of soil: climate, topography, parent material, time, and biologic processes. Soil microbiologists have long recognized that soil organisms play a significant role in the development of their own habitat. This is certainly true, to varying degrees, in other subsurface environments but is most easily observed in soils. Soil formation is initiated by chemical and physical weathering of rocks or sediments. The release of nutrients by these weathering processes allows colonization by algae and lichens. This initiation of primary production leads to the establishment of a heterotrophic bacterial population. In turn, these combined microbial processes speed the weathering processes by increasing the carbon dioxide partial pressure and by releasing organic acids. Once a suitable combination of weathered rock debris and organic matter is achieved, plants are able to initiate growth. This, in turn, speeds organic carbon production, increases the activity of heterotrophic bacteria, and results in more

efficient weathering processes. The development of soil, therefore, may be viewed as a combination of processes that are linked in positive feedback loops. That is, as each process becomes more efficient, the efficiency of the other processes is enhanced.

The net result of soil formation processes is the establishment of a stable, well-defined soil profile at the top of the unsaturated zone (Fig. 7.2). The formation of clay-organic complexes results in the stabilization of clay, sand, and silt particles into aggregates. Aggregate formation is initiated when polysaccharides from roots and microorganisms combine with clays to form organic matter and mineral complexes. Microorganisms live in the pores of the soil aggregates, generally attached to particle surfaces. The porosity and size of pore throats in the soil is an important structural feature because it forms living space and access to nutrients.

Moisture and Gas Content The coexistence of aqueous and gas phases in the unsaturated zone has important consequences to microbial processes. All microorganisms require water for cell maintenance and growth. The lack of water is therefore extremely detrimental to microbial activity. On the other hand, if pore space is completely filled with water, the transport of metabolically important gases such as oxygen is greatly decreased. Thus, too much water or too little water will both depress microbial processes.

The presence of a distinct gas phase is a feature unique to the unsaturated zone. The most abundant gases are those found in the atmosphere: nitrogen, oxygen, and carbon dioxide. In well-aerated sediments, the oxygen content is near that of the atmosphere (~20%) and the carbon dioxide content is less than 2%. However, high rates of microbial activity can remove oxygen from the gas phase of the unsaturated zone and increase the carbon dioxide as high as 19%.

The vertical diffusion of gases is governed by Fick's law

$$q = D\, dC/dz$$

where q is the rate of diffusion (mmoles cm^{-2} sec^{-1}), D is the diffusion coefficient (cm^2 sec^{-1}), C is the concentration of the gas (mmoles cm^{-3}), and z is depth. In the unsaturated zone, however, the diffusion of gases is considerably more complex than implied by Fick's law. For example, gaseous diffusion will depend upon the percentage of pore space actually filled with gas (as opposed to capillary water) and this is subject to rapid changes with time. Also, biologically active gases such as oxygen or carbon dioxide may be consumed or produced as diffusion proceeds. A more accurate description of gaseous transport in the unsaturated incorporating these complexities was given by van Balvel (1951) as

$$q = R + YP_gD\, dC/dz$$

where q, D, and dC/dz are as given in Fick's law and where R is the production or consumption of a gas (mmole cm^{-3} sec^{-1}), Y is a factor that takes the tortuosity of the sediment into account (dimensionless), and P_g is the gas-filled porosity of the sediment (dimensionless).

A quantitative treatment of gas transport in the unsaturated zone of several sites in the western United States has been given by Thorstenson et al. (1983) and Wood

and Petraitis (1984). These studies showed that oxygen concentrations decreased while carbon dioxide concentrations increased with depth, reflecting oxygen consumption by aerobic microorganisms and consequent carbon dioxide production. The unsaturated zone is extremely dynamic in terms of CO_2 production and diffusion. For example, the CO_2-depth profile for one site in North Dakota shows that in May the pCO_2 is higher deep in the unsaturated zone than at land surface. Consequently, there is a diffusive flux of CO_2 from the unsaturated zone to the atmosphere. Later in the summer, however, CO_2 production in the root zone reverses the diffusive flux and there is a net transport of CO_2 from the soil zone downward toward the water table. Although year-round data from this site are not available to document the timing of the diffusive gradient reversal, it is clear that it occurs in the winter when root zone CO_2 production is depressed by cold weather. This site provides an excellent example of the dynamic nature of gas transport in the unsaturated zone.

7.2.2 Biomass Measurements in Soil Microbiology

Many of the methods commonly used in subsurface microbiology were developed by soil microbiologists. For this reason, a brief survey of some techniques commonly used in soil microbiology is of practical as well as historical interest.

Biomass is defined as the living part of soil organic matter exclusive of roots and soil animals larger than 5,000 cubic micrometers in size. In soils, biomass consists of both bacteria and protozoa. Below the soil zone, however, biomass consists largely of bacterial cells. While protozoa are commonly found deeper in the unsaturated zone, their total biomass is small compared to that of the bacteria.

Measurements of biomass in soils has long been a preoccupation of soil microbiologists. The most important reason for this is that there is a direct relationship between soil biomass and soil fertility. There are many techniques for measuring biomass, each of which has certain advantages and disadvantages.

Direct Microscopy Winogradsky, the founder of soil microbiology, was the first investigator to successfully attempt direct microscopic examination of soils. The basic method developed by Winogradsky is simplicity itself: a microscope slide is placed into the ground, soil is packed carefully around it, and it is left for several weeks. After the incubation time has passed, the slide is removed and stained, and the microorganisms that have adhered to the slide observed directly. This technique provides a qualitative view of microbial interaction in situ.

More recently, the scanning electron microscope (SEM) has been used to observe microorganisms in soils. This technique works well for observing fungi and actinomycete structures, but is less useful for observing bacteria. This is because bacteria are difficult to unequivocally distinguish from small soil particles.

A common technique designed specifically for counting microorganisms is epifluorescence microscopy. This technique is notable because it has been widely adapted to enumerating bacteria in samples from the deep subsurface. A sediment sample is dispersed in a blender or shaker, sometimes with the help of a detergent (tween 80), and a small volume of the suspension is filtered onto a membrane. The membrane is then stained with a fluorescent dye, and bacteria enumerated with an epifluorescent microscope. This type of microscope illuminates the stained sample

with ultraviolet light, causing the fluorescent dye to emit visible light that can be observed by the microscopist. Acridine orange is the most widely used fluorescent stain. Acridine orange binds to nucleic acids, RNA and DNA and the resulting complex fluoresces at a characteristic wavelength of UV light. Fluorescein isothiocyanate (FITC) binds to sulfhydrl groups of proteins in cell walls and is sometimes used. After enumeration of the stained sample, the number of bacteria in the sample is calculated from the volume of sample filtered, the area of the filter, and the area of filter actually enumerated.

There are significant practical problems with obtaining reproducible counts of bacteria using the epifluorescence method. It is impossible, for example, to be sure that all bacteria have been desorbed from sediment grains by the shaking process. Also, acridine orange is a cationic dye and binds to negatively charged clay mineral surfaces. Thus, there is generally a large amount of interference in counting samples containing large amounts of clay. Despite these problems, direct counting procedures have been widely used.

Chemical Techniques The most widely used chemical technique for determining soil biomass is the *soil fumigation method.* Chloroform ($CHCl_3$) under pressure is forced through the soil in order to completely saturate it. The chloroform destroys the cell membrane of living cells allowing the cytoplasm to leak into the soil. After the fumigation has lysed the bacteria, the soil is inoculated with a small amount of soil containing living microorganisms, and the mixture incubated for 10 days along with untreated controls. Biomass is then calculated as

$$B_c = (CO_{2\,tr} - CO_{2\,un})/K_c$$

where $CO_{2\,tr}$ is the amount of carbon dioxide evolved from the cloroform-treated sediment, $CO_{2\,un}$ is the carbon dioxide evolved from the untreated soil, and K_c is the percentage of biomass carbon mineralized to carbon dioxide (often assumed to be 0.41).

A chemical technique that has been used to measure biomass in estuarine and marine environments is to quantify the abundance of phospholipids (Gillan, 1983; White, 1983). This method has been applied to biomass measurements in soils (Federle et al., 1986) and in other subsurface environments (White et al., 1983). The phospholipid technique takes advantage of the fact that bacterial membranes (as well as the cell membranes of some eucaryotes) contain phospholipids and that these compounds are unstable once the cell dies. Thus, the phospholipid content of sediments is directly proportional to biomass. Phospholipids can be extracted from sediments using chloroform, hydrolized into their various fatty acid components, and quantified using gas chromatography. Given the average amount of phospholipid present in bacterial cells (generally on the order of 50 micromoles/g bacteria), phospholipid content can be converted to bacterial numbers (Gehron and White, 1983).

A significant advantage of the phospholipid method over other measures of biomass is that the kinds of fatty acids liberated from sediment samples gives some information about microbial diversity. This reflects the unique phospholipid content of each bacterial strain. Also, because some phospholipids are found only in procaryotic organisms and some found only in eucaryotic organisms, the method can help distinguish the biomass component attributable to each class. More important,

in deep subsurface sediments where eucaryotes are rare, the method gives information on bacterial diversity that cannot be gathered using culture techniques.

Another chemical technique for estimating biomass in soils is by ATP assay. In this method, the cells in a soil sample are lysed, often by chloroform fumigations as discussed earlier, and the ATP content determined. This determination is based on the reaction

$$\text{D-Luciferin} + \text{ATP} + \text{Luciferase} + O_2 \rightarrow \text{AMP} + CO_2 + \text{Luciferase} + \text{light}$$

The light released by this process can be measured by a photometer and related to ATP content by comparison to standard curves. This technique is widely known as the "firefly" assay because firefly tails are a ready source of D-Luciferin and Luciferase.

Activity Measurements The essence of heterotrophic microbial metabolism is the oxidation of organic carbon compounds to carbon dioxide, with the microorganisms using the energy obtained for cell maintenance and growth. Thus, measurement of carbon dioxide production from soils has been widely used as an indicator of microbial activity. As with methods for estimating biomass, this method has been widely adapted for activity measurements of subsurface environments other than the soil horizon (Chapelle et al., 1988). The most straightforward method of estimating carbon dioxide production from soils is to place a sample in a closed vial, and monitor CO_2 production with time. Carbon dioxide is most conveniently quantified by gas chromatography equipped with a thermal conductivity detector (McMahon et al., 1990). However, CO_2 can also be stripped from incubation vials by a stream of nitrogen gas and trapped in a NaOH solution. The carbonate ions generated by the trapping procedure are then quantified by titration with $BaCl_2$ and precipitation of $BaCO_3$.

The accuracy and sensitivity of quantifying respiratory activity by measuring CO_2 production can be significantly enhanced by use of radiotracers. For example, amending a soil sample with ^{14}C-labeled glucose and quantifying the rate of $^{14}CO_2$ evolved is a powerful tool for measuring metabolic rates in soils. This radiotracer technique is particularly useful for determining relative decomposition rates of different organic compounds by indigenous microorganisms. For example, numerous radiotracer studies have shown that aliphatic compounds degrade much faster than phenolic compounds. This has been interpreted as reflecting the relative difficulty soil microorganisms encounter in degrading compounds containing relatively stable benzene rings.

A significant problem with both the CO_2 and $^{14}CO_2$ methods is that they tend to overestimate respiration rates. This may reflect soil disturbance due to sampling procedures, enhancement of activity by addition of artificial nutrients, or a host of other possibilities. For this reason, these procedures are most effective when used to determine the activities of different soils relative to each other. In this way, sampling and experimental protocols can be standardized, and the relative activities of soil samples determined fairly accurately.

Other Measures of Biomass and Activity A large amount of work has been invested into alternative methods of measuring biomass and microbial activity. One of these methods, that of measuring the activities of specific enzymes in soils, has

been widely applied. Because microorganisms catalyze a wide variety of metabolic functions enzymatically, the activity of a particular enzyme should reflect the rate of metabolism. Some of the enzyme activities used for this purpose include oxidoreductases such as catalase, dehydrogenase, glucose oxidase, and oxidase as well as hydrolases such as amylase and cellulase.

Dehydrogenases, enzymes used by microbes to transfer hydrogen ions, have been widely used as a direct measure of metabolic activity. Dehydrogenase activity is measured by determining rates of 2,3,5-triphenyl tetrazolium chloride reduction to triphenyl formazan in soil under anaerobic conditions. The triphenyl formazan can then be extracted and measured spectrophotometrically. Significantly, a positive correlation between dehydrogenase activity and respiration rates determined by CO_2 or $^{14}CO_2$ production is not commonly observed. This has been interpreted as reflecting the nonactive state of much of the ambient soil microflora.

One variation of the enzyme activity method is to measure the products of an enzymatic process, such as hydrolosis, that may be carried out by a wide variety of enzymes. For example, fluorescein diacetate (FDA) is hydrolyzed by many different enzymes, such as lipases, proteases, and esterases. Thus, the rate of FDA hydrolosis in soil slurries is a qualitative measure of a broad spectrum of enzymatic activity and thus bacterial activity (Schnurer and Rosswall, 1982). The FDA hydrolosis technique has been applied to a number of soils. A notable example was given by Federle et al. (1986) who showed that microbial activity was present well below the soil zone and pointed out that this activity could well impact the transport of contaminants. These authors also showed that FDA activity correlated well with the abundance of phospolipids, a measure of biomass.

Another method long used in soil microbiology as a measure of microbial activity is heat output. This was first proposed by Hesselink van Suchtelen in 1931, and significant advances in the technique, termed *microcalorimetry,* have been made recently in applying this concept to soils (Sparling, 1981).

Given the wide variety of methods available for measuring microbial activity in soils, it is reasonable to wonder how well these techniques agree with each other. In fact, the methods do not always agree as well as one might expect. One such comparison was given by Sparling (1981), who showed that heat output consistently correlated with respiration, ATP content, amylase activity, and biomass. Curiously, however, heat output did not correlate well with dehydrogenase activity. In addition, there was little correlation between the various activity measurements and soil properties such as pH, carbon content, or nitrogen content.

The lack of consistently strong correlations of the various measures of soil biomass and microbial activity troubled microbiologists for many years. Recently, however, a consensus has emerged that no one technique is capable of giving the "answer" as to soil biomass or microbial activity. Each of these techniques, however, is suited for answering particular questions. Thus, the technique selected generally depends more on the question being asked than on the "accuracy" of the technique. If, for example, the question deals with relative respiration rates between the soil zone and sediments deeper in the unsaturated zone, CO_2 or $^{14}CO_2$ production rates are suitable techniques. If, on the other hand, the question is how much of the soil organic matter is tied up in microbial biomass, the chloroform fumigation or ATP techniques may be more appropriate. Finally, if the question deals with the diversity of the microbial community, the phospholipid technique is most appro-

priate. This generality applies to biomass and microbial activity measurements in all subsurface environments. The measurement technique largely depends on the question being asked.

7.2.3 Distribution of Bacteria in the Unsaturated Zone

The unsaturated zone is inhabited by bacteria, viruses, actinomycetes, cyanobacteria (formerly called blue-green algae), fungi, and algae as well as by microscopic arthropods. Bacteria, however, are the most numerous of the microorganisms in this environment and their metabolism is the most broadly based. For these reasons, bacterial processes probably have the largest impact on the chemistry of infiltrating ground water. No attempt will be made here to discuss the distribution of microorganisms other than the bacteria.

The Soil Zone A great deal is known about the types and distribution of bacteria in soils. Much less is known about bacterial abundance and distribution in the unsaturated zone below the soil. Very early on, it was observed that total numbers of bacteria decreased sharply with depth in the soil (Waksman, 1932). Because of this, it was easy for soil microbiologists to conclude that bacteria ceased to exist in appreciable numbers below the soil zone. This reasoning, while inaccurate, was perfectly logical based solely on the distribution of bacteria in the soil zone. For example, if one fits an exponentially decreasing function to a bacterial profile like that of Waksman (1952), it is found that the numbers of bacteria approach zero at a depth of only a few meters. Based on this type of reasoning, it's easy to see why some soil microbiologists earlier in this century dismissed the possibility that bacteria could live deep in the unsaturated and saturated zones.

The Intermediate Unsaturated Zone While studies of bacteria in the saturated portion of the subsurface have become fairly common in the last twenty years, relatively little attention has been paid to the unsaturated zone. A notable exception to this was a study at a site in Oklahoma (Balkwill and Ghiorse, 1985). These investigators carefully noted the saturation status of samples taken from the subsurface and reported them as being from the unsaturated zone, from the interface of the saturated and unsaturated zones (i.e., the capillary fringe), or from the saturated zone. Total counts of bacterial cells (determined by acridine orange direct count) were in the range of 10^6 to 10^7 cells per gram of dry sediment. The highest counts were found just below the soil zone and were somewhat lower in the capillary fringe. Interestingly, there was no significant change of bacterial numbers from the capillary fringe into the saturated zone.

Balkwill and Ghiorse (1985) also reported the total numbers of distinct colony types observed on several types of growth media. The number of distinct colony types observed to grow on dilute PTYG (peptone, tripticase, yeast extract, glucose) agar declined noticeably with depth. In contrast, samples plated onto SSA (surface soil extract agar) media showed no noticeable decrease in numbers of colony types with depth. While it's tempting (and probably inappropriate) to overinterpret these data, it does suggest that bacteria from deeper in the unsaturated and saturated zones grew most efficiently on carbon sources readily available from indigenous sources.

The types of bacteria identified by Balkwill and Ghiorse (1985) from the unsaturated and saturated zones were dominated by isolates of the genera *Pseudomonas* and *Arthrobacter*. Significantly, members of these genera are typically observed to be the dominant types of bacteria present in soils. It is entirely possible that the types of bacteria present in the unsaturated zone and shallow saturated zone largely reflects bacterial populations present in the overlying soil zones. However, data are presently insufficient to firmly decide this interesting question.

The Deep Unsaturated Zone Significant areas of the earth are characterized by arid or semiarid climates. These areas often exhibit unsaturated zones that are tens or even hundreds of meters thick. The microbiology of these deep unsaturated zones has not been extensively characterized. However, one such study has been reported by Colwell (1989) that focused on unsaturated sediments from the high desert of Idaho.

In the Idaho desert study, surface soils exhibited direct counts of 10^6 cells/gram, whereas unsaturated sediments from a depth of 70 meters showed direct counts of about 10^5 cells/gram. However, viable counts of bacteria made by plating sediment slurries onto PTYG agar showed a much more dramatic decrease with depth. Surface soils showed about 10^5 cells/gram, whereas unsaturated sediments from a depth of 70 meters showed viable counts of 50 and 21 cells/gram, respectively. This finding contrasts markedly with studies of saturated sediments in which there are typically only two or three orders of magnitude difference between total and viable counts of bacteria. This, in turn, suggests that the bacteria in these deep sediments are under considerable stress.

One unusual finding reported by Colwell (1989) is that of 32 strains of aerobic, heterotrophic bacteria recovered from these deep unsaturated sediments, 84% were gram positive. This again contrasts with studies of saturated subsurface sediments where gram-negative strains typically predominate. It is possible that a major component of the stress in this environment is the lack of moisture. If this is the case, the relatively thick peptidoglycan layer in the cell wall of gram-positive bacteria may confer upon them a competitive advantage over gram-negative bacteria. In any case, the observed presence of nearly atmospheric levels of gaseous oxygen in these deep unsaturated sediments certainly reflects the extremely low indigenous activity of microorganisms in this environment.

7.3 LOCAL FLOW SYSTEMS

A local system of ground-water flow is defined as having its recharge area at a topographic high and its discharge areas at an adjacent topographic low (Fig. 7.1). From a practical perspective, local flow systems often correspond to shallow water table aquifers that are recharged directly from precipitation and that provide base flow to adjacent streams or rivers. Local flow systems are hydrologically very active. In humid temperate climates, like those of the eastern United States where annual rainfall reaches 130 to 150 cm, as much as 25 to 50 cm of water may recharge local flow systems. For example, in a regional simulation of ground water flow of the Atlantic Coastal Plain physiographic province, an estimated 90–98% of recharge to the water table was subsequently discharged to adjacent surface water

bodies. In areas of such high recharge and discharge, rates of ground water flow may be as high as a meter per day.

The depth that local flow systems penetrate into the subsurface is largely dependent upon topographic relief. For example, Toth (1963) shows that for the case of a homogeneous aquifer, the depth of the local flow system varies directly with topographic relief. In one example similar to that shown in Figure 7.1, Toth showed that if topographic relief was about 15 meters, then the local flow system would penetrate about 150 meters into the subsurface. If, on the other hand, topographic relief was increased to 61 meters and all other parameters held constant, then the local flow system penetrated all the way to the impermeable lower boundary.

This is an extremely important result, and it illustrates why depth is such a poor predictor of the kind of flow system predominating at any site. For high-relief topographies, local flow systems may penetrate hundreds of meters into the subsurface. For topographic settings with little relief, local flow systems may penetrate only a few tens of meters into the subsurface.

The other important variable that influences the depth of local flow systems is subsurface permeability variation (Freeze and Witherspoon, 1967). In the case of a low-permeability (K = 1) surface material underlain by a high-permeability (K = 100) material Freeze and Witherspoon showed that the local flow system penetrated about 75 meters into the subsurface. If, on the other hand, there was a layer of intermediate permeability (K = 10), the local flow system penetrated only about 25 meters into the subsurface. In general, high-permeability sediments underlying low-permeability sediments tend to restrict the depth to which local flow systems penetrate. This situation tends to carry water past adjacent recharge-discharge areas so that intermediate flow systems occupy a greater thickness of the section relative to local flow systems.

7.3.1 Local Flow Systems as a Microbial Habitat

Local flow systems are very active hydrologically. This is to say that water from precipitation events quickly passes through the unsaturated zone, recharges local flow systems, and is readily discharged to surface streams or transpired by phreatophytic plants that tap the saturated zone. It's not uncommon for the water table of local flow systems to rise or fall several meters over time periods of only a week or two. It is generally considered that residence times of water in local flow systems are on the order of days to years.

The dynamic nature of local flow systems has important consequences as to the microbial habitat that is generated. Fast rates of recharge and discharge mean that local flow systems are relatively open to sources of nutrients from the surface or from the unsaturated zone. For example, dissolved inorganic carbon generated in the unsaturated zone may be quickly transported into local flow systems. Figure 7.3 shows a conceptual model of DOC production, transport through the unsaturated zone, and delivery to the water table (Thurman, 1985). DOC that is transported into local flow systems, while often consisting of high-molecular-weight compounds that are not easily assimilated by microorganisms, may provide an important source of nutrients to bacterial populations.

Fast rates of recharge and the relatively free exchange of gases within the unsaturated zone encourages the transport of dissolved oxygen to local flow systems.

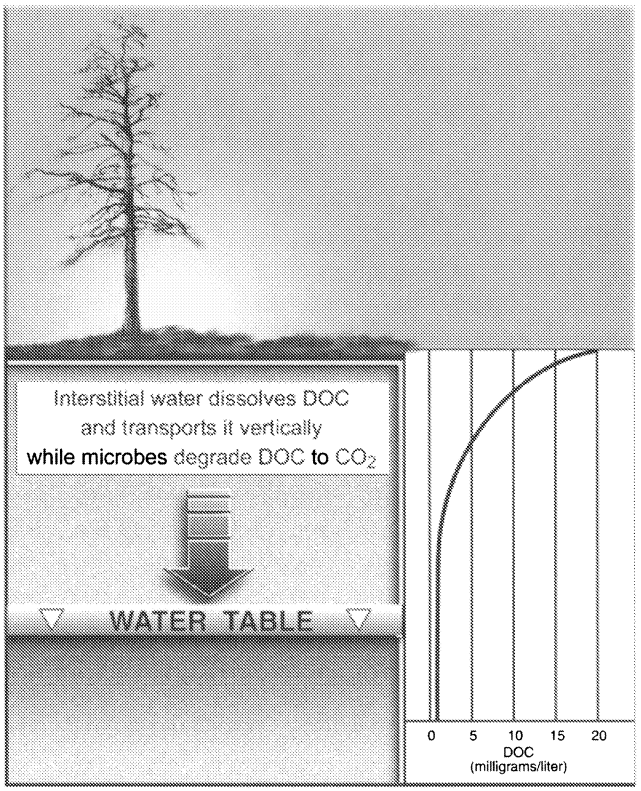


Figure 7.3. The production of DOC in the soil zone and it's transport to shallow ground-water systems.

Because aerobic bacteria obtain more energy per mole of organic material oxidized than bacteria using other electron acceptors (such as ferric iron or sulfate), local flow systems are often dominated by aerobic bacteria. Such aerobic activity can be sustained only if there is a steady flux of oxygen into the system, and this requires the close connection with the unsaturated zone characteristic of local flow systems.

Recharge to local flow systems that passes through the unsaturated zone may also facilitate the transport of dissolved nitrogen species in the form of nitrate or ammonia. Because nitrogen fixation, ammonification, and nitrification are processes characteristic of the soil zone, recharge moving through soils to local flow systems is a potential source of organic nitrogen. Because nitrogen is required for protein synthesis, it is an important nutrient requirement for bacterial growth. In many subsurface environments, nitrogen is the principal limiting factor for bacterial growth. In this respect, however, local flow systems have less limitation than intermediate or regional flow systems.

The proximity to land surface and the dynamic nature of ground-water movement in local flow systems makes them relatively accessible to chemical pollutants from human activities. These pollutants come from a variety of sources and include a dizzying variety of compounds. From the standpoint of a microbial habitat, such pollution is often a boon for bacterial growth, as it may provide carbon (gasoline,

sewage wastes) or nitrogen (fertilizers, septic effluents) sources, each of which is often limiting in subsurface environments. Although precise figures are difficult to compile, it is probable that as much as 80% or 90% of all documented cases of ground-water pollution occur in local flow systems. Thus, our experience with the microbiology of ground-water pollution and bioremediation strategies (Chapters 12 and 13) is largely confined to local flow systems.

7.3.2 Distribution of Bacteria in Local Flow Systems

Systematic studies on the distribution of bacteria in shallow water table aquifers, herein termed local flow systems, are relative latecomers to subsurface microbiology. Whereas the distribution of bacteria in unsaturated sediments (the soil zone) and in regional flow systems (petroleum reservoirs) have been studied since the early twentieth century, studies on local flow systems began only in about 1980 (Ghiorse and Wilson, 1988, p. 126). To a large degree, this neglect simply reflects a lack of pertinent questions concerning microbial processes in local flow systems. In the case of the unsaturated zone (soils), the obvious connection between soil fertility and the abundance of soil microbes presented investigators with a wide range of important questions. Addressing these questions has spurred research in soil microbiology for 100 years. In the case of regional flow systems (largely petroleum reservoirs), it was recognized in the 1920s that bacterial processes affected petroleum production. This led to many studies on bacterial corrosion of well casings, biofilm plugging of reservoir rocks, and metabolism of hydrocarbons (Davis, 1967).

However, there was no similar impetus for studying microbial distributions in shallow water table aquifers. The increasing awareness that some human activities contaminated water table aquifers to the point of their having to be abandoned as sources of potable water changed this in the early 1970s. It was widely thought, for example, that simple dilution by uncontaminated water was the most practical method of renovating contaminated water supplies. Since many surface water bodies are completely turned over in a matter of days or weeks, the "dilution solution" was deemed a workable remediation strategy. By this criterion, however, ground-water reservoirs could not be salvaged once contamination took place. Even local flow systems are recharged very slowly relative to surface water bodies, and the conventional wisdom was to simply write off contaminated ground water as a loss.

This conventional wisdom did not stand up to closer examination. It became a common observation in the consulting industry, for example, that the size of plumes resulting from gasoline spills often tended to shrink over time. How could such observations be explained? To microbiologists, an obvious question to ask was whether microbial metabolism could be involved. This question was systematically attacked by a group of researchers brought together by the U.S. Environmental Protection Agency working out of Ada, Oklahoma (Dunlap and McNabb, 1973; McNabb and Dunlap, 1975; Wilson et al., 1983; Ghiorse and Balkwill, 1983). These investigators not only showed the presence of bacteria in shallow aquifers, but also that the bacteria were capable of degrading a variety of organic pollutants (Wilson et al., 1983). This realization rapidly changed the conventional wisdom that pollution in ground-water reservoirs was irreversible, and led to an explosion of interest in subsurface microbiology.

The distribution of bacteria indigenous to a local flow system near Lula, Oklahoma, as measured by direct microscopy as well as aerobic plate count methods determined by Balkwill and Ghiorse (1985), were all in the 10^6 cells per gram (dry weight) range and were remarkably consistent. The plate counts, on the other hand, varied widely. The lowest counts were found on nutrient-rich peptone-tripticase-yeast extract-glucose (PTYG) media. Higher counts, performed on duplicate sediment samples, were found using dilute (1:20 dilution) PTYG media and surface soil extract (SSE) media. The fact that higher counts were consistently found using dilute media was interpreted by Balkwill and Ghiorse (1985) as reflecting the acclimation of subsurface bacteria to low-nutrient conditions. This acclimation apparently tends to lower the organisms' tolerance to high-nutrient conditions, and hence the lower counts on the rich media. Anaerobic plate counts were generally one to four orders of magnitude less than aerobic counts.

Just as interesting was the demonstration that the total counts were consistently higher than the plate counts. Two possible explanations for this observation include (1) the presence of a large number of inactive organisms or (2) the inability of some of the organisms present to effectively grow on the media offered. Balkwill and Ghiorse (1985) tended to lean toward the second possibility because plate counts on SSE media consistently approached (within an order of magnitude) the direct counts. However, that particular question may never be resolved adequately.

Perhaps the most extensive characterization of bacterial distributions in local flow systems has been at the Savannah River Site (SRS) in South Carolina (Sinclair and Ghiorse, 1989; Balkwill, 1989; Fredrickson et al., 1989). The geology of this site is extremely complex and represents a series of overlapping deltas that have been deposited since Late Cretaceous Time (~80 MY). The hydrology of the site is similarly complex. Figure 7.4 shows a generalized stratigraphic column together with counts of bacteria and eucaryotic organisms at one location (site P24) at SRS (Sinclair and Ghiorse, 1989). Local topographic relief shows elevation changes of 25 to 50 meters between ridges and stream bottoms. This relatively high relief, combined with the high permeability of the surface sediments, allows the local flow systems to penetrate deeply into the subsurface at this site. At site P24 (Fig. 7.4), the local flow system penetrates about 75 meters into the subsurface and grades into an intermediate system below the low-permeability Williamsburg formation.

Within the different geologic horizons in the local flow system, counts of bacteria by direct microscopy (AODC) are all in the 10^6–10^8 cells per gram range. As was observed at the Lula, Oklahoma, site, plate counts of bacteria showed much more variation than direct counts. Interestingly, these sediments contained a significant population of eucaryotic organisms, with numbers ranging from below detection to as much as 10^3 cells per gram of sediment. There was little variation of eucaryotes with depth, and no measurable change between the local and intermediate portions of the flow system. The most consistent predictor of the numbers of eucaryotes was clay content, with high-clay samples containing the least numbers of cells. While present throughout the column at the SRS, numbers of eucaryotes were always 3 to 5 orders of magnitude less than bacterial numbers. These are the most comprehensive data yet compiled showing that bacterial numbers far exceed numbers of eucaryotes in subsurface sediments below the soil zone.

A major finding of the microbial characterization at the SRS was that numbers of bacteria correlated inversely with the amount of clay in the sediments (Fig. 7.4).

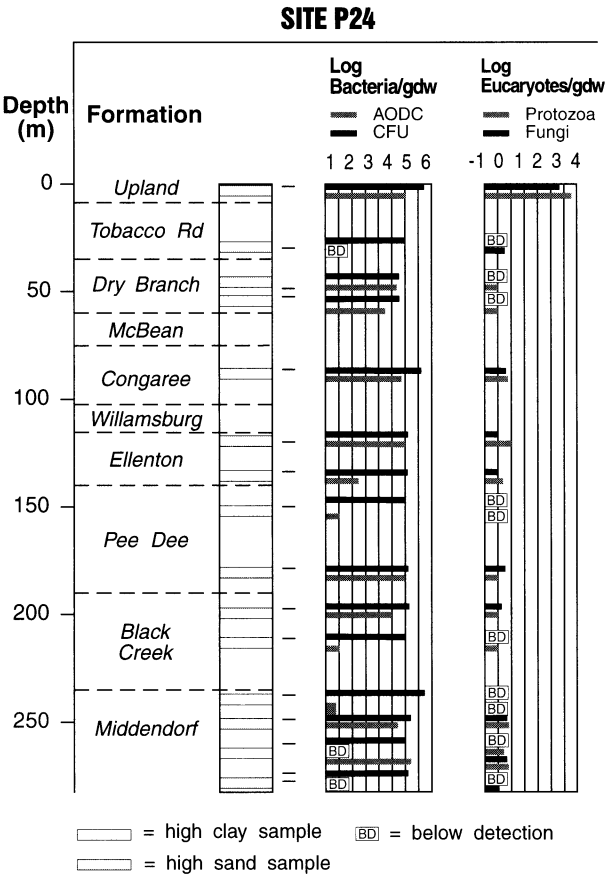


Figure 7.4. Numbers of bacteria, protozoa, and fungi in coastal plain aquifers that grade from a local flow system (<75 m) to an intermediate flow system (>75m). (From Sinclair and Ghiorse, 1989, with permission courtesy of Taylor and Francis Publishers.)

Fredrickson et al. (1989) attribute this to the lower hydraulic conductivity of clay-rich sediments. However, these authors also point out that the pH of pore water in clays was lower than in the sands and that this might also contribute to the observed trend.

Not only were bacterial numbers lowest in the clay horizons, but bacteria activities were correspondingly low (Phelps et al., 1989a). For example, clayey samples from borehole P24 oxidized ^{14}C-labled acetate to $^{14}CO_2$ at rates of 100–500 disintegrations per minute (dpm) per day whereas sandy sediments ranged from 10,000 to 50,000 dpm/day. Not only were rates of substrate oxidation greater in sandy sediments, but other measures of bacterial activity, such as ^{14}C acetate incorporation into lipids and [^{3}H]thymidine incorporation into DNA, were consistent with this finding.

The aquifers underlying the SRS, both those in the local flow system above the Ellenton Formation and those of the deeper intermediate flow system, are largely aerobic, with dissolved oxygen concentrations varying between 1 and 6 mg/L. In spite of this, anaerobic bacteria are part of the ambient microflora (Jones et al.,

1989). Numbers of sulfate-reducing bacteria ranged from undetectable to as high as 10^4 cells/gram of sediment. While these numbers are much lower than numbers of aerobic bacteria, it does show a significant presence. Similarly, there was methane production from a number of zones. The presence of anaerobic bacteria in a predominantly aerobic aquifer is not entirely unexpected. Aerobic soils, for example, often contain significant populations of anaerobic bacteria. This is often interpreted as reflecting anaerobic microenvironments in the sediment where the diffusion of oxygen is insufficient to overcome oxygen consumption. The actual activity of anaerobic bacteria in the local flow system of the SRS is probably fairly low. Concentrations of methane in the ground water are generally less than 0.01 micromolar and sulfate concentrations show no decreasing trend along aquifer flow paths. The documented presence of anaerobic bacteria in these sediments, however, confirms the similarity of local flow systems with sediments from the unsaturated zone.

The distribution and abundance of bacteria have been studied in local flow systems developed in other geologic settings. For example, in the midwestern United States, rivers with beds incised into Paleozoic bedrock often have narrow (1–10 km) alluvial aquifers associated with them. These aquifers are termed buried-valley aquifers, since they represent Pleistocene river channels that have subsequently been filled with sediment. In a survey of microbial populations in Kansas, Sinclair et al. (1990) have shown that many of the microbial characteristics found in coastal plain sediments of the SRS also occur in buried valley aquifer sediments. For example, total counts of bacteria range between 10^6 and 10^8 cells per gram of sediment, which is the range found at the SRS. Also, counts of eucaryotes were much lower than those of bacteria. Finally a similar trend of decreasing bacterial abundance with increasing clay content was found.

The statistical correlating matrix prepared by Sinclair et al. (1990) demonstrated several other patterns. For example, there was a strong ($r = 0.64$) positive correlation of number of colony types (NCT) with the total number of colony-forming units (CFU). This suggests that those zones characterized by the greatest numbers of bacteria also exhibit the greatest diversity of bacterial types. This trend was also evident in the correlation between NCT and most probable number of protozoa (MPNP), suggesting that protozoa are most abundant where bacteria were most abundant. From an ecological point of view, these observations make intuitive sense. One would expect that given a favorable environment, several different types of microbes, both procaryotes and eucaryotes, should thrive. Intuition, however, is notoriously unreliable, and it is comforting to see these trends confirmed by hard data.

An interesting feature of the aquifers studied by Sinclair et al. (1990) is the presence of an apparent redox boundary indicated by a change of sediment color from tan (oxidized) to gray (reduced). There was no noticeable change in total numbers of bacteria across this boundary, but there was a drop in the numbers of viable bacteria as evaluated by plate count enumeration. One possible explanation for this is that a higher percentage of cells present in the gray sediments were strict anaerobes. They would therefore be countable by direct microscopy, but not countable by aerobic plate counts. Many local flow systems are well-oxygenated, and thus the predominant types of bacteria are aerobic. However, anaerobic zones are not uncommon, and these conditions can be expected to change the kinds of bacterial processes that are carried out in situ.

7.4 INTERMEDIATE FLOW SYSTEMS

The defining characteristic of an intermediate flow system is that one or more topographic low areas separate recharge areas from discharge areas (Fig. 7.1). In practice, intermediate flow systems generally correspond to confined aquifer systems of moderate (<300 meters) depth. Because intermediate flow systems produce a high percentage of the ground water used for human consumption, animal consumption, and irrigation in the United States, they are economically very important.

Intermediate flow systems differ from local flow systems primarily in the extent of connection with the surface and with the unsaturated zone. Because of this relative lack of connection, recharge rates are much lower than in most local flow systems. For example, digital modeling studies of Atlantic Coastal Plain aquifers show that average recharge rates to the confined part of the system (i.e., the intermediate flow system) are typically in the 1 to 3 cm/yr range (Leahy and Martin, 1986). This contrasts with recharge rates to shallow local flow systems of 30 to 50 cm/yr. In particularly humid climates characterized by particularly permeable aquifer sediments, such as the Floridan aquifer system of the southeastern United States, recharge rates to intermediate flow systems are somewhat higher and are in the 10 cm/yr range (Bush and Johnston, 1986). Recharge rates to the High Plains aquifer systems in the Central USA, where the climate is arid, are as low as 0.1 cm/yr.

Partly because of these low recharge rates, intermediate flow systems are characterized by relatively slow rates of ground water flow. Typical rates of ground water flow in intermediate flow systems are on the order of 1 meter per year (Chapelle and Lovley, 1990).

7.4.1 Intermediate Flow Systems as a Microbial Habitat

The low rates of recharge and ground water flow characteristic of intermediate flow systems creates a microbial habitat very different from that of local flow systems. Available evidence suggests that the delivery of DOC from the soil zone to local flow systems is fairly low. This being the case, delivery of DOC from surface soils to intermediate flow systems is often negligible. Because of this, microbial populations in intermediate flow systems are largely dependent upon organic material present in the sediments as a primary carbon source. These carbon materials are often refractory in nature, and this severely limits the metabolic and growth potential of microbes.

For example, McMahon (1990) showed that sedimentary organic material present in the Black Creek aquifer of South Carolina consists largely of complex aromatic and aliphatic polymers. The ^{13}C nuclear magnetic resonance (NMR) spectra of such materials show that carbohydrate carbon is largely absent, having been removed by prior microbial processes. McMahon (1990) also showed, however, that indigenous bacteria are able to metabolize this residual organic carbon, concentrating on the aliphatic fraction. Evidence for this was provided by evaluating the NMR spectra of organic material cored from the subsurface before and after a long-term incubation. In one instance, the NMR spectra of organic carbon cored from a depth of 58.5 meters showed two large peaks, one corresponding to aliphatic carbon compounds and the other to aromatic compounds. The aromatic:aliphatic ratio (R) of the peak areas for the freshly cored material was 1.28. However, after

being incubated in the lab for one year, a procedure that allowed indigenous bacteria to metabolize the material, the ratio increased to 1.70. This indicates that indigenous bacteria are capable of metabolizing the refractory carbon compounds available in the aquifers and that they preferentially oxidize the aliphatic portion. Because this pool of available carbon is so refractory, intermediate flow systems are typically very oligotrophic (nutrient-poor) environments.

Due to these oligotrophic conditions, rates of bacterial matabolism in intermediate flow systems are slower than those in local flow systems. Rates of bacterial metabolism as measured by CO_2 production in a local flow system underlying Hilton Head Island, South Carolina, were in the 10^{-2} mmoles per liter per year (mmole/L/yr) range (Chapelle et al., 1988). In contrast, rates of bacterial metabolism in the intermediate flow systems of the Black Creek and Middendorf aquifers were three orders of magnitude slower, in the 10^{-5} mmole/L/yr range (Chapelle and Lovley, 1990).

In addition to being strongly carbon-limited, microbial processes in intermediate flow systems are limited by the availability of organic nitrogen. Delivery of dissolved nitrogen compounds to intermediate flow systems from soil water is limited for the same reasons that delivery of DOC is limited. Not surprisingly, therefore, concentrations of nitrogen species in ground water of intermediate flow systems are generally less than 0.1 mg/L.

7.4.2 Distribution of Bacteria in Intermediate Flow Systems

The presence of bacteria in intermediate flow systems has been recognized for much of the twentieth century (Gurevich, 1962). However, data documenting the occurrence and distribution of bacteria in particular hydrologic systems has not generally been available until the 1980s.

Early work showing the presence of bacteria in intermediate flow systems concentrated on documenting the presence of bacteria in water produced from the aquifers. For example, Leenheer et al. (1976) showed that methanogenic bacteria were present in ground water produced from coastal plain aquifers of North Carolina. Godsy (1980), using a broth-antibiotic disc method, showed the presence of methanogenic bacteria in water produced from the Floridan aquifer.

Studies of bacterial distributions in local flow systems had clearly shown that a large percentage of indigenous bacteria were attached to sediment particles rather than living free in interstitial water. However, by the late 1980s there were no systematic studies of bacteria in sediments of intermediate flow systems. The reason for this lack of attention, as was the case with local flow systems prior to 1970, was that there were no pressing scientific questions that required such investigations. In the case of local flow systems, this impetus was supplied by concerns about ground-water pollution. However, because of the low recharge rates, chemical contamination of intermediate flow systems was a rare occurrence. Thus, microbial studies of intermediate flow systems were not mandated by concern about ground-water pollution.

The primary motivation for studying bacterial processes in intermediate flow systems came from another, somewhat unlikely, source. In 1983, techniques for studying the ground-water geochemistry of large-scale aquifer systems was codified in a series of papers by L. N. Plummer, D. C. Thorstenson, and D. L. Parkhurst of

the U.S. Geological Survey (Plummer et al., 1983). This series of techniques, loosely termed "geochemical modeling," provided geochemists a means of quantifying the amounts of minerals and gases that reacted along flowpaths of individual aquifers. As geochemical modeling studies progressed in many different aquifer systems, it quickly became apparent that production of CO_2 gas in aquifers was one of the most important processes affecting ground-water chemistry (Thorstenson, 1979; Plummer and Back, 1980; Chapelle, 1983). The problem in 1980 was that there was no known process capable of generating this CO_2 in deeply buried sediments below the soil zone. This led Chapelle and Knobel (1985) to resurrect a hypothesis originally put forth by Cedarstrom (1946) that the metabolism of bacteria in these confined aquifer systems was the ultimate source of the observed incoming CO_2.

Cedarstom's hypothesis was strengthened when Chapelle et al. (1987) documented the presence of viable bacteria in ancient coastal plain sediments penetrated by a 200-meter-deep core hole in southern Maryland. This core hole penetrated the Aquia (Paleocene) and Patapsco (Upper Cretaceous) aquifers, both of which function as intermediate flow systems in that part of Maryland. This aquifer system was much different from the local flow systems that had been studied previously. The most important difference was that ground water in both the Aquia and Patapsco aquifers is anaerobic. Not surprisingly, counts of total bacteria were in the 10^6–10^5 cell per gram range, about an order of magnitude less than found in aerobic aquifers. Furthermore, the viable cell counts were very low, on the order of 10^4 cell/gram, which is at the detection limit of the direct viable count procedure used for the measurements. Most significantly, the presence of viable sulfate-reducing bacteria in the sediments, as had been postulated forty years earlier by Cedarstrom (1946), was confirmed.

The primary significance of this work was to show that bacterial metabolism was a plausible source of CO_2 to ground water. As often happens, however, these data raised more questions than they answered. For example, how diverse were the bacterial communities? What were the interactions between different types of bacteria? Were there bacteria present that were in any way unusual? Did bacterial numbers and diversity vary with sediment properties?

At present, these questions have been considered only in a few intermediate aquifer systems. One of the most intensely studied intermediate aquifer systems is the Upper Cretaceous section of South Carolina. This work, performed at the Savannah River Site (SRS) in South Carolina, has been discussed previously in reference to local flow systems. The three deepest units present at the SRS—the Pee Dee, Black Creek, and Middendorf aquifers—largely function as intermediate flow systems. Unlike many intermediate aquifer systems these aquifers are aerobic, and therefore they are somewhat unusual. However most aquifer systems exhibit some unusual characteristics. A "typical" intermediate flow system probably does not exist.

Figure 7.4 shows the distribution of bacteria and eucaryotes in the intermediate part of the flow system, consisting of the Pee Dee, Black Creek, and Middendorf aquifers, in a core hole drilled at the SRS in South Carolina (Sinclair and Ghiorse, 1989). As was found in the shallower sediments comprising local flow system at the SRS, the most consistent predictor of microbial abundance was sediment texture. Sands generally contained the greatest numbers of microbes and clays con-

tained the lowest numbers. Significantly, the diversity of bacteria also correlated with sediment type, with the greatest diversity found in the sandiest sediments.

Not surprisingly, the aerobic activity of bacterial populations behaved in a similar manner. Hicks and Fredrickson et al. (1989) investigated the metabolism of ^{14}C-labled acetate, 4-methooxybenzoic acid, and phenol in cored sediments over a 21-day incubation period. Acetate and phenol were metabolized relatively easily in the sandy sediments, whereas 4-methooxybenzoate was metabolized less readily. In general, the highest rates of mineralization were observed in the sandy sediments and the lowest in the clays for each compound.

The dependence of bacterial activity on the clay content of sediments is a general observation and is found in other intermediate flow systems with quite different geochemical properties. For example, in the anaerobic portion of the Black Creek and Middendorf aquifers, rates of ^{14}C-labled acetate and glucose oxidation were orders of magnitude higher in sandy aquifer sediments than in clayey confining bed sediments (Chapelle and Lovely, 1990). There are probably many mechanisms that contribute to this effect, but one important factor is the small size of pore throats in clays relative to sands. Figure 7.5 shows that the average pore-throat diameter in clays, as measured by mercury injection, is less than 0.05 microns. In sands, on the other hand, average pore-throat diameters are much greater and are in the 2–20-micron range. Because bacteria generally have diameters of 0.1 to 1.0 microns, the small pore throats of clays must greatly restrict the ability of bacteria to move about and reproduce effectively. Similarly, helium injection into sediments shows that the interconnected porosity of sands is in the 90–95% range, whereas in clays it is much lower. This would tend to restrict the mobility of nutrients to active cells as well as restricting the movement of waste products away from cells. Again, this would tend to depress bacterial activity in clays relative to sands. Geochemical effects, such as lower pH of confining bed pore water, may also contribute to this effect (Fredrickson et al., 1989).

While data on the abundance and distribution of bacteria in intermediate flow systems are too sparse to allow comparisons between systems in a meaningful way, data on the activity of bacterial processes are available for three different systems. Figure 7.6 shows the ranges of bacterial activity, as measured by rates of CO_2 production, for the Madison aquifer system of the western United States (Plummer et al., 1990), the Patapsco aquifer system of southern Maryland (Chapelle et al., 1987), and the Black Creek–Middendorf aquifers system of South Carolina. The notable feature of these data is how similar they are, with CO_2 production rates in the 10^{-4} to 10^{-6} mmole per liter per year range. Furthermore, the ranges of CO_2 production are considerably lower than those in recent marine sediments. In fact, the only known environment with similarly low rates of CO_2 production are the nutrient-deprived waters of deep ocean basins. These data serve to illustrate that, while bacteria and bacterial activity are evident in many intermediate flow systems, overall rates are extremely low. In fact, these aquifer systems are among the most oligotrophic microbial environments that have ever been described.

There is some evidence that some intermediate flow systems lack bacterial activity entirely. For example, dissolved oxygen in concentrations of 6–8 mg/L are present in deep (~400 meters) alluvial-basin aquifers of the southwestern United States (Winograd and Robertson, 1982). This oxygenated water has been dated as being 20,000 to 30,000 years old, which implies that rates of bacterial oxygen

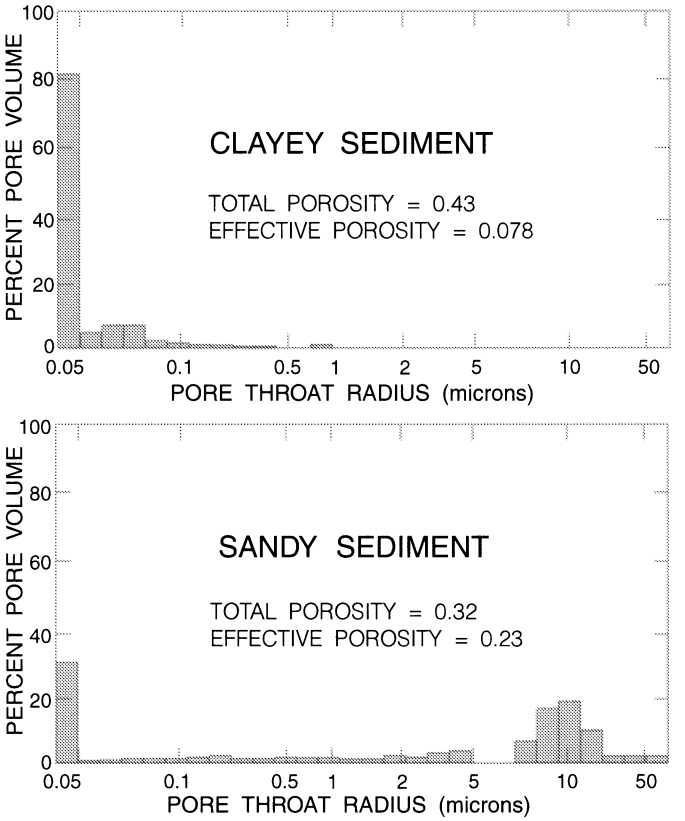


Figure 7.5. Pore throat radii in sandy and clayey sediments from an intermediate flow system. The smaller radii characteristic of clays may depress the mobility and activity of microorganisms. (Modified from Chapelle and Lovley, 1990, with permission courtesy of the American Society for Microbiology.)

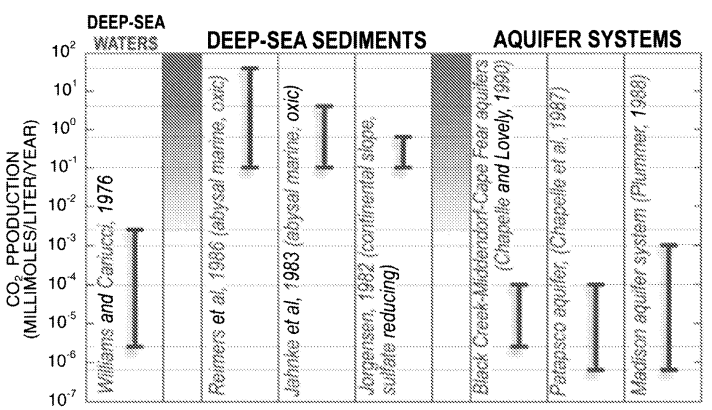


Figure 7.6. Ranges of CO_2 production, an indicator of overall rates of microbial metabolism, in aquifers (intermediate flow systems), deep-sea sediments, and deep-sea waters. (Modified from Chapelle and Lovley, 1990, with permission courtesy of the American Society for Microbiology.)

uptake are less than 10^{-7} mmoles per liter per year. In those samples where dissolved oxygen concentrations were still at atmospheric saturation, bacterial respiration rates must be virtually zero. The reasons for this lack of bacterial activity are not difficult to understand. These sediments were deposited under arid conditions and the amount of organic carbon present in the sediments is extremely low. The lack of a suitable organic carbon substrate evidently precludes the metabolism or growth of bacteria. However, studies on the distribution and abundance of bacteria in this system are not available to confirm or refute this.

7.4.3 Microbial Processes in Confining Beds

While it is generally observed that microbial activity in clayey confining beds is much less than that in more permeable aquifers, activity is often not completely absent. Furthermore, this activity can have important impacts on the chemistry of water in adjacent aquifer sediments in intermediate flow systems.

The first systematic evaluation of microbial processes in confining beds, and how they affect ground-water chemistry, was given by McMahon and Chapelle (1991). This study pointed out some unusual patterns in the concentrations of organic acids in aquifer and confining-bed pore water. Specifically, confining-bed pore water contained fairly high concentrations of acetate and formate (50–70 μM) compared to the fairly low concentrations found in the pore water of sandy aquifer sediments (1–10 μM). Furthermore, the clayey confining bed sediments showed much greater hydrogen (H_2) production than did sandy aquifer sediments. Since hydrogen, acetate, and formate are central to anaerobic food chains, these data were exactly opposite of what one might have expected given reported low rates of microbial activity in clayey sediments (Phelps et al., 1989; Chapelle and Lovley, 1990).

The reasons for the observed accumulation of organic acids in confining beds relative to aquifer pore water were clarified by means of radiotracer experiments. When confining-bed sediments were amended with ^{14}C-labled glucose, a fermentable substrate, it was observed that most of the ^{14}C accumulated in formate and acetate rather than being oxidized to CO_2. In sandy aquifer sediments, however, all of the ^{14}C was oxidized rapidly to CO_2. In anaerobic sediments, complete oxidation of fermentable substrates such as glucose requires the symbiotic action of fermentative and respirative bacteria. Fermentative bacteria partially oxidize the substrate with the production of organic acids and hydrogen. These simpler compounds are then utilized by respirative bacteria. The observed accumulation of organic acids, therefore, indicated that fermentation outpaced respiration in clayey confining-bed sediments.

This finding has important implications for the microbial cycling of carbon in ground-water systems. The concentration differences of organic acids between confining beds and aquifers appears sufficient to drive a net diffusive flux of organic carbon to aquifers. This, in turn, provides a constant supply of simple organic substrates for respirative bacteria in the aquifers.

The model proposed by McMahon and Chapelle (1991) is shown in Figure 7.7. Complex organic carbon material, including both aliphatic and aromatic compounds, in confining beds is subjected to slow continuous microbial fermentation. Respiration in confining beds, for reasons not well understood, is inhibited, leading

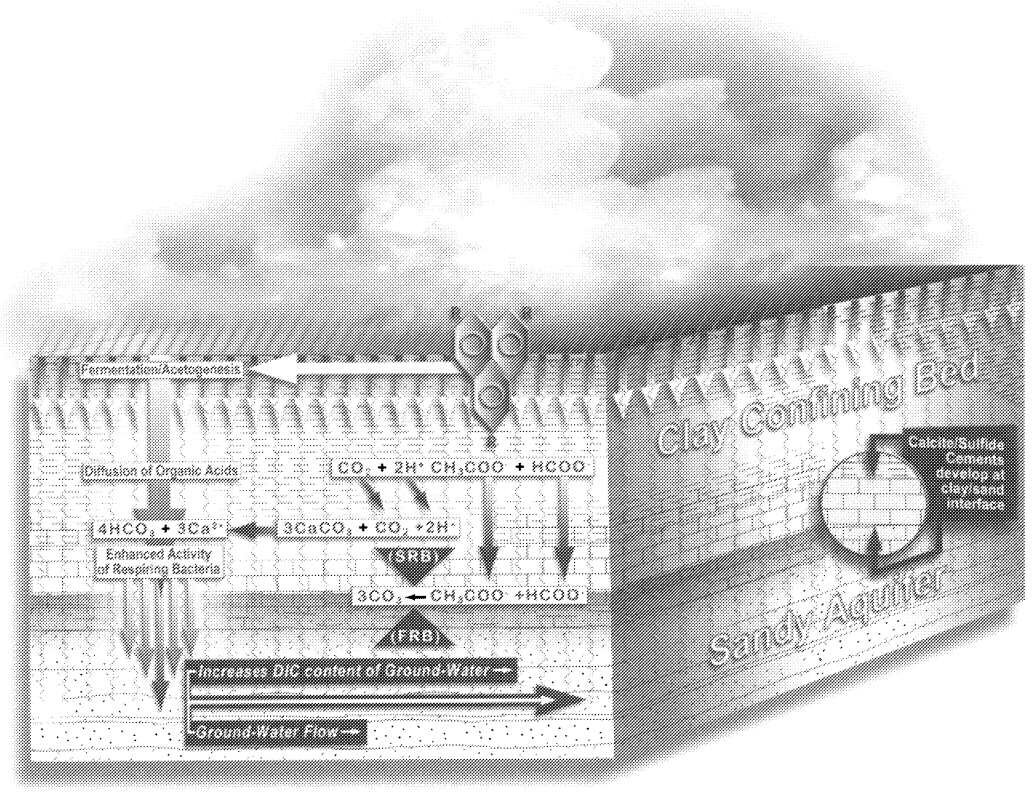


Figure 7.7. Model showing how acetogenic fermentation of complex organic matter in clayey confining beds can lead to the diffusion of acetate (CH_3COO^-) and formate ($CHOO^-$) to sandy aquifers. These processes can support the respiration of sulfate-reducing (SRB) or Fe(III)-reducing bacteria (FRB) in sandy aquifers and lead to the accumulation of dissolved inorganic carbon (DIC) in ground water.

to the accumulation of organic acids in the pore water. These organic acids then slowly diffuse to aquifers, where they are consumed by respirative bacteria such as sulfate reducers (SRB) or Fe(III) reducers (FRB). This model is important because it is the first proposed mechanism that effectively links the large pool of organic carbon often present in confining beds with microbial processes in aquifers.

The next logical question to ask concerned the kinds of fermentations occurring in confining beds. This question was undertaken by Chapelle and Bradley (1996), who also considered confining-bed sediments of the Atlantic coastal plain. They showed that high organic acid concentrations in confining-bed pore waters were a generally observed phenomenon, and that organic acid concentrations tended to increase with depth and were higher in thicker confining beds than in thin confining beds (Fig. 7.8). Furthermore, it was shown that a particular fermentation known as acetogenesis was an important microbial process in these sediments. Acetogenic bacteria are widely dispersed in the environment (Drake, 1994) and have the ability to derive energy by converting molecular hydrogen (H_2) and carbon dioxide (CO_2) into acetate according to the stoichiometry:

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$$

Like methanogenic bacteria, acetogens can use H_2 as an electron donor and CO_2

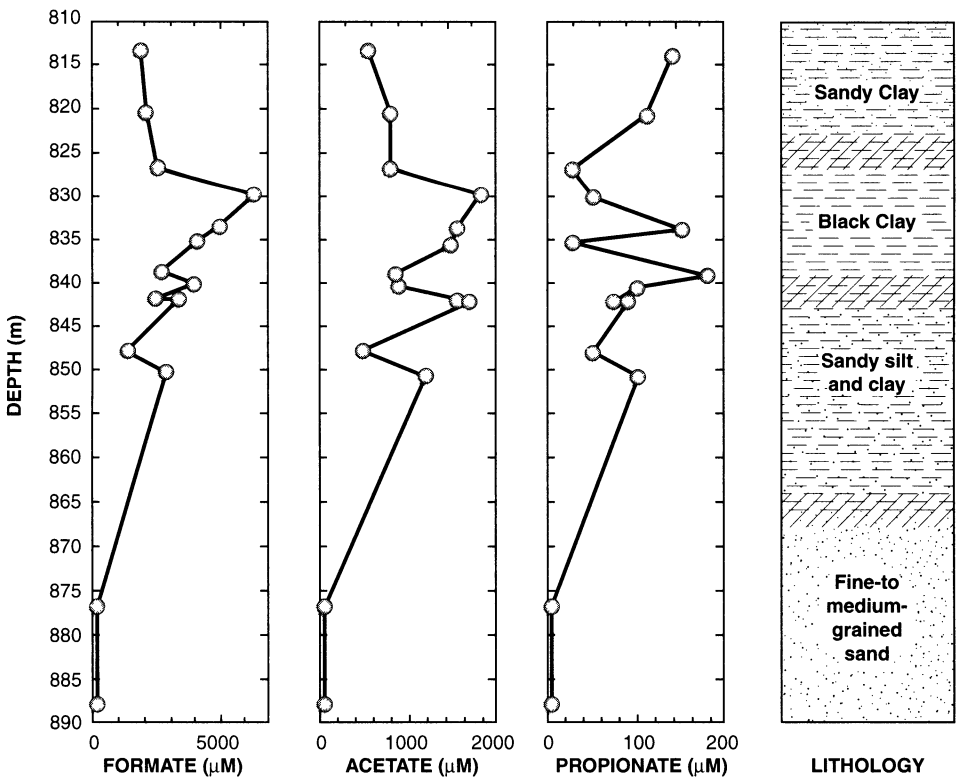


Figure 7.8. Clayey confining beds contain pore water with much higher concentrations of formate, acetate, and propionate than adjacent sandy aquifers in deeply buried coastal plain sediments.

as an electron acceptor. However, with acetogens, acetate is the end waste product. This process was demonstrated by showing that radiolabled $^{14}CO_2$ was converted to $^{14}CH_3COOH$ in experimental microcosms constructed with confining-bed material and incubated with a H_2 headspace. Interestingly, there was no observed generation of methane in these microcosms. This implies that methanogenic bacteria were absent from confining beds, which is consistent with previous observations (Beeman and Suflita, 1987).

The reasons why fermenting bacteria, such as the acetogens, are able to inhabit confining beds whereas respirative bacteria such as Fe(III) reducers, sulfate reducers, and methanogens do not is not known. There are, however, several possibilities. The acetogens cultured from confining-bed sediments by Chapelle and Bradley (1996) were gram-positive. One ecological advantage of the gram-positive cell wall is that it helps microorganisms deal with the lack of water. This is probably one reason why gram-positive bacteria are so abundant in the unsaturated zone (Colwell, 1989). Water in highly compressed confining beds is held tightly at the surface of clay minerals. Thus, while confining bed sediments are water-saturated, this water is much less available to microorganisms. It is possible, therefore, that the gram-positive cell walls of acetogenic bacteria provide them with a competitive advantage over gram-negative respiring bacteria in confining beds. This, in turn, may explain the predominance of acetogenic metabolism in confining-bed environments.

The importance of confining bed–aquifer interactions is not limited to coastal plain sediments. In a study of consolidated rocks of Cretaceous age in New Mexico, Krumholz et al. (1997) showed that sulfate–reducing activity was much higher at shale–sandstone contacts then elsewhere in the rock column. This study used a sensitive in situ assay for measuring relative rates of sulfate reduction. In this method, a silver foil is placed on intact rock and used to collect radiolabeled sulfide ($^{34}S^{=}$) generated by $^{34}SO_4$ reduction. This investigation confirmed the hypothesis originally proposed by McMahon and Chapelle (1991) that confining beds are important sources of organic carbon for aquifers, and that the movement of carbon between confining beds and aquifers has important effects on ground-water chemistry.

7.5 REGIONAL FLOW SYSTEMS

A regional flow system is defined as having its recharge area at a ground-water divide and its discharge area at the bottom of the regional basin. A regional flow system is characterized by long flowpaths and extremely slow rates of ground-water flow (Fig. 7.1). Because of the long residence times of ground water in regional systems, the water is often highly mineralized and unsuitable for human consumption, animal consumption, or irrigation. Also, the great depth of many regional flow systems, often greater than 300 meters, makes water-well drilling prohibitively expensive. For these two reasons, regional flow systems are not often tapped for supplies of potable water.

The deep sedimentary basins in which regional flow systems develop are, however, very important economically because they are often sites of hydrocarbon accumulation. It was recognized very early on that bacterial processes such as sulfate reduction and methanogenesis had important impacts on the production of

oil and gas from wells. In addition, it was soon recognized that bacterial processes may have been involved in petrogenesis, the processes that convert buried organic matter into petroleum. For these and other reasons, studies of bacterial processes in regional flow systems were undertaken early in the twentieth century and have continued to the present.

7.5.1 Early Observations from Petroleum Reservoirs

The beginning of the petroleum industry in the United States is usually traced back to 1859, when Edwin Drake drilled his first oil well in Pennsylvania. Throughout much of the nineteenth and early twentieth centuries, prospecting for petroleum reservoirs and the production of oil and gas was a decidedly unscientific enterprise. Perhaps the classic example of this was the discovery of the Spindletop oil field near Beaumont, Texas, at the turn of the century. A gentleman named P. Higgins decided, on the basis of some remarkably poor geologic mapping, that a large pool of oil was present under Spindletop. In fact, Higgins got almost everything wrong about the geology except one: There really was oil under Spindletop!

The first oil wells drilled at Spindletop were spectacular gushers. It was common for oil to spurt hundreds of feet into the air once the "cap rock," or upper confining bed to the reservoir, was breached. Spindletop set off a wild oil rush, and literally thousands of wells were drilled in a short period of time. Beaumont became a boomtown, and oil was produced frantically in an effort to make as much money as quickly as possible. Unfortunately, this led to massive overpumping. Salt water moved into the oil-bearing strata, and within a few years the reservoir was ruined.

The experience at Spindletop and other fields made a deep impression on the fledgling petroleum industry. It gradually became clear that the best way to produce consistent profits in this high-risk business was by knowing as much as possible. The major oil companies that emerged from these early years began to invest in scientific studies to determine the origin, transport, and accumulation of petroleum in the subsurface. These studies soon led to consideration of microbial processes in regional flow systems.

The single largest problem for a petroleum prospector is obviously how to find accumulations of oil. To this end, enormous effort was invested in understanding the geology, hydrology, and geochemistry of known petroleum reservoirs in order to identify leads for finding new reservoirs. One such lead was that brines associated with petroleum tended to be characterized by low concentrations of sulfate and high concentrations of sulfides (Rogers, 1917). The practical importance of this was that the simple analysis of water for sulfate and sulfide was a possible indicator of the presence or absence of nearby petroleum accumulations. This observation by itself, however, was unsatisfactory until its cause could be identified. This was accomplished by Bastin (1926) when he showed that sulfate-reducing bacteria were present in oil-field brines and that the activity of these microorganisms could account for the observed phenomenon.

The best example of how this information was useful in petroleum prospecting is the case of the Carrizo Sand in Texas (Davis, 1967). The Carrizo is a confined aquifer system that grades from a local flow system where it crops out at land surface to a regional flow system in downdip areas (Fig. 7.8). Throughout its re-

gional extent, Carrizo aquifer ground water contains 12–52 mg/L of sulfate except in the vicinity of a fault-controlled accumulation of petroleum (Fig. 7.8). Near the petroleum (well no. 6) sulfate concentrations are less than 1 mg/L. In this case, the lack of sulfate in ground water is a clear indication of a nearby petroleum accumulation. Davis (1967), as did Bastin, was able to relate this phenomenon to the presence of sulfate-reducing microorganisms, which were present in ground water at concentrations between 10^3 and 10^4 cells per liter. Davis also noted the presence of facultative anaerobic bacteria as well.

There is much literature concerning the presence of microorganisms and microbial processes associated with petroleum reservoirs. A good deal of this work was performed by Russian scientists (Kuznetsov, 1950; Ekzertev, 1951; Ekzertsev and Kuznetsov, 1954; Al'tovskii et al., 1962). This tradition has been continued and extended in more recent years (Belyaev and Ivanov, 1983).

7.5.2 Distribution of Bacteria in Regional Flow Systems

Sampling sediments from regional flow systems presents extreme technological problems. While auger drilling rigs are easily adapted for aseptically sampling local flow systems, and mud-rotary rigs can be used to aseptically sample local and intermediate flow systems, aseptically sampling sediments from deep regional flow systems is much more difficult. One of the most obvious problems is cost. Drilling into regional flow systems is expensive under any circumstances and coring sediments is prohibitive. While cores are routinely taken in such environments, they are usually taken for stratigraphic control or for analysis of organic carbon content. In addition, procedures for obtaining cores that are not contaminated by drilling fluids are difficult to apply. For these reasons, microorganisms in regional flow systems are most commonly recovered from ground water.

Culture Techniques As in local and intermediate flow systems, various culture techniques have been used to investigate the presence and kinds of microorganisms in regional flow systems. The work of Bastin (1926) and Davis (1967) is fairly typical of this approach. More recent examples of how the distribution of microorganisms in regional flow systems were given by Dockins et al. (1980) and Olson et al. (1981), who documented the presence of methanogenic and sulfate-reducing bacteria in water produced from the Madison aquifer system of Montana. The work of Dockins and Olsen is particularly notable in that the investigators showed that the bacteria grew at the temperatures of the aquifers (~70°C) but would not grow at room temperature (~20°C). This showed that the bacteria were acclimated to the specialized conditions of the aquifer and ruled out the possibility that the observed bacteria were contaminants from the near surface.

Because of the slow movement of fluids in regional aquifers and because of their isolation from the surface, there has been considerable interest in using them as repositories of hazardous and radioactive wastes. Due to the potential for microorganisms to affect waste mobility, evaluations of such flow systems as waste repositories has led to microbiological studies. One such study was described by Pedersen and Ekendahl (1990). These investigators showed that ground water produced from a deep (860 m) granitic aquifer contained on the order of 105 cells/

ml total bacteria, of which between 10^2–10^3 were cultureable under anaerobic conditions. Sulfate-reducing and methanogenic bacteria were part of the ambient microoflora as well as members of the genera *Shewanella* and *Pseudomonas*.

Pedersen and Ekendahl (1990) were microbiologists; they approached the problem of microorganisms in the deep granitic terrain using classic microbiologic techniques. When confronted with the problem of determining which microbial processes were active in situ, however, it was clear that the mere presence of certain microorganisms was equivocal. Consequently, these investigators, as had the earlier petroleum geologists, turned to evidence provided by ground-water chemistry. They noted that sulfate (and sulfide) was present in the ground water and that sulfate reducers largely exclude the activity of methanogens. The conclusion, therefore, was that sulfate reduction was the most likely predominant process in situ.

Geochemical Methods There are obvious problems with relying solely on culture methods to study the distribution of microorganisms in regional flow systems. In addition to the problems of media selectivity and determining which processes are most active in situ, there is the problem of how representative microorganisms in ground water are of the sediment-bound population. Davis (1967) recognized this problem explicitly, as did Pedersen and Ekendahl (1990). In both cases, it was suggested that ground-water geochemistry gave important information as to in situ microbial processes.

In practice, much of what is known about the distribution of microbial processes in regional flow systems has been deduced from ground-water chemistry data. The Carrizo sand (Fig. 7.9) is one example of this that uses fairly simple water chemistry patterns (lack of sulfate, presence of sulfide) to describe distribution of microbial processes. The development of geochemical modeling to deduce operative geochemical processes (including microbial activity) has made this procedure more quantitative.

One of the first examples of this was given by Thorstenson et al. (1979). The system under study was the Fox Hills/Basal Hell Creek aquifer. This aquifer ranges from being a local flow system near the recharge area, an intermediate flow system

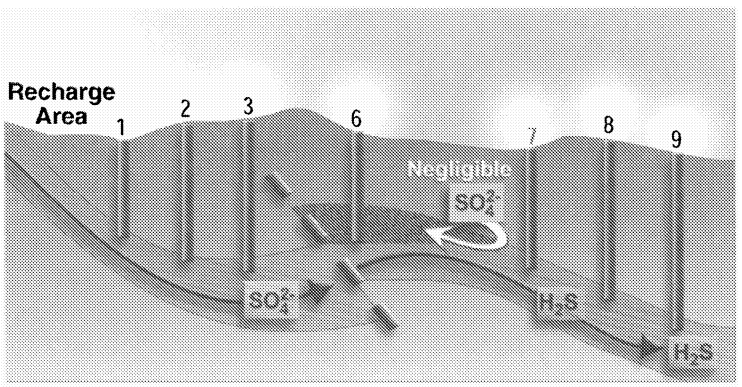


Figure 7.9. Depletion of sulfate near a fault-controlled petroleum accumulation in the Carrizo Sand. (Modified from Davis, 1967, with permission courtesy of Elsevier Publishing Company.)

downgradient of the recharge area, to a regional flow system in the most downgradient parts of the system. Using ground-water chemistry data and a computer program for making equilibrium chemistry calculations, Thorstenson et al. (1979) identified sulfate reduction as the predominant microbial process in the intermediate portion of the flow system, which graded to methanogenesis as the predominant process in the regional portion of the flow system. This paper showed that geochemical modeling was one of the best available methods for deducing the distribution of microbial processes in inaccessible regional flow systems.

Molecular Methods Recent advances in molecular methods for analyzing microbial communities without culture methods have been applied to intermediate and regional flow systems. An example of this was given by Fry et al. (1997). This study used molecular methods to study the microflora of an intermediate flow system (~300 meters below land surface) and an underlying regional flow system (~1,200 meters below land surface) of the Columbia River Basalt aquifer. Microorganisms from the two aquifers were collected by filtering pumped ground water. Nucleic acids from these samples were extracted, and the microbial community examined using oligonucleotide 16S RNA probes supplemented by analysis of rDNA analysis. The results of this study indicated that, while members of the domain Bacteria dominated the microflora (92–64%), members of the Archaea (~2%) and Eucarya (5–14%) were also present. A substantial percentage of gram-positive bacteria (7–12%) were present, and possibly indicate the presence of acetogenic microorganisms.

The ground-water chemistry of the two sampling locations suggests that the shallower aquifer was dominated by methanogenic respiration (methane concentrations $\sim 10^{-4}$ M; sulfate concentrations <0.05 mg/L) and the deeper aquifer dominated by sulfate reduction (methane concentrations $\sim 10^{-6}$ M; sulfate concentrations ~140 mg/L). Interestingly, there was little apparent difference in the presence of methanogens (*Archaea*) or sulfate reducers (*Desulfovibrio*) between the two sampled horizons. This may reflect bias introduced into the analysis by considering free-living microorganisms as opposed to rock-bound microorganisms, or by bias inherent in the primers and oligonucliotide probes used. Alternatively, it may reflect a reality that both methanogens and sulfate reducers were equally abundant in each horizon, but that their activity differed. At present, there are too few studies using this approach to make reliable conclusions. Nevertheless, the molecular approach holds much promise for understanding the complex microbial ecology of regional aquifer systems.

7.7 CHAPTER SUMMARY

In dealing with microbial processes in subsurface environments, it is first necessary to classify the kinds of environments that are most commonly encountered. In this chapter, a classification is given that divides subsurface environments on the basis of their hydrologic attributes and is independent of depth. The two broad environments recognized are (1) the unsaturated zone characterized by the presence of both gas and water in interstices, and (2) the saturated zone where water fills all available interstices. The saturated zone is further subdivided into local, interme-

diate, and regional flow systems. These divisions are made on the basis of quantitative considerations of ground-water flow in closed basins as described by Toth (1963). Local systems are characterized by abundant local recharge, fast ground-water flow velocities (meters/day), and are generally aerobic. Intermediate systems are not directly affected by transient precipitation events, have slower flow velocities (meters/year), and are generally anaerobic. Regional systems are largely isolated from surface influences, often have very sluggish flow velocities (meters/century), and are ubiquitously anaerobic.

These different subsurface environments are characterized by different microbial processes. The unsaturated zone and local flow systems are dominated by aerobic heterotrophic bacteria that are present in numbers of between 10^6 and 10^8 cells/gram sediment. These systems may contain measurable numbers of eucaryotic microorganisms as well. Intermediate flow systems often contain very diverse populations of anaerobic and facultatively anaerobic microorganisms in numbers ranging from 10^4 to 10^6 cells/gram sediment. Regional flow systems often contain microorganisms as reflected in their presence in ground water. However, little information is available on the numbers or kinds of microorganisms present in the rocks or sediments of these deeply buried systems. Geochemical modeling is a useful tool for studying microbial processes in regional flow systems. However, the methods of molecular microbial ecology show promise for increasing our understanding of these inaccessible subsurface ecosystems.

QUESTIONS TO CONSIDER

1. How important is depth in distinguishing among local, intermediate, and regional flow systems?
2. Why do you think gram-positive bacteria are so much more numerous than gram-negative bacteria in the thick, unsaturated zone sediments underlying the Idaho desert?
3. Does recharge moving through the unsaturated zone to local flow systems always contain high concentrations of dissolved organic carbon? Why or why not?
4. What microbial processes serve to transport organic carbon present in clayey, low-permeability confining beds to microorganisms present in sandy, high-permeability aquifers?
5. Describe how concentrations of sulfate in ground water can be an indication of the presence or absence of petroleum accumulations.

CHAPTER 8

MICROBIOLOGICAL SAMPLING OF SUBSURFACE ENVIRONMENTS

Subsurface environments, whether they are shallow local flow systems or deep regional flow systems, share a common characteristic: They are relatively inaccessible and therefore very difficult to sample. Simply obtaining materials from subsurface environments, which may consist of unconsolidated sediments or fully lithified rocks, is difficult under most circumstances. However, if the additional requirement is made that these samples must be free of chemical and biological contamination induced by the sampling process, this becomes an even more formidable undertaking.

Early work on the microbiology of deep subsurface environments, such as the isolation of sulfate-reducing bacteria by Bastin (1926), relied on water produced from oil or water wells as a sample source. This is certainly the easiest and most direct method for sampling the deep subsurface. Unfortunately, there are many potential problems with such methods. The most serious is that the drilling process, for either water wells or oil wells, can potentially contaminate the environment under study. Drilling fluids and grouting materials contain substantial quantities of microorganisms, and these can "inoculate" subsurface environments during drilling and well construction. When sampling wells, therefore, it is difficult to judge whether the microorganisms recovered were indigenous or introduced by the drilling process.

A second problem with using wells for sampling the subsurface is that the construction of a well introduces a new "environment" that did not exist prior to drilling. This new environment includes the borehole, the gravel pack, the casing and screen, and the grout. Since all of this material is artificially introduced, it may substantially alter conditions in the vicinity of a well. This alteration of the physical conditions may significantly affect the microbial processes near the well. Perhaps the best example of this is the potential introduction of oxygen to the subsurface. Wellbores, being open to the surface, may transport oxygen into previously anaerobic environments. This, in turn, may support growth of aerobic bacteria that are

not active under undisturbed conditions. Clearly, the possibility of such alteration must be carefully accessed when using wells for microbial sampling.

A third problem with using water from wells for microbiologic analysis is that many microorganisms in the subsurface tend to be sediment-bound. Thus, the kinds and diversity of free-living microorganisms may be significantly different from those attached to sediment surfaces. This possibility, together with the other problems associated with sampling well water, introduces considerable uncertainty into the data obtained.

For these reasons, modern studies of microbial processes in subsurface environments have tended to emphasize direct sampling of subsurface materials. Significant advances have been made since the early 1970s in techniques for obtaining uncompromised samples of unconsolidated sediments from subsurface environments for microbiological analysis. These sampling techniques have probably been more important in the investigation of microbial processes in subsurface environments than any other factor. Much less attention has been paid, however, to sampling consolidated rock materials. In this chapter, different methods of sampling subsurface environments for microorganisms and microbial processes are outlined.

8.1 SAMPLING THE UNSATURATED ZONE

The unsaturated zone, as was discussed in Chapter 7, is characterized by a negative hydraulic pressure. That is, water in the unsaturated zone tends to be bound to sediments by capillary forces and does not flow readily to wells. This characteristic has a major impact on methods for sampling sediments from the unsaturated zone. The relative lack of water pore pressure means that the sediment framework is supported by grain-to-grain contacts rather than by hydrostatic pressure. This greatly increases the cohesiveness of the sediment and makes it much easier to sample.

A sampling system for subsurface sediments must simultaneously solve three problems. First, and most obviously, the system must be able to bore a hole from the surface to the horizon of interest. Second, the system must be capable of removing sediments from the hole as it is bored. This sediment retrieval may or may not be coupled to the sampling process. Third, the system must be able to maintain the sides of the borehole so that it does not collapse. The cohesive nature of sediments in the unsaturated zone allows each of these problems to be solved with relatively simple technology.

8.1.1 Hand Augering

By far the simplest method of obtaining sediment samples from the subsurface is by the use of hand augers. A typical hand auger is equipped with a simple blade system that enables the tool to cut into unconsolidated sediments. The head of the auger also is designed to capture and hold sediments so that they can be retrieved. The sediments that are retrieved serve both to deepen the hole and as the sample material as well. Thus, for hand-augering systems, there is no distinction between hole construction and sample recovery. The stem of the hand auger is equipped with a handle that enables the head of the auger to be twisted into the ground.

Once the head of the auger is twisted into the ground, the handle serves as a means of pulling the head out of the hole. The handle of the auger, and thus the length of the auger stem, can be extended by additional lengths of rod.

The usual procedure for obtaining sediment samples by means of a hand auger is as follows. First, the auger is twisted into the ground the approximate length of the auger head; this is usually about 6 or 8 inches. Second, the sample is retrieved by pulling the head of the auger back to land surface. Finally, the sediment recovered is subsampled by means appropriate to the problem at hand. If the sediments are to be used for microbiologic analyses, subsampling is accomplished using flame-sterilized instruments and the subsamples placed into appropriate sterilized containers.

It is generally possible to sample subsurface sediments to a depth of about 20 or 30 feet using hand augers. Beyond 20 feet or so, the weight of the augers as well as the difficulty in twisting them into the ground and retrieving quickly becomes limiting. It is often difficult to hand-auger below the water table. The increased pore pressure of the water tends to separate the sediment grains, thus decreasing the sediment cohesiveness. Sand-sized sediments will usually collapse once the auger is removed so that it becomes impossible to deepen the hole. Even in clayey sediments that are more cohesive, pulling the auger out of the sediments tends to collapse the hole. In general, hand augers are not suitable for routine sampling of sediments below the water table.

The advantage of hand augering is the technical ease of obtained subsurface sediments and its applicability to a number of unsaturated subsurface environments. However, because of the limited depth obtainable with this method, the inability to penetrate hard or indurated sediments, and the difficulty of sampling saturated sediments, its applicability to many subsurface environments is greatly reduced.

8.1.2 Air Drilling and Coring

In arid regions of the western United States, hydrologic systems are often characterized by extensive unsaturated zones that may be hundreds of feet thick. Because of the unsaturated nature of these materials, even unconsolidated sediments tend to be relatively cohesive and therefore do not require technology for holding a bored hole open. For these unsaturated zone sediments, the use of a core barrel for sample recovery together with compressed air for removing cuttings from the hole is an effective sampling method.

In air drilling and coring, a large compressor is used to force air down the drillpipe, out the bit, and up the outside of the borehole. As the core barrel cuts the subsurface materials, the air serves to blow the cuttings out of the hole as well as to cool the core barrel. Often, a small amount of water or surfactant is injected into the airstream to control dust and to aid with cooling the bit. However, use of these materials increases the possibility of contamination of the recovered sediments.

An example of the use of air coring for recovering unsaturated sediments for microbiologic analysis has been given by Colwell (1989). In this case, air coring was considered to be the sampling method of choice because it minimized the possibility of sample contamination by circulating fluids. To reduce the possibility of airborne microorganisms compromising the sampling procedure, the air being

circulated into the borehole was first filtered though a 0.3 micron filter. Because water or surfactants were not used to help cool the core barrel, drilling had to proceed fairly slowly in order to avoid overheating the core barrel. Clearly, if the core barrel was allowed to become too hot, the microbiological integrity of the samples could be compromised. The main disadvantage of this method, therefore, is that sample recovery must proceed fairly slowly. The advantage is that the probability of sample contamination by the coring process is fairly low.

8.2 SAMPLING LOCAL FLOW SYSTEMS

Water-saturated sediments, which by definition occur below the water table, are much less cohesive than unsaturated sediments. When a hole is bored into saturated unconsolidated sediments, therefore, there is much more tendency for the hole to collapse. A major technical problem in sampling saturated sediments is to be able to hold a borehole open. There are numerous solutions to this technical problem with saturated sediments. By far the most common technique in relatively shallow water table aquifers (local flow systems) is the use of auger drilling combined with split spoon and shelby-tube core sampling.

8.2.1 Split-Spoon Sampling

A widely used method for sampling sediments from relatively shallow subsurface environments is by means of hollow-stem auger drilling combined with split-spoon coring. The split-spoon technique was developed for use by civil engineers in foundation studies for buildings, bridges, and roads. The dual purpose of this technique was to provide estimates of the in situ strength of the subsurface materials and to provide samples for laboratory testing. Because of the ease of using this technique in relatively shallow systems, however, it has been widely adapted for groundwater applications. In turn, this technique has been widely adapted for microbiologic sampling of subsurface environments.

A typical system for hollow-stem coring includes hollow auger flights, a plug for closing off the hole in the auger flights, a cutter bit on the auger flights, a pilot bit on the plug, and rods that are coupled to the plug and pilot bit. Both the pilot and auger cutter bits are designed to penetrate unconsolidated sediments.

The usual procedure for obtaining sediment samples with an auger system is to drill to the desired horizon and then remove the plug by pulling the rods. The plug is then replaced with a split spoon. A typical split spoon consists of two halves of an 18-inch cylinder $1\frac{1}{2}$ inches in diameter that are machined to fit together. The lower part of the spoon is threaded to accommodate a bit. This bit is generally fitted with a core retainer that is designed to keep the core from slipping out of the barrel during sample retrieval. The upper part of the spoon is threaded for attachment of the drilling rods used to lower and retrieve the spoon. When the spoon is at the bottom of the hole, it is driven into the ground ahead of the auger flight. The most common method for driving the spoon is by means of a 140-pound hammer that is repeatedly dropped from a height of 36 inches. The number of "blows," or number of hammer drops needed to drive the spoon 6 inches, is related

to the foundation strength of the material being cored and is typically recorded in engineering applications of this method.

Once the split spoon has been driven the 18 inches of its length, the sediment-containing spoon is removed from the hole, generally by upward tapping with the hammer. Once the spoon has been retrieved, it is removed from the string of drilling rods. The bit of the spoon is removed, and the spoon "broken" in half by gentle tapping. Excessive pounding to separate the spoon halves can disturb the core. Once the spoon is broken in two, the sediment recovered is accessible for whatever sampling procedure is to be used. The plug is then recoupled to the drilling rods, inserted into the auger flights, and drilling to the next sample depth can commence. This sampling procedure is then repeated.

The main advantage of the hollow-stem auger method of drilling is that the auger flights remain in place during sampling, thus preventing collapse of the borehole. Also, because the screw action of the auger flights is what removes cuttings from the borehole, circulating fluids are not required. This greatly decreases the potential for contamination of the sampled sediments.

The principal disadvantage of the split-spoon method is that the depth of sampling is greatly limited. Because of their great weight, the number of auger flights that can be manipulated by the drilling rig is limited. A typical drilling rig designed for hollow-stem augering has a depth limitation of about 100 feet. If more auger flights are added, the rig would be incapable of holding the added weight and the drill string would be lost. Another disadvantage of this method is that the diameter of recovered sediment samples is typically limited. A sample diameter of $1\frac{1}{2}$ inches, the diameter of core recovered by a standard split spoon, is the minimum size for obtaining sediments for microbiological analysis. In clayey-textured sediments that are fairly cohesive, this sample size is not a significant problem. In some coarse sandy sediments, however, the lack of cohesion often makes it difficult to obtain or manipulate uncompromised sediments. In such sediments, or for applications where more sample material is needed, other drilling techniques must be used.

8.2.2 Push-Tube (Shelby Tube) Sampling Methods

For many applications, particularly involving aseptic technique for microbiologic analysis, the limited sample diameter provided by split-spoon sampling is insufficient. An alternative is provided by push-tube sampling methods. These methods are notable from a historical perspective because they were among the first used to obtain subsurface sediments that were uncontaminated by surface-sediment microorganisms (Dunlap et al., 1977).

An example of this kind of aseptic sampling procedure was described by Wilson et al. (1983). A diagram of the sampling apparatus is shown in Figure 8.1a. It consists of a core retainer, core barrel, and adapter much like a split-spoon apparatus. In this system, however, the core barrel consists of a single tube that cannot be broken in order to retrieve the sample. Instead, the sample is removed from the core barrel by a hydraulic cylinder. During sample extrusion, the outside of the sediment sample is removed by a paring device (Fig. 8.1b). Thus, the sample can be extruded directly into a sterile container, which reduces the possibility of contamination.

In order to obtain samples with this type of coring device, the hole is augered to the desired depth for sampling, and the auger flights are removed from the hole.

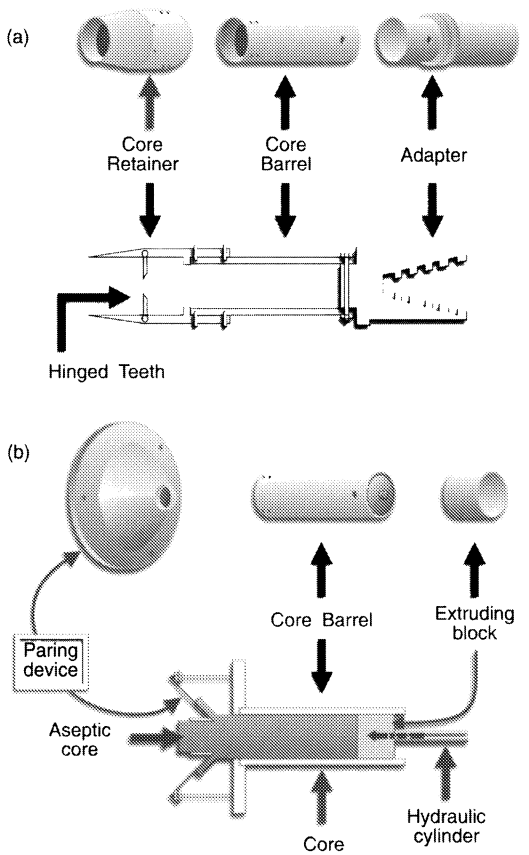


Figure 8.1. Sampling apparatus for recovering aseptic sediment samples using hollow-stem auger drilling including (a) diagram of core barrel and retainer, and (b) core extrusion and paring device. (From Wilson et al., 1983, reprinted courtesy of the National Ground Water Association.)

Next, the core barrel is lowered to the bottom of the hole with drilling rods, and the barrel pushed into the sediment by the drilling rig. It is also possible, and sometimes necessary, to drive the sample tube into the sediment with a hammer apparatus such as that used in split-spoon sampling. Once the tube has been driven into the sediment, it is retrieved with the drilling rig and brought to the surface for subsequent processing.

A *shelby tube* is a specialized sampler often used for soil science or foundation engineering applications. The dimensions of a shelby tube are 3 inches in diameter by about 20 inches long. The purpose of a shelby tube is to obtain sediments in an undisturbed condition. The wall of a shelby tube is relatively thin, requiring the tube to be pushed rather than pounded into the sediment. Once it is retrieved, the ends of the tube are often sealed by dipping them into melted wax or with special caps, and the entire tube returned to the laboratory for testing. This method is designed primarily for clayey or silty sediments and is not suitable for recovering sandy sediments.

An important limitation of the push-tube methods for obtaining subsurface sediments is that the auger flights must be removed prior to sampling. This, in turn, requires the sediments to be sufficiently cohesive so as to prevent the borehole from caving in as the flights are withdrawn. This is possible in some, but not all, locations.

An ingenious modification of this method, which circumvents possible borehole-caving problems, has been described by Leach et al. (1989). In this method, the plug at the head of the auger flights is replaced by a set of telescoping plates that can open outward (that is, downhole) but cannot open inward (allowing sediment into to the hollow stem of the auger). Using this system, the hole can be augered to the desired depth, and the augers lifted a few inches to allow the telescoping plates to open. Next, the sample tube is lowered down the hole without removing the augers. The sample tube pushes through the plates and can then be pushed into the sediment for sample recovery. After sample retrieval, the augers are pulled from the hole and the telescoping plates reset. The augers are then returned to the hole and drilled to the depth of the next sample. This method is more technically difficult and consumes more time than split-spoon sampling, but it allows recovery of larger-diameter samples while solving the hole-collapse problem.

8.2.3 Direct Push Sampling

A significant advance in sampling shallow unsaturated zone and local flow system sediments was made in 1988 with the development of direct push methods. Unlike augering or drilling, direct push methods use static pressure combined with percussion hammering to advance the sampling tool into soils or sediments. Rather than removing cuttings from the hole, the combined action of pressure and hammering pushes sediments aside and compresses them in order to advance the tool into the ground. This generally works well with unconsolidated soils and sediments, but not with indurated or cemented materials.

Most direct push methods, such as Geoprobe, are designed for sampling depths between 30 and 60 feet (9–18 meters). However, it is possible to sample as deep as 100 feet (90 meters). Depending on the kinds of tools used, it is possible to obtain sediment cores, soil-gas samples, and ground-water samples. For sampling soils or other unsaturated zone sediments, the open-tube method can often be used (Fig. 8.2a). With this method, a core barrel equipped with a plastic liner is simply driven into the ground, filling the barrel with sediment so that it can be retrieved. For sampling saturated zone sediments that often collapse, a closed-piston method is used (Fig. 8.2b). With this method, the core barrel is equipped with a retractable point that allows the closed barrel to be driven to the desired depth. Next the point is retracted, and the core barrel filled by driving it into the sediments as with the open-barrel method. In both cases, sediments obtained are subject to various kinds of contamination, which must be carefully considered when sampling for microbiologic purposes.

8.2.4 Aseptic Technique With Split-Spoon, Shelby Tube, and Direct Push Sampling

The goal of sediment sampling for microbiologic analysis is the recovery of materials that are representative of the subsurface environment under study. In order

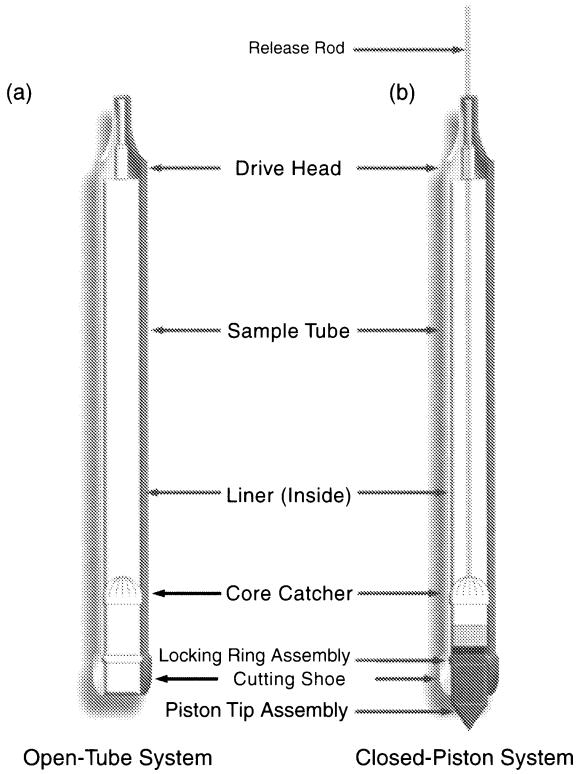


Figure 8.2. Diagram of a direct-push coring device (*a*) and open tube system useful for surface sediments, and (*b*) a closed-piston system used for capturing sediments below the water table.

to accomplish this, the most obvious requirement is that the samples not be contaminated by microorganisms from other horizons. This is particularly important for subsurface sediments that contain fewer microorganisms than surface soils or aquatic sediments. Because of this, the possibility for contamination needs to be carefully assessed in each individual application.

Tool Contamination There are numerous possible sources of contamination when using any kind of soil or sediment sampling device. First, there is possible contamination from the sampling tool itself. This possibility has been emphasized by Dunlap et al. (1977) and Wilson et al. (1983), who stress that the tools must be sterilized prior to sampling. Wilson et al. (1983) favor flame sterilization, in which the tool is rinsed with alcohol and then ignited until the alcohol is completely burned off. This is probably the most convenient method of sterilizing sampling tools and is common in field applications.

Another method of sterilizing sampling tools is with the use of compressed steam. It is common practice, when drilling sites characterized by chemical contamination, for drillers to steam-clean auger flights and sampling tools in order to prevent cross-contamination between holes. These steam cleaners operate at tem-

peratures in excess of 100°C and are capable of sterilizing the tools as well as cleansing them of chemical contamination. Because the steam is delivered at atmospheric pressure, there is some doubt that bacterial spores can be inactivated.

Down-Hole Contamination Another possible mode of sample contamination is from down-hole contamination. This is to say that materials that originated from the surface or from the side of the borehole fall to the bottom of the hole and are recovered by the sampling tool. Such contamination is often readily apparent upon sample recovery by the presence of loose sediment at the top of the sample container.

Down-hole contamination presents two technical problems. The first is, obviously, that the top portion of the recovered core is not representative of the sample interval of interest. The generally accepted solution to this problem is to discard the upper 4 or 5 inches of a recovered core as a matter of standard procedure (Wilson et al., 1983). The second technical problem is that because the sampling tool must pass through a layer of down-hole contamination before undisturbed sediments are reached, the tool itself becomes recontaminated. Thus, even if the tool has been properly sterilized at the surface, it will under most circumstances not be sterile during actual sampling.

The accepted method for dealing with this potential contamination is to assume, as a matter of course, that the outside of a sediment is contaminated. This outside portion of the core must be either removed (Fig. 8.1) or the inside of the core be sampled separately with sterilized instruments.

8.3 SAMPLING INTERMEDIATE AND REGIONAL SYSTEMS

The technology involved with split-spoon, push-tube, or direct push sampling is relatively simple and straightforward. However, it is rarely feasible to use these methods for sampling sediments deeper than about 100 feet. It is possible, with special equipment, for auger rigs to penetrate as deep as 250 feet. However, this is rare and seldom done in practice. When dealing with these deeper systems, which generally include intermediate and regional ground-water systems, more sophisticated technology is required. This technology introduces several more problems related to aseptic technique that must be carefully evaluated.

8.3.1 Mud Rotary Drilling

In mud-rotary drilling, the hole is bored by a rotating bit, as was the case with auger drilling. However, the twin problems of cuttings removal and hole stabilization (that is, preventing the hole from collapsing) are solved by the use of drilling fluid. Drilling fluids are continually circulated in the hole to remove cuttings and to apply pressure to the borehole in order to keep it from collapsing. This technique has been widely used in microbiologic investigations of deep subsurface environments, and considerable effort has been invested in solving the sample-integrity problems encountered with this technique.

A mud-rotary rig has a mast (also called a boom), which can be raised for drilling and lowered for traveling by means of hydraulic cylinders. The hole is cut

by a bit that has ports to allow drilling fluid to be pumped into the hole. The drilling fluid is pumped into the drilling rods through the water swivel (since the rods are rotating) with a specially designed mud pump. The fluid travels down through the rods, out of the bit, and back up the hole. As the fluid moves up the hole, cuttings are carried out of the hole. The cuttings are then separated from the fluid, and the fluid is reused.

Mud-rotary drilling is most commonly used to construct a borehole for later construction into a well. However, mud-rotary technology can also be used for coring applications. For subsurface microbiology applications, mud-rotary coring is the most common method for obtaining samples of relatively deep sediments (100–3,000 feet). This method has been extensively used in studying the microbiology of coastal plain sediments (Chapelle et al., 1988; Fredrickson et al., 1991).

8.3.2 Drilling Fluids

Drilling fluids are generally referred to as "mud" by drillers and geologists. This term, while being a fairly accurate description, gives little hint as to the technological sophistication involved. Drilling fluid is a marvel of applied chemistry and has undergone extensive research and development since its invention in the nineteenth century.

Drilling fluids are designed to accomplish four main functions:

1. *To remove cuttings from the hole during drilling.* After cuttings are generated by the drill bit, it is critical to immediately remove them from the hole. If the cuttings are allowed to settle around the drill bit, they will prevent the bit from turning freely. Ideally, cuttings are immediately entrained by the fluid stream and carried out of the hole.
2. *To keep the borehole from collapsing.* When sediments, particularly sandy sediments, are saturated by water, they have little cohesive strength. Under most conditions, a borehole drilled into sandy sediments will not remain open without some sort of support. Drilling fluid provides this support by virtue of the pressure it exerts on the borehole wall. If the pressure exerted by the drilling fluid is greater than the pressure in the formation, the borehole will not collapse. A borehole that is prevented from collapsing with drilling fluid is referred to as a "stable" hole.
3. *To cool and lubricate the bit.* Drilling bits, due to friction with the sediments being drilled, generate considerable amounts of heat. Drilling fluids prevent a buildup of heat in the drilling tools by lubricating the bit, thus minimizing heat generation, and by transferring heat away from the bit.
4. *To control fluid loss into the formations being penetrated.* When drilling in highly permeable formations, drilling fluids will tend to migrate from the borehole into the formation. Drilling fluids are designed to control this fluid loss by generating a filter cake on the outside of the borehole. This filter cake is made primarily from low-permeability clays that inhibit migration of fluid out of the borehole.

Each of these functions is addressed by one or a combination of drilling fluid properties. The principal properties of water-based drilling fluids are listed in Table 8.1.

TABLE 8.1 Principal Properties of Water-Based Drilling Fluids

1. Density (weight)	4. Gel strength
2. Viscosity	5. Fluid-loss-control effectiveness
3. Yield point	6. Lubricity (lubrication capacity)

Source: Reprinted courtesy of Johnson Division, St. Paul, Minnesota.

Density The density of a drilling fluid is defined as the weight per unit volume. Thus, it is common practice to use the terms "density" and "weight" interchangeably. Density is usually expressed in terms of pounds per gallon (lbs/gal) or pounds per cubic foot (lbs/ft^3).

The pressure exerted by drilling fluid on the walls of the borehole is a function of density and the length of the fluid column. In order to prevent the borehole from collapsing, the pressure of the drilling fluid must be greater than the pore pressure of the formation being drilled. Thus, if very unstable sediments are penetrated near land surface, the drilling fluid must be denser in order to prevent hole collapse than if those same sediments were penetrated at greater depth. It is considered good practice for the drilling fluid pressure to exceed formation pore pressure by about 5 pounds per square foot (psi) in order to maintain a stable hole. The hydrostatic pressure (H_p) exerted by drilling fluid equals the product of the fluid density (F_d) and the height of the fluid column (F_h)

$$H_p = F_d \times F_h$$

where H_p is in psi, F_d is in lb/gal, and F_h is in feet.

When drilling aquifer systems that are under water table conditions (i.e., the pore pressure at the top of the aquifer equals atmospheric pressure), dense drilling fluids are not needed in order to maintain a stable hole. However, when aquifer systems exist under artesian conditions (i.e., the pore pressure at the top of the aquifer exceeds atmospheric pressures), denser drilling fluids are required for borehole stability.

The density of drilling fluids is adjusted by the use of clay and soluble salt additives. Bentonites have a specific gravity of between 2.6 and 2.7, which often provides sufficient density for drilling shallow (< 500 feet) boreholes. If unusual pore pressures are encountered or if deeper formations are being drilled, it is often necessary to use fluid densities greater than can be obtained with just clay additives.

The most important high-density additive used in drilling fluids is barite (barium sulfate), which has a specific gravity of 4.2 to 4.35. By varying the amount of barite added to the drilling fluid, it is possible to obtain fairly high density. When drilling in formations with high hydrostatic pressure, progressively more barite is added to the drilling fluid. When drilling formations with lower hydrostatic pressure, which includes most shallow formations, little or not barite is added to the mud. Other soluble salts, such as sodium chloride and calcium chloride, are also used to adjust drilling fluid density. Figure 8.3 shows the density ranges obtainable using water with different additives.

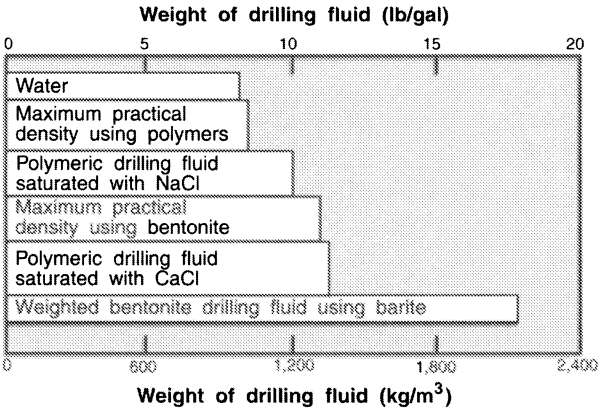


Figure 8.3. Range of densities possible with water-based drilling fluids. (Reprinted courtesy of Johnson Filtration Systems, St. Paul, Minnesota.)

Viscosity The viscosity of a drilling fluid refers to the sheer stress that it can exert on surfaces or particles. The ability of drilling fluids to lift cuttings from the hole are a function of fluid viscosity and fluid velocity. Curiously, viscosity and density are not functionally related to each other and are measured in different units.

In the field, the viscosity of drilling fluid is measured with a Marsh funnel. This is a funnel that carries a standard volume and has a standard-sized opening at the bottom. Viscosity is expressed as the number of seconds required for the fluid to drain from the funnel. A Marsh viscosity reading of 35–40 seconds is sufficient to remove most fine sands from the hole. Coarser sediments, such as coarse gravels, may require a Marsh viscosity of 75–85 seconds.

The viscosity of most drilling fluids is adjusted by the addition of clay minerals. In fact, the early practice of adding clay minerals to drilling fluids used for drilling oil wells is where the term "drilling mud" originated. When clay minerals such as montmorillonite are added to water, they tend to swell as much as tenfold. This swelling leads to the clay particles occupying more area and, therefore, leads to a more viscous fluid. Nonswelling clays can be used to adjust viscosity of drilling fluids as well as swelling clays. However, swelling clays are much more compact when they are dry and are more convenient to work with. Bentonite, which is the rock name for the swelling clay montmorillonite, is a basic component of most drilling fluids and is added primarily to increase viscosity.

Another method of varying the viscosity of drilling fluids is by addition of synthetic polymers such as polysaccharides. Plain corn starch was the first such additive used in this manner. In more recent years, very sophisticated polysaccharides have been developed for use in drilling fluids. The structure of one such polymer, which is called guar gum, is shown in Figure 8.4. This polymer consists of two kinds of sugar molecules, D-mannose and D-galactose. The D-mannose molecules are linked together to form a chain and the D-galactose molecules are linked to, on average, every other D-mannose molecule.

One of the advantages of using guar gum as a viscosity-enhancing additive is that the shear strength is a function of fluid velocity. When a colloidal suspension

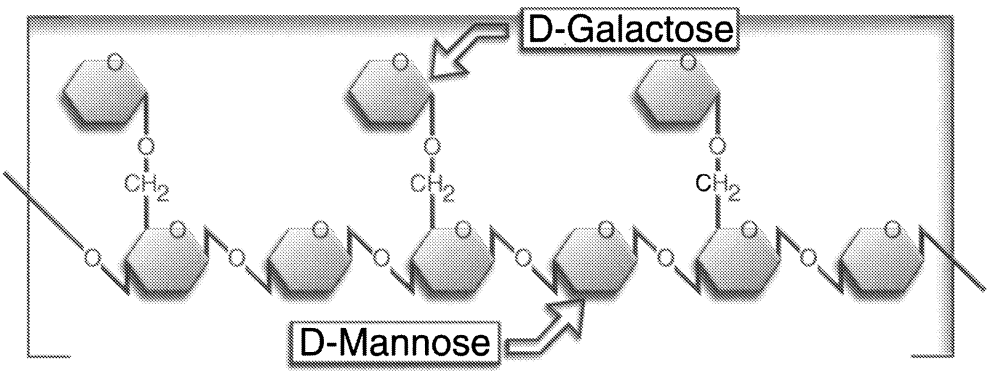


Figure 8.4. Molecular structure of guar gum, a polymer used to enhance the viscosity of drilling fluids. (Reprinted courtesy of Johnson Filtration Systems, St. Paul, Minnesota.)

of guar gum is at rest, the polysaccharide chains tend to coil and become entangled with each other, thus increasing viscosity. When shear stress is increased by pumping the suspension, the coils are stretched out and untangled, thus decreasing viscosity. This property enables the driller to stop mud circulation without allowing sediment particles entrained in the drilling fluid to settle out and possibly trap the string of drilling tools in the borehole.

The use of organic polymers as drilling fluid additives when drilling or coring for microbiological applications must be carefully considered. Most of these polymers are biodegradable—in fact, many of them are designed to be biodegradable, and this property may significantly alter conditions in the hydrologic systems being drilled. For example, if there is substantial penetration of the drilling fluid into a formation, biological activity will in most cases be stimulated. This stimulation may alter conditions in the formation to the point that they are no longer representative of the natural system. In general, biodegradable drilling fluids should be avoided when drilling for microbiological investigations.

Yield Point Water is referred to as a Newtonian fluid because it deforms in direct proportion to an applied stress. Drilling fluids that contain clay minerals and other additives, however, tend to behave as plastics. This is to say that they do not deform until a significant amount of stress is applied. The yield point of a drilling fluid is the pressure at which the pump is able to move the drilling fluid, and the fluid begins to behave as a Newtonian fluid. Below the yield point, the viscosity of the drilling fluid changes with increasing stress. Above the yield point, the drilling fluid viscosity does not increase with increasing stress.

Gel Strength Gel strength is a measure of a drilling fluid's ability to support particles when the fluid is not moving. In clay-based drilling fluids, gel strength is provided by the electrical interaction of the clay particles. The flat surfaces of clay particles are typically positively charged whereas the edges are negatively charged. When the fluid is at rest, the attraction between clay particles provided by these charge differences provides its gel strength. Gel strength can also be enhanced by means of polymer additives such as guar gum.

The gel strength of a drilling fluid is maximized if the clay particles and polymers are well-dispersed, allowing them to align themselves according to their electrical and or chemical properties. For this reason, a good driller takes care to thoroughly mix clays and polymers in the fluid prior to use.

Fluid-Loss Control An important function of drilling fluid additives is to prevent fluid migration into the formations being drilled. Because fluid pressure in the borehole must exceed the hydrostatic pressure in the formation to prevent borehole collapse, the tendency is for drilling fluids to migrate into the formation. This is undesirable for two reasons. First of all, drilling fluids are expensive and loss during drilling is a significant economic drain on the driller's operation. Second, the presence of substantial quantities of drilling fluid in a water-producing formation can degrade water quality, rendering any well useless for water-chemistry studies or for water supplies.

A combination of clay minerals and organic additives are generally used to control fluid loss during drilling operations. When the drill bit penetrates a formation, some amount of drilling fluid flows into the formation due to the pressure difference between the borehole and the formation. However, the clay minerals and/or organic polymers present in the drilling fluid tend to be trapped in the pore throats of the formation materials. As this process continues, a filter cake is deposited on the borehole surface that becomes less and less permeable to fluid penetration. Eventually fluid penetration virtually ceases.

Clay minerals added to drilling fluids to increase viscosity confer significant mud-cake-forming properties. Several organic polymers, such as guar gum (Fig. 8.3), are also effective in preventing fluid loss. One of the advantages of using organic polymers to prevent fluid loss is that they can be subsequently removed by chemical or biological action. For example, in order to develop a well to maximum capacity, it is obviously desirable to remove the mud cake from the borehole. Organic polymers can be removed by addition of oxidizing agents (chlorine) or by natural biological activity. Clay mud cakes, on the other hand, require mechanical methods such as hole-scrubbing techniques to effect complete removal.

Lubricity One function of drilling fluid is to lubricate and cool the bit during drilling operations. The flat structure of clay minerals used in drilling fluids confers excellent lubricating properties. When physically pressed together, clay minerals tend to align themselves along their flat, positively charged surfaces. Because the positive charges repel each other, there is little tendency to stick together, and the clay surfaces slide easily past each other. The lubricating characteristics of drilling fluids maximizes the efficiency of drilling and minimizes down-hole heat buildup.

8.3.3 Mud-Rotary Coring

The technology that has been most extensively utilized for microbiological sampling of intermediate and regional flow systems is mud-rotary coring. This coring technology has developed parallel to mud-rotary drilling methods and utilizes many of the same tools and drilling fluids. However, there are significant philosophical differences between drilling and coring technologies. The primary goal of drilling technology is the production of a borehole that can be used for constructing a well.

In coring, however, the primary goal is the recovery of sediment samples that have been disturbed as little as possible, and the borehole itself is generally of less importance. The net affect of these differences is that coring technology has become a narrowly focused specialty in the drilling trades that is practiced by relatively few individuals. It is important for the subsurface microbiologist to recognize that successful coring operations require a coring specialist and cannot be carried out by just any drilling contractor.

Equipment Used for Mud-Rotary Coring The essential features present on a coring rig include a boom for raising and lowering the drill string, a rotary table for turning the drilling tools, and a mud pump for circulating drilling fluid. In all of these particulars, a coring rig is virtually identical to any mud-rotary rig. The primary differences are in the tools that are used.

The drilling rig, operated by the Branch of Eastern Regional Geology of the U.S. Geological Survey, uses coring tools designed and manufactured by Christensen, Inc. The Christensen system, which has been extensively used for subsurface microbiology investigations (Phelps et al., 1989b; Chapelle and Lovley, 1990), is generally referred to as a wire-line system. This is because the coring tools are lowered and retrieved by means of a wire line operated inside the drilling rods.

The basic tools involved in wire-line coring are shown in Figure 8.5. The outer barrel (Fig. 8.5a) of the coring system attaches directly to the drilling rods and carries a drilling bit designed to cut semiconsolidated sediments. The outer barrel rotates with the drilling rods and actually cuts the borehole. The bit on the outer barrel has ports though which drilling fluid is circulated. The inner barrel (Fig. 8.5b) fits into the outer barrel and is raised and lowered by the wire line. The function of the inner barrel is to trap the sediments as the outer barrel drills downward. When lowered down-hole, the inner barrel locks into the outer barrel. However, the inner barrel is supported by a swivel equipped with ball bearings. This enables the outer barrel and bit to rotate while the inner barrel remains stationary. This, in turn, minimizes stress on the sediments in the inner barrel and helps avoid core disturbance. The top of the inner barrel is equipped with a latching apparatus. This latch allows the retrieval tool on the wire line to attach to inner barrel. This attaching mechanism simultaneously unlocks the inner barrel from the outer barrel so that it can be retrieved and brought to the surface.

The inner barrel does not actually cut the formation and is not equipped with a bit. The end of the inner barrel, however, is coupled to what is termed a shoe (Fig.

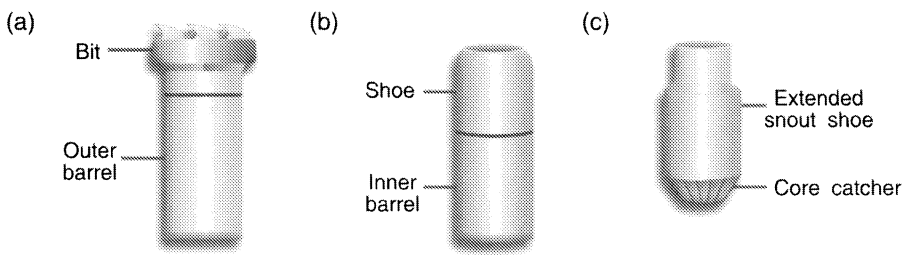


Figure 8.5. Tools used for mud-rotary coring: (*a*) outer barrel and cutting bit, (*b*) inner barrel and shoe, and (extended snout shoe and core catcher.

8.5b). The shoe is designed to allow core into the inner barrel during drilling, but to prevent the core from falling out as the inner barrel is retrieved. There are two separate design features of the shoe that can be employed to prevent core loss during retrieval. The shoe itself is slightly tapered so that it is narrowest at its tip. Inside the shoe, a split metal sleeve is fit that also is tapered. During coring, this sleeve is pushed upward where the shoe is widest, allowing the sleeve to expand slightly and admit cored sediments to move easily past it. However, when the inner barrel is retrieved, the sleeve is pushed down in the tapered shoe, and the sleeve contracts around the core and holds it.

The shoe may also be equipped with a metal or plastic core catcher (Fig. 8.5c). This core catcher is identical in function to catchers used during split-spoon coring, allowing the sediment into the core barrel but discouraging it from falling back out. The coring system used by the U.S. Geological Survey Branch of Eastern Regional Geology has been modified to use both the contracting sleeve and plastic core catcher simultaneously. This, in turn, has greatly increased core recovery efficiency.

Under most drilling conditions, the shoe fits slightly behind the drilling bit on the outer barrel. However, when drilling through coarse sandy materials, drilling fluid pumped out of the drilling bit may tend to wash the sediments away before they can be trapped in the inner barrel. The Christensen system solves this problem by having a series of shoes of different length. For most coring situations, the short shoe (Fig. 8.5b) that fits behind the drilling bit is used. For sandier sediments, a shoe that protrudes slightly ahead of the bit is used (Fig. 8.5c). For very coarse sediments, an even longer shoe may be utilized. This system greatly increases sediment recovery when drilling sandy sediments. Because the sandy sediments typically comprise the aquifer portion of subsurface environments, this system has greatly aided investigations of aquifer materials.

After a core has been drilled, the inner barrel is retrieved by the wire line and the shoe removed from the inner barrel. Once the shoe is removed, the core is extruded from the inner barrel. For sandy sediments that do not expand significantly when removed from the ground, simply removing the shoe often allows the core to slide out of the inner barrel. Other sediments, notably clays, tend to expand and become tightly trapped inside the inner barrel. In these cases, the core must be extruded using a rod or, in some cases, hydraulic pressure must be used.

The Role of the Driller It is difficult for the casual observer to readily appreciate the skill involved in mud-rotary coring. When watching a coring operation, one sees the physical work of raising and lowering the inner barrel, adding rods to the drilling string, and manipulating the rig's controls. These physical activities are by far the easiest and least important aspects of the driller's job. Far more important are things that are not immediately apparent.

When coring a particular "run," as a length of core barrel is called, the driller continuously adjusts three variables:

1. Down-hole pressure on the drilling bit.
2. Drilling fluid pressure at drill bit.
3. Rate of drill bit rotation.

The driller must exert enough down-hole pressure on the bit to enable it to cut though the sediments. However, if too much pressure is exerted, the sediments will be cut faster than they can be removed by the drilling fluid and the borehole will become clogged. Similarly, the driller must provide enough drilling fluid pressure at the drill bit to effectively remove cuttings. If the pressure is too great, however, the drilling fluid will wash away the sediments before they are pushed into the core barrel. In this case there will be no sediment recovery at all. Clearly, the driller must adjust the drilling bit pressure and drilling fluid pressure so as to maximize core recovery and coring rate. Typically, subtle adjustments need to be made continuously in order to obtain the best results. This, in turn, requires considerable skill on the part of the driller.

The third factor that the driller varies is the rotation speed of the drill bit. In general, the size of the drill shavings cut by the drilling bit is proportional to the relative hardness of the materials being drilled. Hard materials are not cut as easily and therefore produce smaller shavings. Thus, in order to drill hard materials, it is efficient to increase the rate of bit rotation in order to increase the rate of drilling. Softer materials, of course, can be efficiently drilled with progressively lower rotation rates.

Each of these three factors—drill bit pressure, drilling fluid pressure, and rotation rate—are interrelated during drilling. It is much like having one equation with three variables. Once drill bit pressure and the rotation rate are fixed, there is an optimum drilling fluid pressure that must be used in order to maintain optimum drilling rate with optimum recovery. Deviations from this optimum will result in poorer core recovery and/or slower drilling progress. Furthermore, because the properties of the material being drilled often changes rapidly, the optimum drilling parameters also must be constantly changed.

When watching an experienced driller work, it is possible to follow some of the constant adjustments as they are being made. When the driller glances at his return flow of drilling fluid, he is judging the efficiency with which the fluid is removing the cuttings. Having established that he's getting return flow, he might then look at the drilling fluid pressure gauge. If that pressure is fairly high in order to maintain fluid flow, he might ease some of the pressure on the drilling bit slightly. This will allow the fluid pressure to drop and keep the fluid from washing the core away. This adjustment is made by moving a lever perhaps a quarter of an inch. This whole process, which might be critical to core recovery, generally takes a matter of seconds and is repeated over and over again. Many drillers do not like to be spoken to while drilling. This is due to the tremendous concentration required by the job.

It is important for the geoscientist and microbiologist working with cored sediments to be at least cursorily familiar with the drilling problems involved in core recovery. Many of the same factors that enter into successful core recovery also are involved with potential core contamination by drilling fluids. For example, many drillers tend to keep as much drilling fluid pressure at the drilling bit as possible. This is because if the fluid jets become clogged with sediments, the entire string of rods must be removed in order to clean them. For a drilling string 1,000 feet long, this could easily become a one- or two-day job. However, high fluid pressures also increase the possibility that drilling fluids will penetrate into the core and contaminate them. When informed of the special requirements of microbio-

logical sampling—that is, samples that are not contaminated with drilling fluids—most drillers will adjust their techniques to accommodate the more exacting standards. Communicating these special requirements, however, it is much more effective if the scientists understand the many technical problems facing the drillers.

Drilling Fluid Technology and Coring Proper application of drilling fluid technology is critical to successful coring operations. In simple borehole drilling, drilling fluids are mixed to maximize drilling rates and to minimize use of expensive drilling fluid additives. Thus, a borehole driller may choose to work with a less viscous drilling fluid and make up for cutting removal efficiency by increasing the rate of fluid circulation in the hole. As we have already seen, however, the coring driller often does not have this extra flexibility, as excessive fluid velocity inhibits core recovery.

Drillers engaged in core recovery often try and maximize drilling fluid viscosity in order to minimize the drilling fluid pressure needed for efficient cuttings return. This practice also has the effect of minimizing fluid penetration into cored sediments and is thus consistent with the needs of microbiological sampling.

One technical problem encountered in coring that is not encountered in borehole drilling is when working in sediments—generally clean sands—that lack cohesive strength. Such sediments may tend to clump together when pushed into the inner barrel, creating a "sand lock." This sand lock prevents any other sediments from entering the core barrel and usually results in lost recovery. One method of dealing with this problem is the addition of organic polymers to the drilling fluid. One such additive often used in coring has the trade-name Quik Trol and is manufactured by Baroid. Quik Trol is an organic polymer that forms a thin coat on the core as it enters the inner barrel. This coat provides just enough cohesion so as to minimize sand lock problems and increase core recovery.

8.4 DRILLING-FLUID CONTAMINATION OF CORED SEDIMENTS

The use of drilling fluids in coring operations introduces a significant source of possible chemical and biological contamination in recovered sediments. The possibility of such contamination must be carefully evaluated when using cored sediments for microbiological or pore-water chemistry investigations.

8.4.1 Down-hole Saturation Contamination

There are several ways in which drilling fluids can penetrate and contaminate cores during coring operations. First, if the pressure of the drilling fluid at the bit face is excessive relative to the permeability of the formation, the fluid will saturate sediments several inches below the bit. When this drilling-fluid-saturated sediment is subsequently recovered by the inner barrel, the entire sample is always contaminated. This type of contamination, which is referred to as *down-hole saturation contamination,* is very common when coring sandy or gravelly material.

There are several techniques that an experienced driller can use to minimize or eliminate down-hole saturation contamination. The most important technique, and one that a good coring driller practices as a matter of course, is to minimize the

drilling fluid pressure at the bit. When coring silty or clayey sediments, this practice is often sufficient to prevent down-hole saturation.

When drilling permeable sandy sediments, however, even minimal drilling fluid pressure can result in contamination. The most obvious, and also most dangerous, drilling technique for avoiding down-hole saturation in sandy sediments is to simply stop fluid circulation during coring. The lack of circulation fluid will greatly reduce the possibility of contamination. However, the lack of circulation fluid makes it easy for the fluid jets on the coring bit to become clogged. Once clogged, fluid cannot be circulated and the entire string of drilling rods must be pulled in order to unclog the bit. Also, if cuttings are not removed from around the outer barrel, it may become trapped. For these reasons, halting circulation is a last resort that drillers prefer to avoid. However, this method can be used to sample a particularly important sandy horizon if no other option is available.

A more technologically advanced technique for minimizing down-hole saturation contamination is the use of the Christensen extended shoe design (Fig. 8.5c). By extending the shoe of the inner barrel as much as 2 inches below the drilling bit, it is possible to recover sediments below the zone of down-hole saturation. By combining the proper length of drilling shoe and by minimizing drilling fluid pressure, it is possible to recover uncontaminated sediments even in very coarse sandy sediments. It is not an exaggeration to say that the development of the extended shoe design is the most important advance in drilling technology to modern studies of microbial processes in deep hydrologic systems.

8.4.2 Core Seepage Contamination

A second kind of contamination is called core seepage. When cored material is pushed into the inner barrel, some drilling fluid is present on the outside of the core and can seep into the center of the core. This type of contamination can become particularly severe if the driller maintains an incorrect ratio between fluid pressure and drill stem pressure. When coring sandy material, excessive fluid pressure relative to drill stem pressure results in the core being slightly smaller than the diameter of the inner barrel. This, in turn, allows excessive quantities of drilling fluid in with the core and encourages drilling fluid seepage into the core.

Core seepage occurs to some extent with all cores recovered by mud-rotary drilling, and the outside portion of any core must be considered contaminated. The extent to which such contamination occurs, however, varies widely. The degree of core contamination is largely dependent upon sediment texture. Sandy sediments often experience severe seepage contamination during coring, whereas clayey sediments generally experience much less seepage contamination. The reason for this difference is related to the distribution of porosity in each sediment type. In sandy sediments, typically 70–80% of the total porosity is interconnected. This allows fluids, including drilling fluid, to move through it with relative ease. In clayey sediments, on the other hand, less than 10% of the total porosity is interconnected, which retards penetration of drilling fluids.

8.4.3 Core-Fracture Contamination

Another common way for cored sediments to become contaminated by drilling fluids is by core fracturing. It is fairly common for cored sediments to come under

considerable compressional stress as they are pushed into the inner barrel. If some portion of the sediment has less compressional strength than another portion, the sediment can fracture. Once the core is fractured, drilling fluid from the outside of the core can seep inward and contaminate the inner portion of the core. Core fracture is the most common way that clayey sediments can become contaminated.

Cores can become fractured by means of extensional stress as well as compressional stress. When the inner barrel containing a freshly recovered core is lifted, extensional stress can develop along the length of the core. As before, if a particular portion of the sediment is relatively weak, a fracture can develop and drilling fluid seepage along the fracture trace can occur.

In both cases, compressional and extensional core fracturing, the problem can be minimized by careful application of proper drilling technique. To minimize compressional stresses on the core during drilling, the down-hole pressure on the drilling string and the drilling fluid pressure must be properly balanced. If the drilling fluid pressure is insufficient, the core will be of slightly greater diameter and increase the compressional stress as it is pushed into the core barrel. A good driller always examines each core as it is retrieved in order to make sure string pressure and fluid pressure are properly balanced.

Extensional core fracturing can also be minimized by good drilling technique. At the end of a core run, the sediment in the core barrel is still attached to sediments at the bottom of the hole. The driller can effect a clean break of the sediment at the bit by alternately spinning and stopping the drill string. If this break is made successfully, there will be less extensional stress applied to the core as the inner barrel is retrieved.

8.4.4 Evaluating Drilling Fluid Contamination

Using cores recovered by mud-rotary techniques for biological or pore-water chemistry studies requires that the extent of drilling-fluid contamination be quantitatively evaluated. As all of the cores are contaminated to some extent, the problem is to determine the extent of contamination and then to judge whether the cores are usable for the purpose at hand.

A number of methods have been used over the years to evaluate drilling fluid contamination. All of these methods involve the utilization of some kind of tracer to track contamination. The characteristics of a good tracer include:

1. It is a substance that is present or can be added to the drilling fluid but that is not present in large amounts in the sediments being cored.
2. It is present or can be added in sufficient quantity to be easily recognizable.
3. It can be readily analyzed with adequate sensitivity.

There are at least four classes of tracers that have been used in subsurface microbiological investigations. These include:

1. The drilling fluid itself
2. Chemical additives to drilling fluid
3. Particulate tracers
4. Biologic tracers

Each of these methods has particular advantages and disadvantages and each has been utilized in subsurface microbiological studies with varying degrees of success.

Drilling Fluid As a Tracer Drilling fluid has a carefully controlled chemical composition and, under many circumstances, makes a perfectly good tracer. For example, drilling fluids that are weighted with barite as an additive typically contain between 10 and 100 mg/L dissolved barium. Because barium is generally present in sediment pore waters at concentrations of less than 10 ug/L, barium can be used as a tracer.

An example of using barium as a drilling fluid tracer was given by Chapelle and Lovley (1990). The formations being drilled were under relatively high pressure and the driller weighted the drilling fluid with barite to about 12 lbs/gal. After the cores were recovered, they were shipped to the laboratory for processing. In the lab, the cores were broken in half and one of the faces sampled along a traverse from the outside obviously contaminated portion of the core to the inside. Sediment samples of 1 ml were mixed with 9 ml of deionized water, shaken, and the supernate analyzed for barium concentrations using plasma atomic emission spectroscopy. The results were normalized to mg/L of pore fluid using measured moisture content. The results (Fig. 8.6) showed that some cores contained measurable barium concentrations into the center of the core. Other sediments showed only the pres-

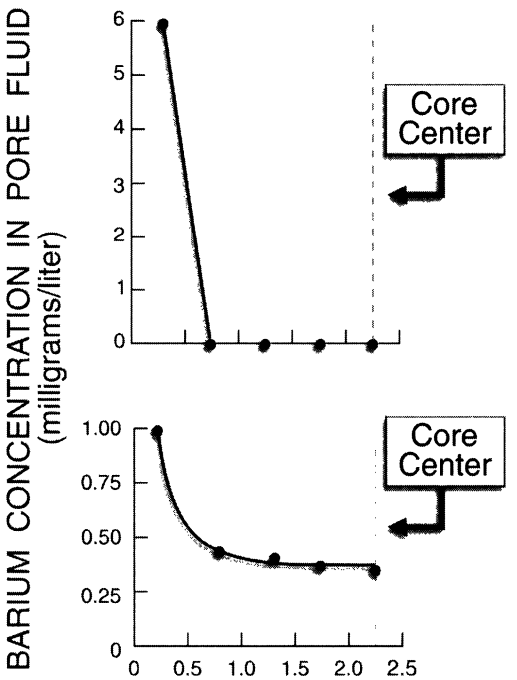


Figure 8.6. Concentrations of barium, an additive of drilling fluid, as a tracer of core contamination: (*a*) contamination present on outside of core but absent in core center, and (*b*) contamination present throughout the core. (Reprinted from Chapelle and Lovley, 1990, with permission courtesy of the American Society for Microbiology.)

ence of barium on the outside of the core. This, in turn, showed that core seepage rather than down-hole saturation was the predominant process leading to core contamination.

In addition to soluble cations present in drilling fluids, soluble anions may also be used. Chapelle and McMahon (1991), in a study of the pore-water chemistry of aquifer and confining-bed sediments, measured sulfate concentrations in sediment pore-waters. The drilling fluid used for the coring operation contained about 5 mM dissolved sulfate. Analysis of ground water from a nearby production showed that sandy aquifer sediments contained between .05 and .1 mM sulfate. The pore water of uncontaminated (as determined using a particulate tracer) sandy sediments showed sulfate concentrations in the 0.05–0.1 mM range—identical to concentrations in well water. The pore water of contaminated sediments contained as much as 2 or 3 mM dissolved sulfate. Thus, the sulfate in the drilling fluid could have been used as a tracer for contamination.

The principal problem with using dissolved constituents of drilling fluids as tracers is that analysis tends to be fairly cumbersome. In the case of the barium tracer, the samples had to be returned to the laboratory before contamination could be evaluated. In the case of sulfate, pore water was collected from the sediments in the field but analysis was performed in the laboratory. It is highly desirable, in terms of both time and effort spent, to be able to screen the cores for contamination in the field. Analysis of cations—usually atomic adsorption or emission spectroscopy—in the field is not generally feasible. Similarly, analysis of anions—usually by ion chromatography—is also not feasible in the field.

Chemical Additive Tracers A number of chemicals may be added to drilling fluid to serve as tracers. Many of these substances have been adapted from other tracer technologies. For example, rhodamine dye, an organic compound that absorbs light at one wavelength (~550 nM) and emits light at another (~590 nM), can be added to the drilling fluid as a tracer. This method has been used by Phelps et al. (1989) in microbiologic studies of deep coastal plain sediments.

The advantages of rhodamine are that it does not occur naturally, it can easily be detected with a portable fluorometer, and it is relatively sensitive. Rhodamine dye in water can be detected in concentrations as low as 100 parts per trillion. The principal disadvantage of rhodamine is that it is degraded by contact with chlorine and chloride—solutes common in drilling fluids—and that turbidity interferes to some degree with analysis. Also, rhodamine is strongly adsorbed by some organic materials. Another disadvantage is that because it alters the color of the drilling fluid, drillers dislike working with it. Drillers keep track of how much formation clay is suspended in drilling fluid by monitoring color changes over time. If these color changes are masked by use of a dye, it makes the driller's job more difficult. Drillers can, however, work within this if use of rhodamine is judged to be necessary.

Fluorescein is another fluorescent dye that has been used as a tracer for drilling fluid contamination. Pedersen and Ekendahl (1989) described the use of fluorescein in a study of bacteria in a granitic aquifer. When drilling hard rock formations such as granites, it is not necessary to weight drilling fluids to hold open the borehole. In the case described by Pedersen and Ekendahl (1990), water produced from a nearby well was used as the primary drilling fluid. Prior to use in drilling, fluoresin

was added to the ground water. During later microbiologic sampling of the wells, the amount of fluoresein present was quantified and the approximate percentage of drilling water contamination assessed. It was found that very low levels, as low as 0.1%, drilling water contamination was measurable using this technique.

In addition to fluorescent tracers, other chemical tracers can be used. Phelps et al. (1989b) and Garland et al. (1991) added potassium bromide to drilling fluids as a tracer. Bromide is present in only trace amounts in the pore fluids of most aquifer systems and can readily flag drilling fluid contamination.

Another class of chemical tracer that has been applied to sampling deep subsurface sediments are perfluorocarbons (PFTs). PFTs are fully fluorinated alkyl substituted cycloalkanes. They are chemically inert, nontoxic, and are detectable in very low concentrations (~1 picoliter/gram of sample) using gas chromatography with an electron capture detector. PFTs are widely used in atmospheric tracing studies and as tracers in oil and gas reservoirs for evaluation of enhanced recovery potential. PFTs have been used for tracing drilling fluid contamination in deep coastal plain sediments (Senum and Dietz, 1991). In this application, differently substituted PFTs were used to separate potential contamination due to drilling of a preliminary test hole from contamination due to the coring operation. These results, which showed some cross-contamination of the two boreholes, indicated that contamination from one borehole can extend several meters away from a borehole. The sensitivity of this method combined with the flexibility provided by using different PFTs for different parts of the drilling operation shows that it is a potentially very useful tool for contamination tracing.

Particulate Tracers In addition to solutes, it is also possible to use particulates as tracers of drilling fluid contamination. The most widely used particulate tracer that has been used in microbiologic investigations are micron-sized polystyrene spheres impregnated with a fluorescent dye. These microspheres, which are manufactured by Polysciences, Inc., come in a number of sizes, chemical characteristics, and fluorescent properties. The sizes available range from 0.1 to 10 micrometers in diameter. Importantly, the polystyrene from which they are made has a density of about 1.05, or very near that of fresh water. Because the spheres are much less dense than sediment particles, they are easily separated for microscopic examination. The microspheres are manufactured with a neutral, positive, or negative surface charges and are available with a variety of fluorescent properties.

Column experiments with sandy sediments have shown that the adherence of microspheres to sediment surfaces is a function of size and surface charge. The smaller spheres tend to adhere to sediment particles more than larger spheres do. Furthermore, since the surfaces of most silicate mineral grains have a net negative charge, negatively charged (carboxylated) microspheres adhere less than those that are neutral or positively charged. Microspheres can be easily detected with epifluorescent microscopy. With this microscopic technique, the sample is illuminated with fluorescent light. Those objects that emit visible light when excited with fluorescent light are then readily observed by the microscopist.

The manufacturer supplies the microspheres in 10-ml vials. The total number of spheres per 10-ml vial is dependent on sphere diameter, the weight percent of solids per ml, and the density of the solids. For 1-micron-diameter spheres, a 10-ml vial

contains 4.5×10^{11} spheres. The final concentration of microspheres in the drilling fluid is dependent on the volume drilling fluid being used and the effectiveness of mixing with the mud. An even distribution of microspheres can be obtained by having the driller add microspheres as drilling fluid is mixed. Typically, a 100-foot borehole (6 inch diameter) uses about 400 gallons of drilling fluid. Each next 100 feet of borehole requires approximately an additional 100 gallons. Thus, for a 200-foot borehole, addition and complete mixing of one 10-ml vial results in a final microsphere concentration of about 10^7 spheres/ml of drilling fluid. Experience has shown that a concentration of 10^5 to 10^6 microspheres/ml drilling fluid is sufficient to trace contamination of cored materials.

The use of fluorescent microspheres as a tracer for drilling fluid contamination of cored sediments has been described by Chapelle and McMahon (1991). For this method, the epifluorescent microscope was set up at the drill site and microspheres added to the drilling fluid for a final concentration of 10^5 spheres/ml. After a core had been recovered, it was broken in half to expose a fresh sediment surface. One of the sediment surfaces was sampled along a traverse from the outside to the middle of the core. Approximately 1 ml of sediment was placed in 9 ml of distilled water and shaken. When the larger sediment particles had settled, one ml of the suspension was filtered onto a nucleopore membrane. The membrane was placed directly on a microscope slide, and the microspheres enumerated by scanning about 10% its total area.

The principal advantage of the microsphere method is that cores can be screened for contamination within minutes of core recovery. This allows contaminated sediments to be quickly identified so that valuable time is not spent processing them.

Biological Tracers Drilling fluids normally contain large numbers (~108 cells per ml) of microorganisms. Because many of these microorganisms are different from the natural flora of deep subsurface sediments, they can be used as a tracer. One application of this general technique was given by Chapelle et al. (1988), who observed that the bacterium Gluconobacter was present in the drilling fluid but not in the sediments being cored. Thus, the presence or absence of this microorganism was an indication of the presence or absence of drilling fluid contamination.

Another example of this kind of tracer was described by Beeman and Suflita (1988). These investigators noted that drilling fluids contained large numbers of coliform bacteria (bacteria that normally inhabit the intestines of animals), whereas subsurface sediments did not. Thus, the presence or absence of coliform bacteria could serve as a "serendipitous" tracer for drilling-fluid contamination.

The principal drawback for using microorganisms as tracers is that it is difficult to use them quantitatively. While they are often useful qualitative indicators of the simple presence or absence of contamination, it is difficult to determine the sensitivity of the method. For quantitative purposes, chemical or particulate tracers are more suitable.

8.5 SAMPLING GROUND WATER FOR MICROORGANISMS

Sampling ground water for microorganisms is fraught with numerous potential contamination problems. Drilling fluid used in constructing boreholes for wells

contain substantial numbers of microorganisms. The drilling process, therefore, effectively "inoculates" any aquifer system being drilled. It may well be that the majority of bacteria introduced by drilling die off with time in their new environment; however, this has never been studied quantitatively. It must be assumed that some microorganisms recovered from well water may represent introduced, rather than indigenous, species.

For some applications, however, it is just as important to consider introduced microorganisms as well as indigenous ones. For example, microorganisms introduced by drilling procedures into wells to be used for water supply are probably of more concern from a health standpoint than indigenous species. For some applications, therefore, sampling ground water may be the procedure of choice.

The procedures used to sample ground water for microorganisms are generally fairly simple. Olson et al. (1981), in a study of methanogenic and sulfate-reducing bacteria in the Madison aquifer of Montana, allowed the completed well to purge for 8 hours prior to sampling. A sterile piece of rubber tubing was then inserted into the discharge pipe, and this tube used to fill the sterile sample bottles.

Hirsch and Rades-Rohkohl (1983) used a more sophisticated setup to sample ground water from a shallow aquifer in Schleswig-Holstein, Germany. For sampling fully penetrating wells, these investigators sterilized a submersible pump and its attached tubing with 70% ethanol alcohol and lowered it into the wells to be sampled. Three casing volumes were then pumped from the well and discarded in order to flush out traces of ethanol and to remove free standing water from the well bore. Ground water was then collected in a sterile 500 ml sampling flask equipped with a screw cap for subsequent plate count and isolation procedures. These procedures included spread-plating 0.1 ml of ground water onto triplicate plates of solid media, followed by standard streak-plate isolation methods.

In addition to sampling fully-penetrating wells in the shallow aquifer, Hirsch and Rades-Rohkohl (1983) sampled a series of multilevel screened wells. As before, about three well-volumes of ground water was removed from each well prior to sampling. Plate counts of this water that had stood in the well bores showed higher numbers of bacteria than present in water pumped directly from the aquifer. Water was lifted from the wells by suction into a desiccator and a 500 ml sterile sampling flask was filled with water for microbiologic analysis.

Most strategies for sampling ground water from wells for microbiologic analysis are similar to those employed by Olsen et al. (1981) and Hirsch and Rades-Rohkohl (1983). It is assumed that free-standing water in the borehole is not representative of aquifer water and so at least 3 well volumes, and often tens of well volumes, are evacuated prior to sampling. After well development, the ground water samples are collected in appropriate presterilized vessels. Similar techniques have been employed in more recent years by Stetzenbach et al. (1986) in the Tucson Basin and by Pedersen and Ekendahl (1990) in a deep granitic aquifer in southeastern Sweden.

8.6 CHAPTER SUMMARY

Subsurface sediments can be sampled for microbiological analysis using a number of methods. The unsaturated zone is most conveniently sampled using a hand auger to a depth of about 20 feet. For relatively thick unsaturated sediments, air drilling

combines ease of sample recovery with relatively low risk of sample contamination. Local flow systems, which are usually relatively shallow, are most easily sampled using hollow-stem augering combined with split-spoon or push-tube methods. Hollow-stem augering is the method of choice for sample depths less than about 100 feet.

Intermediate and regional flow systems, being deeper, often cannot be sampled using auger rigs. In these cases, mud-rotary coring has been most often employed for sampling. Mud-rotary coring is technologically sophisticated and uses a number of highly specialized drilling tools and drilling fluid additives. While coring, the driller constantly adjusts drillstem pressure, fluid pressure, and drill bit rotation. The efficiency of core recovery depends in large measure on the skill of the drill and the degree to which these three variables are optimized. Coring efficiency also may be enhanced by the proper use of specialized drilling fluid additives.

The use of drilling fluid in mud-rotary coring often results in contamination of the core. There are three common ways for cored sediments to be contaminated by drilling fluids. One is down-hole saturation, where drilling fluid saturates the sediments ahead of the core barrel. Another is core seepage, where drilling fluid seeps into the core while it is in the inner barrel. Fracturing of the core once it has entered the inner barrel will also contaminate cored sediments, particularly relatively hard clays or indurated sediments.

Because of the possibility of core contamination by drilling fluids, it is necessary for each core used for microbiologic analysis to be screened for contamination. To this end, a number of different types of tracers can be used. Some components of drilling fluids, such as barium or sulfate from barite additives, are potential tracers. It is also possible to use microorganisms that are present in the drilling fluid but not in subsurface sediments, such as coliform bacteria, as tracers. Often, however, the best indicators of contamination are chemical or particulate tracers added to drilling fluids. Rhodamine dye or bromide are examples of chemicals used as chemical tracers and florescent microspheres are examples of particulate tracers.

The microbiology of subsurface environments can also be sampled by producing ground water from properly constructed wells. While it is fairly easy to preclude water contamination after it has left the well, it is difficult to rule out sample contamination by microorganisms present in drilling fluids or microorganisms growing gratuitously in the well bore. Furthermore, the similarity between waterborne microbial communities and sediment-bound communities is problematic. Nevertheless, these techniques are suitable for some applications.

QUESTIONS TO CONSIDER

1. What three principal difficulties limit the use of hand augers to collect subsurface sediments?
2. Are there any circumstances in which the outside of a split-spoon core can be considered uncontaminated?
3. If tools are sterilized prior to being lowered into a hole to core sediments or rocks, will they still be uncontaminated when they reach the coring horizon?
4. Name the four principal functions of drilling fluid.

5. What is one obvious problem with using polysaccharides to thicken drilling fluid when obtaining cores for microbiological applications?
6. How important is the skill of the driller in assuring core recovery? Why?
7. Is core seepage contamination more common when coring sandy or clayey sediments? Why?
8. What kind of tracers give the most sensitivity in screening for drilling-fluid contamination of cores?

CHAPTER 9

BIOGEOCHEMICAL CYCLING IN GROUND-WATER SYSTEMS

The primary goal of ground-water geochemistry is to understand the processes governing the distribution of chemical species in ground water. If ground-water systems were sterile environments that lacked any sort of microbial life, this goal could be attained solely from fundamental principles of mineral equilibria and chemical kinetics. However, of the hundreds of ground-water systems studied (excluding high-temperature thermal systems), all have contained viable microorganisms in a more or less active state. It is important, therefore, to consider how the biochemistry of microorganisms affects the occurrence and movement of chemical species in these systems.

The term biogeochemical cycling refers to the alternate storage and release of chemical energy as an element moves through the biosphere. Because the chemical characteristics of each element are different, the pathways that cycle them through the biosphere also differ. Biogeochemical cycling in ground-water systems has a significant impact on the speciation and mobility of biologically active elements.

The concept of biogeochemical cycles comes from ecological theory. Ecosystems can be viewed at many different levels, depending on the questions being asked. For example, the relationships among primary producers (plants), grazers, predators, and decomposers are often depicted in a hierarchical fashion (Fig. 9.1a). This type of diagram is useful in illustrating the kind of relationships between different populations. At another level, however, relationships between populations can be expressed as the flow of energy and chemicals through the ecosystem (Fig. 9.1b). In this view, primary producers store energy and biochemically active elements in a form that can be utilized by consumers. The cycle is completed when a specialized group of consumers, the degraders, return the elements to a form that can again be stored by the producers.

Many of the chemical solutes important in ground-water systems—oxygen, organic, and inorganic carbon species, sulfur species, nitrogen species, and iron species—move through microbial food chains in ways analogous to those shown in Figure 9.1b. For this reason, understanding the nature of biogeochemical cycles is

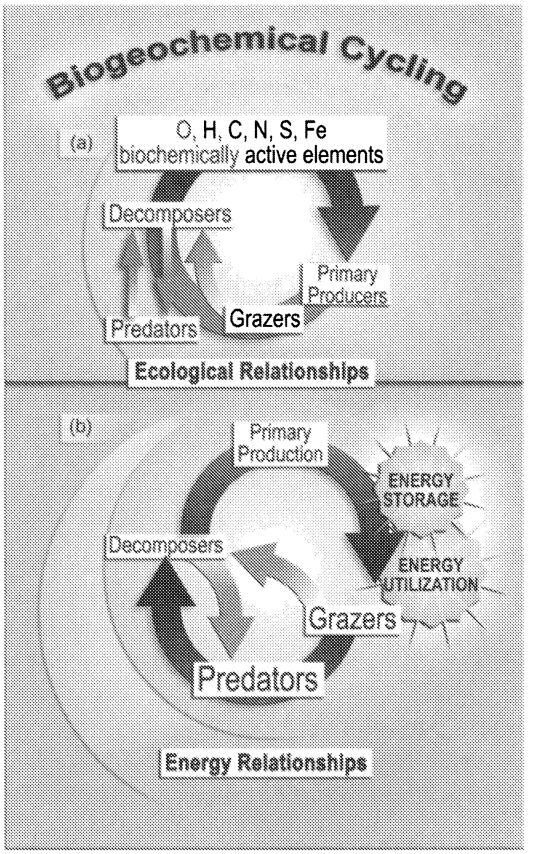


Figure 9.1. The cycling of biochemically active elements in the biosphere.

fundamental to understanding the distribution of chemical species in microbially active ground-water systems. However, because of the unique nature of subsurface ecosystems, the manner in which microbial elemental cycling is expressed is often much different from that observed in other sedimentary environments. In this chapter, the biogeochemical cycling of oxygen, carbon, nitrogen, iron, and sulfur in ground-water systems—and some effects of this cycling on ground-water geochemistry—are considered.

9.1 THE OXYGEN CYCLE

Oxygen is present in the earth's atmosphere solely because of photosynthetic production of reduced carbon compounds from carbon dioxide by green (chlorophyll-bearing) plants and microorganisms. Prior to the evolution of photosynthetic algae (roughly 2 billion years ago) the earth's atmosphere was highly reducing. The overall stoichiometry of oxygen production via photosynthesis is

$$\text{Solar energy} + CO_2 + H_2O \rightarrow CH_2O + O_2$$

where CH_2O represents reduced organic carbon of valence zero. When this reaction proceeds from left to right, it is highly endergonic and requires energy from sunlight in order to proceed. Molecular oxygen produced from this reaction subsequently accumulates in the atmosphere and in surface and ground water. The solar energy that drives this reaction, and which is stored as chemical energy, can be subsequently recovered and utilized by respiring micro- and macroorganisms:

$$CH_2O + O_2 \rightarrow CO_2 + H_2O + \text{chemical energy}$$

On a global scale, the production of oxygen by photosynthesis is exactly balanced by oxygen consumption by respiring organisms as shown in Figure 9.2. This large-scale balance between oxygen production and consumption is referred to as the *global oxygen cycle.*

9.1.1 Oxygen Cycling in Ground-Water Systems

The balance between oxygen production via photosynthesis and oxygen consumption by respiration is maintained only at the global scale. On smaller scales, it is common for imbalances to develop between oxygen production and consumption. For example, in surface water bodies in which photosynthetic algae are active, it is possible for the rate of oxygen production to vastly exceed respiration during daylight hours, resulting in a net accumulation of dissolved oxygen in the water. During the night, however, the activity of respiring heterotrophic bacteria outpaces photosynthesis and oxygen concentrations decrease. The push-pull relationship between oxygen production and consumption at the local scale largely determines the oxygen status of a particular environment.

In many environments, such as ground-water systems and many aquatic sediments, sunlight is excluded and photosynthetic production of oxygen cannot occur. This effectively truncates the oxygen-production component of the oxygen cycle while leaving the oxygen-consuming components intact. In these cases, the oxygen status of an environment is determined by rates of oxygen transport from the atmosphere and by rates of respiration. If rates of oxygen transport into the system

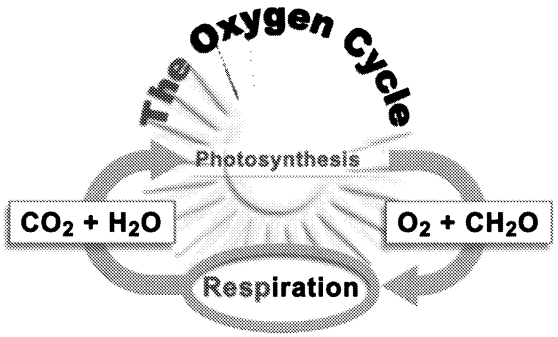


Figure 9.2. The oxygen cycle.

exceed rates of endogenous respiration, oxygen will accumulate and the system will be aerobic. On the other hand, if respiration rates exceed the flux of oxygen, the system will become anaerobic.

The flux of oxygen to all sedimentary environments is severely limited by its low solubility in water. At 20°C, only about 9 mg/L oxygen can dissolve in water. This low solubility affects different systems in different ways. In surface-water systems such as rivers or lakes, water generally moves fairly rapidly and aerobic conditions are common. In aquatic sediments or ground-water systems, however, rates of water movement are much slower. This decreases the available flux of oxygen to these environments and anaerobic conditions are more common.

From a water-quality standpoint, dissolved oxygen is one of the most important geochemical constituents of ground water. Aerobic ground water generally lacks such undesirable constituents as dissolved organic carbon, dissolved Fe(II), sulfides, or methane. For this reason, oxygenated ground water is generally desirable for human and animal consumption.

The distribution of dissolved oxygen in ground-water systems generally follows a predictable pattern. The distribution of dissolved oxygen in the Patuxent aquifer near Baltimore, Maryland (Chapelle and Kean, 1985), is a good example of this (Fig. 9.3). Where the aquifer crops out—and hence where it functions as a local flow system—the ground water contains measurable concentrations of dissolved oxygen. As water flows along the regional gradient and enters the intermediate flow system, however, concentrations of dissolved oxygen decrease below measurable levels.

The pattern of dissolved oxygen changes shown in Figure 9.3 reflects relative rates of dissolved oxygen transport to and oxygen consumption in each aquifer. Local flow systems closely connected to the atmosphere tend to be oxygenated. In these local flow systems, the flux of oxygen from the atmosphere exceeds rates of respiration in the aquifer and hence oxygen accumulates in solution. As ground water moves away from local flow systems and enters confined intermediate systems, the flux of dissolved oxygen is typically less than oxygen consumption, and concentrations decrease.

Aquifer systems vary widely in their rates of respiration and, therefore, rates of oxygen consumption. Some organic-rich aquifers, even where they function as shallow local flow systems, are entirely anaerobic. For example, the Hawthorne aquifer of South Carolina (Chapelle et al., 1988) becomes anaerobic within a few meters of the water-table surface. The rate of oxygen respiration (Ox_{re}) in this system can be calculated from the equation

$$Ox_{re} = \Delta O_2 * R/L$$

where ΔO_2 is the change in oxygen concentrations along a flowpath, R is the average rate of ground-water flow, and L is the length of the flowpath segment. Given the average downward flow rate (R) of 0.23 m/yr, a dissolved oxygen concentration change of 5 mg/L (0.16 mM), and a flowpath length of 6 meters, the rate of oxygen respiration in this system is calculated to be on the order of 10^{-2} mM/L/yr. In contrast, calculated rates of respirative oxygen consumption in the Patuxent aquifer (Fig. 9.3), given an average flow rate of 5 m/yr, a dissolved oxygen change of 10 mg/L (0.32 mM), and a flowpath length of 6 miles (9,654

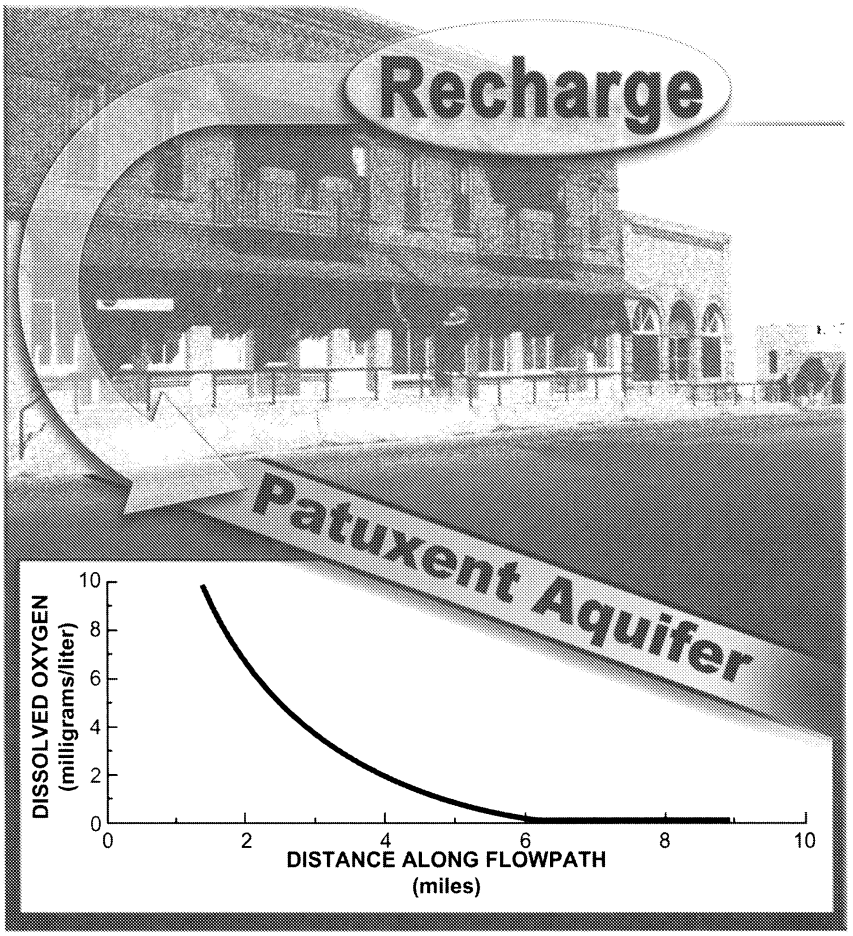


Figure 9.3. How the truncated oxygen cycle leads to oxygen depletion in the Patuxent aquifer near Baltimore, Maryland.

m), indicates a much slower oxygen consumption rate on the order of 10^{-4} mM/L/yr is calculated. It is clear that respirative oxygen consumption rates vary widely between different ground-water systems.

When rates of dissolved oxygen consumption are relatively low, it is possible for low concentrations of oxygen to persist over wide areas of aquifer systems. For many years, analytical methods have been inadequate to study these low-oxygen aquifers. Standard methods for measuring dissolved oxygen concentrations in ground water, the Winkler titration and oxygen-sensitive membrane electrodes, have detection limits of about 0.1 mg/L (~3 μM) dissolved oxygen. This has limited the study of aquifers with fairly low (3.0–0.2 μM) dissolved oxygen concentrations.

A study by White et al. (1990) introduced a more sensitive oxygen-measuring technique based on the oxidation of rhodazine-D. This study showed that oxygen could be reliably measured in concentrations as low as 0.2 μM (0.006 mg/L). Furthermore, it showed that the presence of selenate, an oxidized selenium compound that is relatively mobile as well as being toxic, correlated with the presence of low dissolved oxygen concentrations in a shallow aquifer. It is probable that

application of low detection level techniques for dissolved oxygen will reveal a variety of aquifer systems characterized by low but measurable levels of dissolved oxygen.

Some aquifer systems exhibit virtually no oxygen consumption, even when ground water enters the deeper intermediate and regional flow systems. For example, deep sedimentary basins of the American Southwest, in which ground water is on the order of 10,000 years old, in some cases have oxygen concentrations in the 7–8 mg/L range and thus virtually still at atmospheric saturation (Winograd and Robertson, 1982). In this case, the aquifer consists of alluvial material that is virtually devoid of organic material or other reduced compounds. Consequently, there is little or no consumption of oxygen. This aquifer system is an example of a ground-water system with very low microbial activity. Ground water produced from these aquifers, however, have been observed to contain viable microorganisms (Stetzenbach et al., 1986).

The oxygen cycle illustrates a general principle of biochemical cycling in subsurface environments. This is that the cycles become truncated at one or more places. In the case of oxygen, the truncation of the cycle is due to the lack of energy input in the form of photosynthesis. This, in turn, simply results in the establishment of anaerobic conditions in many subsurface environments. Other elemental cycles may also be truncated by the lack of energy input resulting in the accumulation of particular chemical species. An example of this behavior is the carbon cycle, which is closely intertwined with the oxygen cycle, and this will be considered next.

9.2 THE CARBON CYCLE

The carbon atom, with its ability to maintain several different oxidation states (−4 to +4) and its tendency to form stable covalent bonds, is very efficient at storing and releasing energy. The ability of carbon to alternately absorb (solar) energy by forming reduced organic compounds and then releasing this (chemical) energy through oxidation reactions, is the chemical basis for all life on earth. The biochemical mechanisms and pathways that control the flow of energy through oxidation-reduction reactions involving carbon are known collectively as the global carbon cycle.

A schematic diagram of the global carbon cycle is shown in Figure 9.4. The central compound in this cycle is carbon dioxide (CO_2) present either in the atmosphere as a gas or in water as dissolved inorganic carbon species (CO_2, HCO_3^-, $CO_3^=$). In terrestrial and near-surface marine environments, sunlight is available and CO_2 is reduced to carbohydrates via photosynthesis. The reduction of oxidized carbon in CO_2 (oxidation state +4) to organic carbon present in carbohydrates (oxidation state 0) releases free oxygen and results in aerobic conditions. Much of this organic carbon is aerobically reoxidized via the respiration of plants, animals, and microorganisms and returned to the atmosphere or ocean as CO_2. A significant quantity of this oxidized carbon in marine environments is sequestered by calcium carbonate–precipitating marine organisms.

Certain types of anaerobic bacteria, notably the green sulfur bacteria, the purple sulfur bacteria, and nonsulfur purple bacteria, are also capable of photosynthesis. The biochemistry of this photosynthesis differs markedly from that of green plants

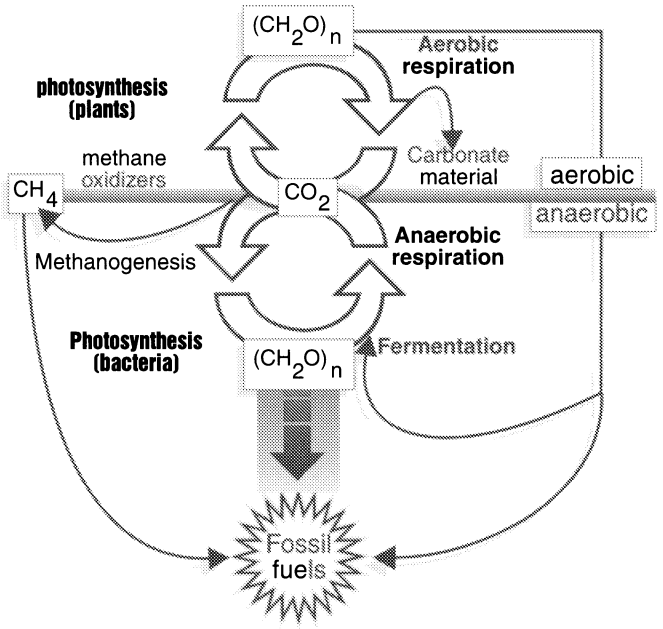


Figure 9.4. The global carbon cycle.

and algae in that molecular oxygen is not produced. Thus, in anaerobic aquatic environments exposed to sunlight, bacterial photosynthesis contributes to the reduction of CO_2 to carbohydrates and other organic compounds but without the production of oxygen.

A large amount of organic carbon produced by plant photosynthesis is cycled back to CO_2 by means of anaerobic oxidation. In anaerobic oxidations, fermentative bacteria incompletely oxidize organic carbon with the production of organic acids, alcohols, and molecular hydrogen. These simple reduced compounds are then completely oxidized by anaerobic repirative bacteria that use mineral electron acceptors such as Mn(IV), Fe(III), and sulfate. The methanogenic bacteria produce methane (CH_4) under anaerobic conditions. Much of this methane subsequently diffuses into aerobic environments where oxidation to CO_2 takes place. A portion of this CH_4 becomes trapped in geologic structures and accumulates. Where aerobic and anaerobic oxidation of organic carbon is incomplete, organic carbon may accumulate over geologic time, forming coal, petroleum, and other fossil fuels.

The observed rise in atmospheric CO_2 levels over the last century reflects a number of human perturbations of the global carbon cycle. In particular, the rapid burning of fossil fuels has oxidized and released large amounts of carbon from storage within the earth. This, in turn, has increased the available pool of CO_2. Because of the complexity of the carbon cycle, however, the effects of this increased pool of atmospheric CO_2 remains controversial. Inspection of Figure 9.4 suggests, for example, that increased availability of CO_2 may stimulate photosynthetic production of organic carbon. This process would act as a negative feedback mechanism for CO_2 accumulation in the atmosphere. However, such increased stimulation of photosynthesis cannot occur if the world's forests are progressively re-

moved by an expanding human population and if oceanic algal populations are depleted by pollution. Possible political solutions to this potential problem will rest, among other things, on a correct interpretation of the global carbon cycle and how it responds to perturbation. This topic is and will remain a major scientific challenge.

9.2.1 The Integrated Carbon, Oxygen, and Hydrogen Cycles

While it is a convenience to consider each elemental cycle individually, it is clear that these cycles are closely intertwined. It is not possible, for example, to discuss the production of molecular oxygen (Fig. 9.2) without also considering the production of organic carbon from CO_2 (Fig. 9.4). Furthermore, the hydrogen cycle, which has not been discussed separately, is also intertwined with the carbon and oxygen cycle.

A schematic representation of the integrated carbon, oxygen, and hydrogen cycles is shown in Figure 9.5. These cycles are driven by energy provided in sunlight. Photosynthesis reduces carbon in CO_2 with the oxidation of oxygen present in

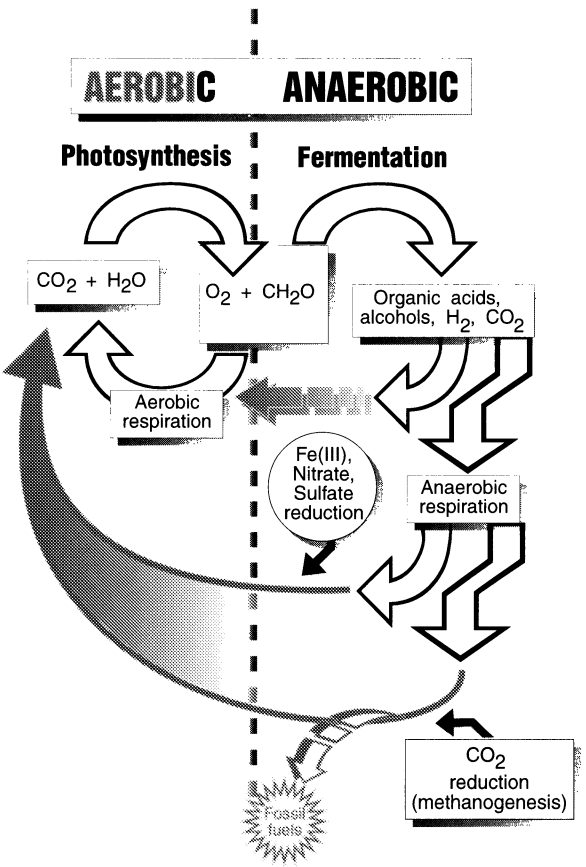


Figure 9.5. The integrated carbon, oxygen, and hydrogen cycles.

water. Under aerobic conditions, much of this reduced carbon is cycled back to CO_2, with oxygen being reduced to water. Under anaerobic conditions, organic carbon compounds may also be oxidized, but these oxidations proceed in stepwise fashion. The oxidation is initiated by fermentative microorganisms that partially oxidize the carbon to CO_2 with the production of reduced compounds such as organic acids, alcohols, and molecular hydrogen (H_2). These reduced compounds can then be completely oxidized anaerobically with Fe(III), sulfate, or carbon (methanogenesis) acting as terminal electron acceptor. Under aerobic conditions, these reduced compounds are cycled directly back to CO_2 and water.

From a microbiologic point of view, the nature of these integrated cycles has a number of important ecological implications. For example, it is clear that the cycling of carbon under anaerobic conditions requires a cooperative food chain in order to achieve complete oxidation. From a geologic point of view, it's interesting to note the steps necessary for the production of biogenic methane gas. First of all, if mineral oxidants such as Fe(III) or sulfate are available, there will be little production of methane. Only in the absence of these mineral oxidants is organic carbon oxidation coupled to the production of methane. This methane may go into storage, creating a pool of potential fossil fuel, or be reoxidized under aerobic conditions. Judging from the relatively small pool of methane trapped in geological structures relative to the scale of the global carbon cycle, it is clear that aerobic reoxidation is by far the most common fate of biogenic methane gas.

9.2.2 Carbon Cycling in Ground-Water Systems

The carbon, oxygen, and hydrogen cycles are driven by two pools of energy (Fig. 9.5). The photosynthetic fixation of organic carbon, which initiates the cycle, is driven exclusively by solar energy. In turn, the oxidation of organic carbon back to CO_2 is driven by chemical energy stored in reduced carbon and hydrogen compounds. A fundamental characteristic of ground-water systems removed from active photosynthesis is that these cycles are effectively truncated. In the case of the oxygen cycle, the truncation of the cycle leads to depletion of oxygen in many ground-water systems. In the case of the carbon cycle, however, truncation would be expected to lead to the accumulation of CO_2 and CO_2-derived carbonate species in ground water.

The accumulation of dissolved inorganic carbon species associated with intermediate and regional flow systems was one of the first chemical attributes of ground water that was systematically investigated. Effervescent spring waters have been known to humanity since prehistoric times. These waters were closely studied over the years, primarily because of their purported medicinal properties, but also out of curiosity about their effervescence. In the early nineteenth century, when rudimentary techniques of analytical chemistry became available, it became fashionable for physicians to perform chemical analyses of spring waters in order to discover the chemical basis of their observed properties.

One particularly well documented case of this was recorded by a physician named John H. Steel (1782–1838). Steel had practiced medicine in the area of Saratoga Springs in New York State for many years. As such, he became familiar with the medical uses of several springs. Being very much a man of the Age of Enlightenment, he resolved to chemically analyze the spring waters and thereby

discover the causes of the medicinal effects. Steel (1838) describes the water of one particular spring, Congress Spring, in this manner:

> The surface of the well is constantly agitated by the escape of gas in fine bubbles, giving the appearance of simmering, not unlike that which water exhibits just before the process of violent ebullition (boiling) takes place. When first dipped, the water is remarkably sparkling, and were it not for the constant escape of gas in innumerable fine points, it would be perfectly transparent.

Steel goes on to describe some affects of the gas issuing from the spring on animals:

> The respiration of all breathing animals is immediately affected by coming in close contact with the surface of this fountain. The gas which issues from it is immediately fatal to the lives of animals which happen to be immersed in it, and even fishes and frogs survive but a short time when placed in it.

Steel observed that this gas, when driven out of the water by heating and collected in an inflatable bladder, was absorbed by "potash of soda" (potassium hydroxide), thus deflating the bladder. This showed that the gas was predominantly "carbonic acid gas" or carbon dioxide. This was a result that explained the observed effects on animals (it suffocated them) and neatly explained the effervescence. Deducing that the gas was carbon dioxide was an accomplishment Steel was greatly proud of. Incidentally, the water also contained relatively high concentrations of magnesium sulfate, which probably also explains the cathartic medicinal properties of the water.

Showing that an accumulation of carbon dioxide explained the effervescent properties of this (and countless other) artesian waters did not explain the source of the CO_2. Steel clearly recognized this problem. In the last paragraph of his book, he writes:

> The production of the unexampled quantity of carbonic acid gas, and the medium through which the other articles are principally retained in solution, is yet, and probably will remain, a subject of mere speculation. The low and regular temperature of the water seems to forbid the idea that it is the effect of subterranean heat, as many have supposed. Its production is therefore truly unaccountable.

The observed accumulation of CO_2 and other dissolved carbonate species, however, may be regarded as a manifestation of a truncated carbon cycle in ground-water systems. In turn, the nature of this cycling is dependent on the properties of the ground-water flow system.

Local Flow Systems Local flow systems are characterized by relatively close interaction with the surface and surface processes. This close connection has a marked impact on carbon cycling in local flow systems that distinguish it from intermediate or regional flow systems.

Figure 9.6 shows a schematic diagram of how carbon is cycled in local flow systems. Photosynthetic production of organic carbon at land surface begins the

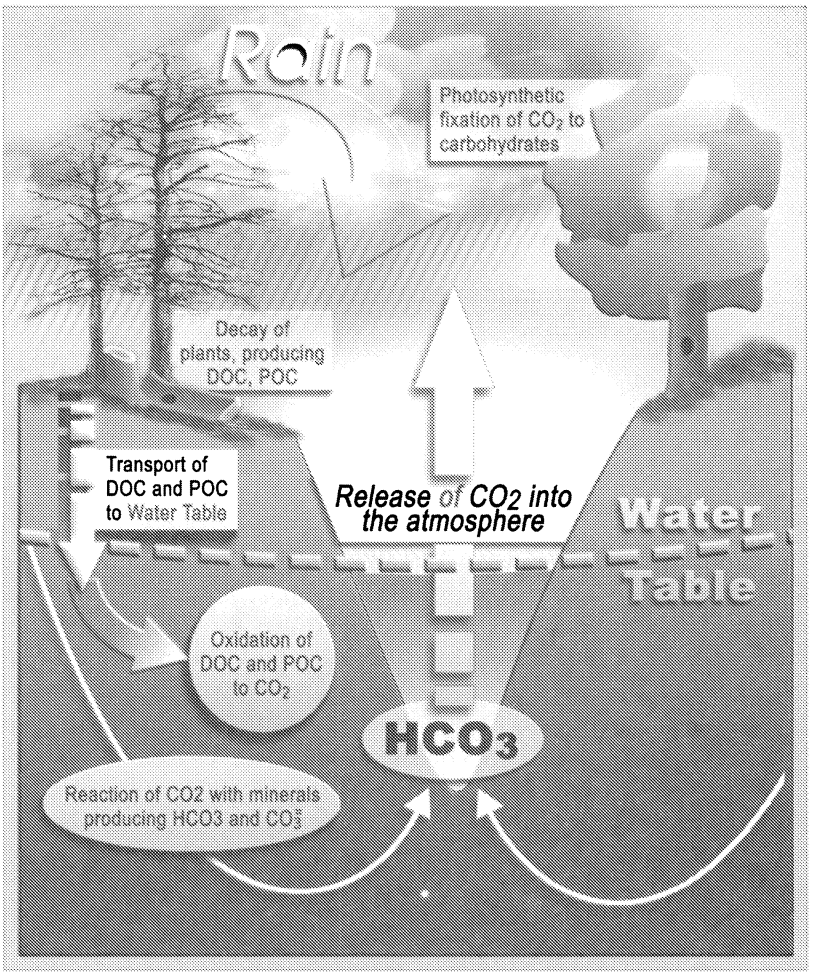


Figure 9.6. Carbon cycling in shallow ground-water systems.

cycle. The decomposition of organic matter near land surface, a designation that includes the soil zone, releases some CO_2 as well as dissolved organic carbon (DOC) compounds and particulate organic carbon (POC). Much of the CO_2 is immediately released to the atmosphere. A small portion of CO_2 as well as some DOC and POC is carried to the water table and into the local flow system by vertically percolating recharge. Much of the DOC and POC transported in this manner are subsequently oxidized to CO_2. However, because the aquifer is under unconfined, water-table conditions, CO_2 in excess of water solubility is readily exchanged with soil gases and ultimately the atmosphere. Most of the CO_2 generated by these processes is transported by ground-water flow to an adjacent recharge area, where it seeps to the surface and escapes to the atmosphere.

While many variations upon this general theme are possible, the basic outline applies to most local flow systems. The outcome of these cycling processes is that there is little net accumulation of CO_2 or CO_2-derived species in ground water. Rather, the close connection of local flow systems with the surface, and therefore

with the atmosphere, ensures rapid and complete cycling of carbon. Carbon cycling in local flow systems closely resembles the kinds of processes that occur in soils, unsaturated zones, and other near-surface environments.

Intermediate and Regional Flow Systems The carbon cycle in intermediate and regional flow systems tends to be much different than in local flow systems. One difference is the relative importance of organic carbon from surface decomposition of organic matter. The ultimate source of organic carbon is, of course, photosynthesis. However, organic carbon originally deposited with the sediments tends to be much more important in intermediate and regional flow systems. This reflects the relatively slow recharge rates from the surface. Because recharge rates are generally slow, DOC or POC fluxes from surface sources also tend to be low.

Some time after deposition, often millions of years, a ground-water flow system is established and slow oxidation of the organic carbon continues. In or near the outcrop areas of the aquifer, oxidation of organic matter may occur aerobically. However, with the consumption of dissolved oxygen, oxidation under anaerobic conditions quickly becomes the dominant process. Oxidation is not confined to the sandy aquifers exclusively, but also occurs in clayey confining beds. It is rare that anaerobic oxidation proceeds only via fermentation. Rather, fermentation and oxidation by mineral electron acceptors tend to be coupled. Because of the presence of confining beds, there is little connection between aquifers and the surface. Therefore, CO_2 and other carbonate species generated by these anaerobic oxidation processes accumulate in solution.

The progress of carbon cycling in intermediate and regional flow systems can have several different outcomes. If the ground water remains isolated from the surface by means of structural traps or other geologic features, then much of the DIC may ultimately be precipitated as carbonate cements. This effectively removes that carbon from the carbon cycle for long periods of time. A more common outcome, however, is the eventual discharge of ground water to the surface by means of springs or by diffuse upward discharge. This type of discharge accounts for the occurrence of mineral springs, such as those at Saratoga Springs, and countless other artesian seeps. The occurrence of CO_2-charged waters at these springs is thus an inevitable manifestation of carbon cycling in these subsurface environments.

On a large scale, in terms of both time and space, it is clear that complete truncation of the carbon cycle (and thus the oxygen and hydrogen cycles) in intermediate and regional ground-water systems is rare. Even dissolved carbonate species from organic carbon oxidation that become sequestered as carbonate cements will eventually return to the cycle when the sediments are uplifted and eroded. The concept of cycle truncation, therefore, is useful only when considering the time frame of human life and history. This time frame, however, is the one of special importance and interest, particularly in cases of anthropogenic contamination.

9.3 THE NITROGEN CYCLE

While the carbon, oxygen, and hydrogen cycles involved both macroorganisms and microorganisms in producing and decomposing organic compounds, the nitrogen

cycle involves only microorganisms and some abiotic processes. Nitrogen in the atmosphere is present primarily as N_2 gas, which is a particularly stable compound. Under ambient atmospheric conditions, oxidized (NO_3) or reduced (NH_3) nitrogen compounds are not produced from N_2. However, such compounds are produced nonbiologically during electrical storms as well as in the higher atmosphere where photochemical processes are important. These nitrogen compounds are then carried to the earth's surface by precipitation. At the earth's surface, however, microbially mediated nitrogen fixation—the production of organic nitrogen compounds from N_2—and microbial nitrogen oxidation and reduction are the sole nitrogen-cycling processes.

A schematic representation of the nitrogen cycle is shown in Figure 9.7. Molecular nitrogen gas is fixed aerobically by a number of microorganisms including *Azotobacter, Rhizobium,* and many species of *Cyanobacteria*. Anaerobically, N_2 is fixed by members of the genus *Clostridium*.

The production of organic nitrogen compounds, such as ammonia, from molecular nitrogen requires a relatively high amount of energy input by microorganisms. The overall reaction of ammonia formation is approximately

$$N_2 + 6H + 16ATP \rightarrow 2NH_3 + 16ADP + 16\ P$$

Thus, a cell must spend about 8 ATP molecules in order to synthesize a single molecule of ammonia. From this it is clear that microorganisms can only afford to actively fix nitrogen if a ready supply of energy, either from photosynthesis (in the *Cyanobacteria*), symbiotic plants (as in Rhizobium), or from organic carbon (as in *Clostridia*). The high "cost" of nitrogen fixation is a crucial factor in nitrogen cycling in ground-water systems.

The crucial enzyme system involved with nitrogen fixation is nitrogenase, a complex molecule consisting of an iron-molybdenum protein and two iron proteins.

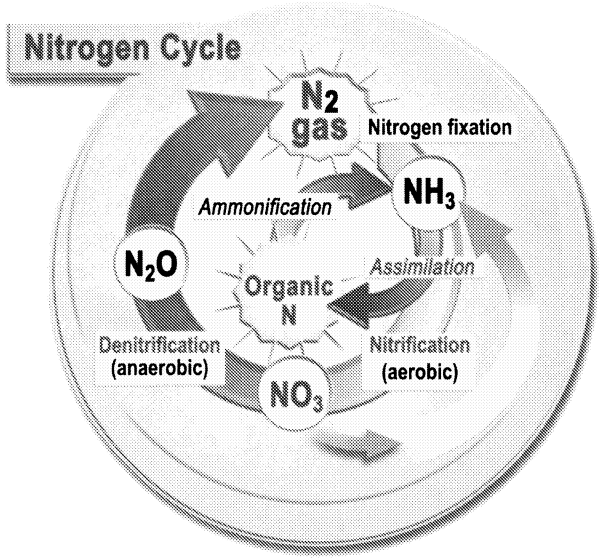


Figure 9.7. The nitrogen cycle.

The general mode of nitrogenase action is for the two iron proteins to reduce the iron-molybdenum protein to a low enough state so that the triple bond of the N_2 molecule may be broken and hydrogens attached to the nitrogen atoms:

$$N_2 + 3H_2 \rightarrow 2NH_3$$

Once the nitrogen cycle is initiated by N_2 fixation, the nitrogen cycle can continue (Fig. 9.7). Ammonia (NH_3) produced by N_2 fixation may be assimilated into proteins by microorganisms and by some macroorganisms such as plants. Upon the death of these organisms, the nitrogen present in proteins may be returned to ammonia via deamination processes.

Under aerobic conditions, ammonia may be oxidized to nitrite (NO_2^-) by speciallized nitrifying bacteria such as *Nitrosomonas*. Nitrite is then further oxidized to nitrate ($NO_3^=$) by other nitrifying bacteria, such as *Nitrobacter*. These combined nitrification processes are the basis of producing nitrate fertilizers from human and animal wastes, procedures that have been used by farmers for millennia. Once complete oxidation of nitrogen is accomplished, the nitrate may be assimilated by a variety of microorganisms and incorporated as NH_2 groups in proteins by either plants or microorganisms. Note that the assimilation into proteins involves the reduction of nitrate. This assimilative nitrate reduction production is a process carried out by a large number of microorganisms.

In addition to being available as a component in protein synthesis, the nitrate ion can function as an electron acceptor in bacterial metabolism. Under anaerobic conditions, nitrate is the most thermodynamically favored electron acceptor and is used by a number of facultatively anaerobic bacteria, including many *Pseudomonas* species. Nitrate reduction coupled to respirative energy production is referred to as *denitrification* to distinguish it from assimilative nitrate reduction. Denitrification occurs in a number of steps, each of which is catalyzed by different microorganisms, in the order $NO_3 \rightarrow NO_2 \rightarrow N_2O \rightarrow N_2$.

As was the case with the oxygen and carbon cycles, the nitrogen cycle is driven primarily by solar energy. The manner in which some microorganisms gain access to the solar energy, however, is interesting and very important for agricultural applications. Members of the bacterial genus *Rhizobium* have evolved a symbioic relationship with several plants, most notably legumes (soybeans and related crops). In symbiotic nitrogen fixation, *Rhizobium* infects the root hairs of the host plant, forming nodules. Within these nodules, the plant supplies sugars to the bacteria. This energy supply gives the bacteria the wherewithal to produce the large amounts of ATP needed to fix nitrogen. This fixed nitrogen is then made available to the plant, which uses it for protein synthesis and growth. It is this symbiotic relationship that is responsible for the relatively high protein content of soybeans and other legumes.

In some cases, the symbiotic relationship between the plant and the microorganism has reached the molecular level. For example, *Rhizobium* is an obligate aerobe, but the activity of nitrogenase is irreversibly destroyed by exposure to oxygen. A specialized protein called leghemoglobin controls access of oxygen to the nodule in order to allow respiration and nitrogen fixation to continue simultaneously. In order to construct leghemoglobin, *Rhizobium* carries the gene for one portion of the protein molecule, whereas the plant carries the gene for another portion. Thus, nitrogen fixation requires both organisms. This is a classic example

of true symbiosis, where each partner requires the other in order to carry out critical life functions.

9.3.1 Nitrogen Cycling in Ground-Water Systems

The behavior of nitrogen species in ground-water systems is strongly affected by three key aspects of the nitrogen cycle. The first of these is that fixation of organic nitrogen from N_2 requires a tremendous input of energy. In oligotrophic ground-water systems, this necessarily places limits on the rate and amount of nitrogen fixation that takes place. Second, the oxidation of organic nitrogen to nitrate (nitrification) takes place only under aerobic conditions. Third, denitrification takes place only under anaerobic conditions. These aspects ensure that nitrate accumulation in ground water requires oxygenated conditions and that nitrate depletion requires anoxic conditions.

In the case of the oxygen and carbon, we saw that the lack of solar energy input in ground-water systems effectively truncated the cycles, resulting in depletion of oxygen and accumulation of carbonate species. In the case of the nitrogen, however, the behavior differs depending on the oxygen status of the ground-water system. Under aerobic conditions nitrification may take place, but the alternate side of the cycle, denitrification, cannot take place. Thus, under aerobic conditions, the cycle truncates after nitrification and nitrate accumulates in solution. Under anaerobic conditions, the reverse is true—nitrate can be converted to N_2 via denitrification, but nitrification is blocked. This generally results in depletion of nitrogen species in anaerobic ground-water systems.

The occurrence of nitrate in ground water is a significant public health issue that has received considerable attention over the years. Excessive intake of nitrate in infants has been linked to methemoglobinemia, often referred to as the "blue baby syndrome," by depleting oxygen in the bloodstream. Furthermore, intake of nitrate has been linked to rates of mortality from gastric cancer. Because of these health concerns, the processes that influence nitrate and nitrogen species mobility in ground water have been closely studied.

As a practical matter, the cycling of nitrogen in ground-water systems is strongly influenced by human activities. Nitrogen fertilizers are widely used in agricultural practice, and the application of these often affects nitrogen cycling in particular systems. Similarly, organic nitrogen is present in a number of waste products, notably sewage effluents, and discharge of these impacts nitrogen cycling. It is convenient, therefore, when considering nitrogen cycling in ground-water systems to distinguish between different kinds of human impacts on ground-water systems. In human-impacted ground-water systems, the principal sources of nitrogen are (1) animal excrement and manure, (2) nitrogen fertilizers, and (3) sewage effluents.

Animal Excrement and Manures Many studies have documented that animal excrements can impact nitrate concentrations in local flow systems. For example, Robertson (1979) showed that nitrate concentrations in a shallow water table aquifer in Delaware tended to be associated with confined feeding operations of poultry or cattle. Furthermore, Robertson (1979) showed that there was a general decrease in nitrate concentrations with well depths.

These observations can be readily interpreted in the context of the nitrogen cycle. Animal manure contains organic nitrogen in the form of ammonia. Under the aerobic conditions at land surface, ammonia is converted to nitrate via nitrifying bacteria. This nitrate is soluble and readily leaches into the shallow ground-water aquifer. This aquifer tends to be aerobic near the water table surface and so nitrate accumulates in solution. However, in deeper portions of the aquifer, where anaerobic conditions are more common, nitrate concentrations decrease due to denitrification reactions. The accumulation of nitrate in this aquifer is therefore primarily dependent on the delivery of nitrate and the presence or absence of oxygen.

The relationship between the delivery of nitrate to local flow systems and the application of manure to fields has also been widely studied. In one such study in eastern Pennsylvania, Gerhart (1985) showed that concentrations of nitrate in a shallow aquifer mirrored recharge events. As water levels in the well rose, reflecting recharge from rainfall events, concentrations of nitrate increased significantly. In general, nitrate concentrations in the ground water were higher during periods of recharge than between periods of recharge.

This behavior reflects the two-step nature of nitrate delivery to shallow ground-water systems. First, the ammonia present in manure must be oxidized to nitrate by nitrifying bacteria under aerobic conditions, often at or near land surface. Second, nitrate produced by this reaction must be delivered to the ground-water system by means of vertically percolating recharge. If the aquifer being recharged is aerobic, nitrogen cycling truncates at this point and nitrate accumulates in ground water. If the aquifer grades from aerobic to anaerobic, as commonly occurs along aquifer flowpaths, the nitrogen cycle continues through denitrification and nitrate does not accumulate in solution. Again, the nitrogen cycle truncates at this point, this time because of the lack of nitrogen fixation.

Sewage Effluents Another important source of nitrogen to ground water in human-impacted local flow systems is by the discharge of sewage effluents. The ground water quality problems generated by sewage effluents have been widely studied worldwide. In the United States, one place where this problem has been studied particularly closely is on Long Island, New York.

Long Island is underlain by coastal plain sediments of the Magothy aquifer, which is in turn overlain by coarse-grained sediments of glacial origin. The coarse, organic matter–poor glacial sediments generally contain dissolved oxygen at or near atmospheric saturation. This allows nitrate to accumulate in solution. Much of Long Island has been dependent on septic drains for disposal of sewage effluent. This has, in some places, resulted in significant accumulations of nitrate in ground water.

The influence of septic systems on nitrate accumulations in ground water of Long Island was studied by Katz et al. (1980). Katz and his coworkers made a statistical comparison of nitrate concentrations in unsewered residential areas, where septic tanks were the predominant method of sewage disposal, and sewered areas where sewage was collected for treatment. Surprisingly, there was no significant difference found in mean nitrate concentrations between sewered and unsewered areas over a 15-year period. The data, however, did indicate some differences. For example, the unsewered areas were much more likely to have nitrate concen-

trations greater than 20 mg/L than the sewered areas. Furthermore, while there was little difference in nitrate concentrations, concentrations of ammonium were significantly lower in the sewered area relative to the unsewered area.

These differences can be interpreted in the context of nitrogen cycling in this ground-water system. For example, a major source of dissolved nitrogen from sewage is the deamination of organic matter to produce ammonia and ammonium ions. In the unsewered areas, therefore, a major portion the nitrogen cycle appears to be deamination followed by nitrification. In the sewered areas, in contrast, deamination was much less important. Katz et al. (1980) suggested that sewage effluents were a more important source of dissolved nitrogen in the unsewered areas than in sewered areas. Furthermore, it was suggested that the lack of significant nitrate differences between the two areas reflected applications of nitrate fertilizers.

Perhaps the most comprehensive study of microbial denitrification associated with sewage effluents in local flow systems was described by Smith and Duff (1988). These investigators measured rates of denitrification in a 4-km-long sewage effluent plume on Cape Cod, Massachusetts. Near the source of the effluent, nitrate concentrations exceeded 50 mg/L. As water in the plume moved downgradient, however, concentrations of nitrate decreased below detectable level.

Smith and Duff (1988) obtained samples of aquifer sediments along the hydrologic gradient and measured rates of denitrification using the acetylene block technique. In this technique the final step of denitrification, conversion of N_2O to N_2, is blocked by adding acetylene to sediment slurries. The accumulation of N_2O gas in the slurry vial thus gives an estimate of the overall rate of denitrification. Using this technique, Smith and Duff found that rates of denitrification decreased as nitrate concentrations decreased along the flowpath. Furthermore, rates of denitrification in the plume were greater than in the pristine portion of the aquifer. Denitrification of nitrate to N_2, rather than nitrate reduction to ammonia (Fig. 9.7), was the predominant reductive process in this system.

Perhaps the most interesting finding of this study was that denitrifying activity in the contaminated plume was not limited by the availability of nitrate. Rather, the limiting factor to denitrification rates was the availability of a suitable organic carbon substrate. In anaerobic aquifers in which measurable concentrations of nitrate persist for any distance along the flowpath, denitrification must be limited by factors other than nitrate availability. In such cases, carbon limitation is often responsible for the observed persistence of nitrate.

Nitrogen Fertilizers Nitrogen fertilizers are a significant source of nitrate contamination of ground water. Nitrogen fertilizers may be composed of a number of nitrogen compounds, including anhydrous NH_3, NH_4NO_3, $(NH_4)2HP_2O_5$, and urea. In general, nitrogen delivered to the soil as NH_3 or NH_4^+ is retained better than nitrogen delivered as nitrate. Ammonium, being a positively charged ion, tends to adsorb to the negatively charged silicate minerals present in soils. The negatively charged nitrate ion, however, is repelled from mineral-grain surfaces and moves freely with percolating pore water, effectively leaching it from the soil.

The impacts of agricultural nitrogen applications on the chemical composition of ground water has been extensively studied. In the United Kingdom, agricultural nitrogen applications to fields overlying a chalk aquifer have been studied by Gray and Morgan-Jones (1980). This study documented the accumulation of nitrate in

three well fields tapping the chalk aquifer over a 40-year period. One of these well fields in particular showed a close correlation between agricultural activity and nitrate concentrations. In the early 1940s, when fertilizer applications were lowered because of World War II, concentrations of nitrate decreased. However, over the next 30 years, as agricultural activity progressively increased, concentrations of nitrate in the ground water increased dramatically. These accumulations reflected the familiar pattern of nitrification in the soil zone, followed by nitrate leaching into and accumulating in an aerobic local flow system.

Nitrate contamination of ground water due to fertilization practices is an important water-quality issue in the midwestern United States. Corn, an important crop in the Midwest, requires large amounts of nitrogen for yields to be economically viable. It is common practice for corn growers to apply as much as 180 lbs N/acre/yr in order to obtain sufficient yields. Nitrogen applied in this manner is rapidly depleted from soil both by plant uptake and by nitrification followed by leaching of nitrate into ground water.

In one study of the impacts of such practices on ground-water quality, Spalding et al. (1978) showed that zones of nitrate contamination in ground water were closely correlated with coarse-textured, well-drained soils. This reflected rates of nitrification, rates of leaching, and the occurrence of oxygenated conditions in the water-table aquifer. This general pattern has been observed in many locations and is characteristic of nitrate contamination of ground water.

Municipal Wastes In addition to animal excrements, sewage effluents, and agricultural fertilizers, municipal wastes that have been disposed in landfills may be important sources of nitrogen contamination to ground water. An example of such nitrogen contamination is given by Baedecker and Back (1979) in describing a municipal landfill in Delaware. The source of the nitrogen species in the ground water was organic nitrogen in the refuse. Near the landfill, ground water was anaerobic and most of the nitrogen was present as reduced organic nitrogen and ammonium. However, as the contaminated water moved downgradient and mixed with the ambient oxygenated ground water, concentrations of reduced nitrogen decreased and concentrations of nitrate increased. Baedecker and Back (1979) described this behavior using a nitrogen index, defined as the ratio of reduced nitrogen species (determined by Kjeldahl digestion) to nitrate. As ground water became progressively more oxygenated, the nitrogen index decreased. This reflected the progressive nitrification of reduced nitrogen with the production of nitrate.

Since anaerobic conditions are more likely in and near landfills of municipal waste, the pattern described by Baedecker and Back (1979) is probably the most common. However, patterns of nitrogen cycling are very sensitive to the availability of oxygen, and other patterns may occur. A very different pattern of nitrogen cycling was described near a municipal landfill and waste-water holding cells in North Dakota (Bulger et al., 1989). This study showed that nitrate was the primary nitrogen species emanating from the landfill under oxidizing conditions. However, once the nitrate plume intersected a plume of high dissolved organic carbon, which was anaerobic, the nitrate was progressively reduced to NH_4^+. This pattern indicates that denitrification, with the conversion of NO_3 to N_2, is not the only pathway of nitrate reduction found in ground-water systems. In this case, nitrate is apparently reduced to NH_4^+ as the plume becomes anaerobic.

Distinguishing Sources of Nitrogen Contamination Because of the numerous sources of nitrogen contamination in ground-water systems, distinguishing whether contamination originated from agricultural fertilizers, animal wastes, or sewage effluents is often difficult. One method for distinguishing different sources of nitrogen contamination that has been widely applied is the use of stable nitrogen isotopes.

Nitrogen occurs as two stable isotopes, ^{15}N and ^{14}N. Ratios of these isotopes are commonly referred to a standard and reported as $\delta^{15}N$. Ranges of $\delta^{15}N$ for nitrogen from different sources are shown in Figure 9.8. Clearly, there is a wide difference between nitrogen present in fertilizers (−4 to +2 per mil) and nitrogen present in animal (and human) wastes (+9 to +18 per mil. All things being equal,

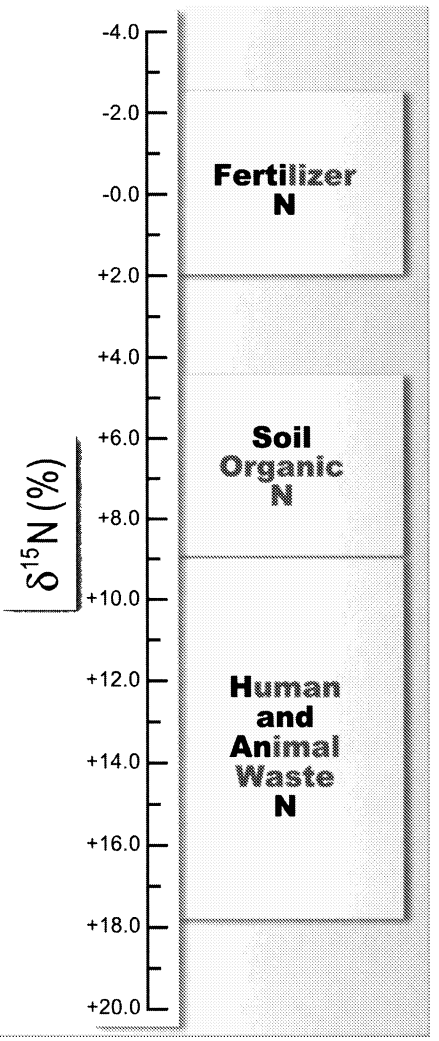


Figure 9.8. Range of nitrogen isotopes characteristic of different sources.

therefore, it should be possible to distinguish fertilizer nitrogen from animal waste nitrogen based solely on $\delta^{15}N$.

However, nitrogen isotopes are subject to a number of fractionation processes in the nitrogen cycle. For example, some ammonia nitrogen applied to soils is subsequently volatilized, resulting in enrichment in ^{15}N. In addition, denitrification also results in a ^{15}N enrichment as microorganisms tend to discriminate against the heavier isotope. These twin processes tend to enrich nitrate with ^{15}N after fertilizer application. For example, Gormly and Spalding (1979) showed that there is an inverse correlation between $\delta^{15}N$ and nitrate concentrations in ground water. This reflects the fact that ground water with lower concentrations of nitrate had undergone more denitrification than the high-nitrate water. However, volatilization and denitrification do not appear to increase $\delta^{15}N$ values above +10 per mil. As such, nitrogen isotopes may be used to distinguish between animal waste nitrogen contamination and fertilizer nitrogen contamination.

This technique has been applied on Long Island, New York, in order to distinguish between animal waste contamination and fertilizer contamination. For example, Flipse and Bonner (1985) showed that the del-15N values of nitrate under a heavily fertilized potato field and under a golf course were in the range of +6.5 to +6.2. While these values were significantly heavier than the fertilizers being applied (−.6 to +.2), they were lighter than the +10 values characteristic of animal wastes. Thus, while fractionation processes altered the isotopic signatures, the technique was still useful for distinguishing between fertilizer contamination and animal waste contamination.

Nitrate Accumulation Due to Dry-Land Farming Practices While nitrogen species in ground water are commonly associated with contamination events, natural processes also contribute to delivery of nitrogen to ground water. In the soil zone, organic nitrogen produced by microbial nitrogen fixation is cycled through deamination, nitrification, and denitrification processes. Because organic nitrogen is often a limiting nutrient for both plant and microbe growth, it is unusual for ground water in pristine local flow systems to accumulate more than 1 or 2 mg/L dissolved nitrate. However, under some conditions, natural processes can contribute substantial nitrate concentrations to ground water.

One example of this was given by Kreitler and Jones (1975). In 1968, several cattle in Runnels County, Texas, died of anoxia from drinking water produced from the water table aquifer. Upon analysis of the water, it was found to contain between 100 and 1,000 mg/L nitrate. This was surprising in that the use of nitrogen fertilizers for agriculture was minimal. What could be the source of the observed nitrate?

One possibility was that animal wastes were the principal source. In order to test this possibility, Kreitler and Jones (1975) examined the $\delta^{15}N$ of nitrate in the ground water. Surprisingly, the observed values were generally less and +10 per mil, indicating that animal wastes were not the principal source. These values, however, did generally match values of soil nitrogen that was present naturally. Prior to 1900, this area was covered with buffalo grass, the roots of which form a symbiotic relationship with nitrogen-fixing bacteria much like legumes. These soils, therefore, were naturally high in reduced nitrogen compounds. The advent of dryland farming practices after 1900, particularly with increased oxygen delivery to

the newly plowed soils, resulted in the oxidation of soil nitrogen and subsequent leaching to the water table. This leaching was greatly accelerated by terracing the fields in order to capture and retain more moisture. The intriguing result, therefore, was the production of a nitrate contamination problem without human or animal application of nitrogen.

This is an excellent example of why considering elemental cycling processes are important to understand some ground-water chemistry problems. The accumulation of biochemically active elements in ground water always reflects the perturbation or truncation of one or more elemental cycles. These may be due to natural causes, such as nitrate accumulating due to truncation of the nitrogen cycle in an aerobic aquifer. On the other hand, the perturbation may reflect human influences, such as adding a nitrogen source (fertilizer) where no source existed before. In either case, the key to understanding the observed phenomenon is to consider the nature of the nitrogen cycle.

9.4 THE IRON CYCLE

Iron in the environment exists predominantly in either the reduced ferrous (Fe(II)) or oxidized ferric (Fe(III)) form. Ferrous iron is relatively soluble in water and is therefore quite mobile. Ferric iron, on the other hand, tends to form insoluble Fe(III) oxyhydroxides and is therefore relatively immobile. The iron cycle in most environments involves alternate reduction (mobilization) of ferric iron followed by oxidation (immobilization) of ferrous iron. Most of these reactions are microbially catalyzed under natural conditions.

A schematic diagram of the iron cycle is shown in Figure 9.9. Under anaerobic conditions, Fe(III) oxyhydroxides are reduced by a variety of microorganisms that use Fe(III) as a terminal electron acceptor. Curiously, the role of microorganisms in iron reduction has only recently been extensively studied. Prior to 1985, most geochemists and microbiologists considered Fe(III) reduction to be an abiological reaction initiated by "reducing conditions." However, with the isolation and characterization of Fe(III) reducing microorganisms in the late 1980s it became clear that this important component of the iron cycle is largely mediated by microbial processes (Lovley et al., 1991). Several microorganisms, including *Geobacter* and *Shewanella putrifaciens,* carry out this metabolic function.

Much of the Fe(II) generated by Fe(III) reduction remains sediment-bound, being sequestered in Fe(II)-bearing minerals such as illite or magnetite. A small percentage (1–5%) of the Fe(II) accumulates in solution, however, and moves in response to diffusion gradients or with flowing water. If dissolved Fe(II) is transported to an aerobic environment, it is subsequently oxidized to Fe(III) oxyhydroxides. This oxidation is highly excergonic and can take place spontaneously without microbial mediation. However, several types of microorganisms have evolved mechanisms to harness this available energy. These include representatives of the genera *Gallionella, Thiobacillus,* and *Leptothrix*. Because spontaneous oxidation of Fe(II) occurs much less rapidly at acidic pH, many of these microorganisms are specifically adapted to acidic environments. *Thiobacillus,* for example, has been widely described in streams impacted by acid-mine drainage. There is relatively little energy to be had from oxidizing Fe(II), and consequently, Fe(II)-

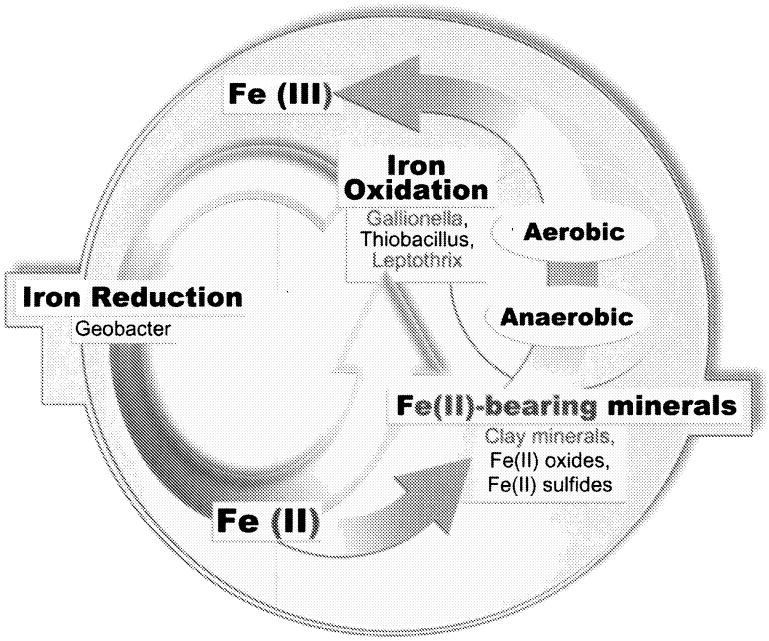


Figure 9.9. The iron cycle.

oxidizing microorganisms need to process large amounts of Fe(II)—and thus produce large amounts of Fe(III) oxyhyroxides—in order to provide enough energy for growth and reproduction.

The microbially mediated mobilization of Fe(II) and subsequent oxidation to Fe(III) is responsible for many economically important accumulations of iron ore. Bog iron ores, for example, occur widely at the margins of swamps and bogs throughout the world. These accumulations of Fe(III) oxyhydroxides result from the mobilization of Fe(II) within the bog, migration of Fe(II) to the aerobic margins of the bog, and subsequent precipitation. Historically, bog iron ores were extremely important in the development of iron technology. The fact that iron ores existed in close proximity to peat, a ready source of fuel, was the basis for many early iron-working technologies such as those in England and Ireland.

9.4.1 Iron Cycling in Aquatic Sediments

Iron is required as a nutrient by most organisms, including microorganisms, plants, and animals. Iron is a critical component of many enzyme systems involved in metabolism and energy storage. For these uses, however, iron is generally needed in only trace quantities. The great importance of iron cycling in aquatic sediments is related more to the availability of phosphorus and other trace elements than to the availability of iron.

Fe(III) oxyhydroxides are very efficient at adsorbing a variety of chemical species, including trace metals and phosphate ions. The growth of photosynthetic algae and cyanobacteria, which form the base of the food chain in many aquatic systems,

are typically limited by the availability of phosphate. When decaying organic matter accumulates at the bottom of an aquatic environment, a considerable amount of the phosphate released is promptly adsorbed by Fe(III) oxyhydroxides in the sediment and thereby removed from the food chain. In the aerobic portion of the aquatic sediment, Fe(III) oxyhydroxides are stable and phosphate ions remain adsorbed. However, when the sediments become anaerobic, Fe(III) oxyhydroxides are reduced with the remobilization of adsorbed phosphate. Fe(II), phosphate, and other previously adsorbed trace elements diffuse into the aerobic zone, and a portion of the phosphate diffuses back into the water column. The cycling of iron between the oxidized and reduced forms provides a mechanism for returning phosphate to the food chain.

While Fe(II) is much more soluble than Fe(III) in aquatic sediments, much of the Fe(II) produced by iron reduction does not enter the aqueous phase. Rather, a considerable portion, perhaps as much as 98%, is sequestered in the form of iron-rich clays such as illite, Fe(II)-bearing oxides such as magnetite or ilmenite, or Fe(II) sulfides such as pyrite. In these forms, iron can be removed from biological iron cycling for long periods of time. However, upon reexposure to aerobic conditions, Fe(II)-bearing minerals are rapidly oxidized with or without microbial mediation, and the iron cycled back to Fe(III) (Fig. 9.9).

9.4.2 Iron Cycling in Ground-Water Systems

In terms of worldwide economic impact, the biological cycling of iron is one of the most important processes in ground-water systems. High concentrations of dissolved iron (Fe(II)) is by far the most common water-quality problem associated with ground water. While dissolved iron is not toxic to humans or animals, the red staining of clothing and plumbing fixtures that it causes is highly undesirable. In addition, iron imparts an unpleasant taste to water, which, while harmless, gives the impression that it is somehow contaminated. Finally, the oxidation of high-iron water as it is applied to cultivated fields can lead to low-pH ferric hydroxide-rich soils that may severely damage agricultural productivity. Treating ground water to remove Fe(II) from municipal, agricultural, and domestic wells is a multimillion-dollar a year business throughout the world.

It has long been recognized that microorganisms play an important role in the oxidation of Fe(II) associated with ground water. However, the role of microorganisms in the other side of the iron cycle, that of Fe(III) reduction, has only recently been worked out. As discussed previously, the first microorganism known to carry out this function, *Geobacter metallireducens,* was isolated in 1987 (Lovley and Phillips, 1988). Two years latter, similar techniques were used to isolate and characterize an Fe(III)-reducing microorganism from a ground-water environment (Lovley et al., 1989). This strain, informally named "172" (the microorganism was isolated from a depth of 172 feet), could perform many of the same biochemical functions as *Geobacter.* However, "172" was clearly a different microorganism. Morphologically, "172" was a short blunt rod, while GS-15 is an elongated rod. Furthermore, the two microorganisms exhibited different temperature optima for growth. Subsequent genetic studies have shown the "172" is a separate species of *Geobacter* that was designated "*Geobacter Chapelleii*" (Fig. 5.14).

The isolation of *Geobacter Chapelleii* was fortuitous, and the manner that it came about has implications for the cycling of iron in subsurface environments.

The upper 150 feet of sediment in the core hole from which *G. Chapelleii* was isolated was predominantly sand of a uniform reddish-brown color, reflecting the presence of ferric oxyhydroxides. Nearby wells screened in these brown sands produced ground water containing 2–6 mg/L of dissolved oxygen and little dissolved iron. Below about 180 feet the sediment remained predominantly sand but was a grayish-white color, reflecting the lack of ferric oxyhydroxides. Wells screened in these gray sands produced anaerobic water that often contained between 0.3 and 1.0 mg/L of dissolved iron. The cored interval between 170 and 180 feet was neither uniformly reddish brown nor grayish white. Rather, it was a mottled mixture of red and white that also contained streaks of purple sediment. These purple streaks appeared to be zones where dissolved Fe(II) had been oxidized from solution and concentrated in the sediment. In addition, this mottled interval clearly occurred at the interface between the aerobic and anaerobic zones of this aquifer system.

As Fe(III)-reducing microorganisms had never been recovered from subsurface environments before, there were no precedents available for determining which horizons to sample. Luckily, however, a sample was taken at 172 feet that turned out to contain Fe(III)-reducing microorganisms. Samples in the overlying aerobic zone and in the underlying anaerobic but Fe(III)-lacking sediments did not yield viable Fe(III) reducers. Apparently, the "172" microorganism required both anaerobic conditions and the presence of grain-coating Fe(III) oxyhydroxides in order to maintain viability. The presence of purple Fe(III) precipitates implied that Fe(III) reduction and Fe(II) oxidation occurred in close proximity to each other. Interestingly, strain *G. Chapelleii* exhibited much more tolerance to oxygen (although it would not reduce Fe(III) in the presence of oxygen) in laboratory studies than did the strict anaerobe *Geobacter metallireducens*.

These observations suggest that the mottled zone is actively involved in iron cycling on a small scale. Under anaerobic conditions, grain-coating Fe(III) oxyhydroxides are reduced and Fe(II) mobilized. Some of this Fe(II) evidently enters the anaerobic portion of the ground-water system and remains in solution. This would explain the presence of high-iron concentrations in the deeper wells. Evidently, however, some of the Fe(II) comes into contact with dissolved oxygen and is reprecipitated as Fe(III).

Wells and Iron-Oxidizing Bacteria If one were to query a roomful of well field operators as to what was the single most important microbial problem that they face, the answer would overwhelmingly be "iron bacteria." When used in this context, the term "iron bacteria" refers to those species of aerobic bacteria that oxidize ferrous iron as a source of energy.

There are several reasons why iron-oxidizing bacteria are so troublesome for well operators, and these are related to their physiology. For one thing, aerobic oxidation of Fe(II) yields a relatively small amount of energy. For the reaction

$$Fe^{2+} + H^{+} + 1/4\ O_2 \rightarrow Fe^{3+} + 1/2\ H_2O$$

only about 40 kcal of free energy is available per mole of reactant. In contrast, complete oxidation of hydrogen sulfide yields about 160 kcal. So in order for iron oxidizers to maintain their cellular structure and accumulate enough energy for

reproduction, they must oxidize a large amount of Fe(II). Fe(III) is relatively insoluble and tends to form oxides and hydroxides

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+$$

The combination of iron oxidizers producing an insoluble waste product and their tendency to produce a lot of it, tends to clog wells and water lines very efficiently.

In addition to these physiological properties, the ecological niche filled by iron oxidizers works against well field operators. Iron oxidizers are adapted to living at the interface of anaerobic environments, where dissolved Fe(II) is available, and aerobic environments, where dissolved oxygen is available. When a well is drilled into and anaerobic aquifer, an aerobic-anaerobic interface is automatically created. This, in turn, creates the perfect environment for iron oxidation.

Examples of the havoc that iron oxidizers can wreak on a municipal well system are numerous. One particularly interesting case occurred in Florence, South Carolina. The ground water, which was pumped from the Middendorf and Cape Fear aquifers, generally contains 1 or 2 mg/L of Fe(II). However, because the oil-lubricated turbine well pumps used to pump the water tended to minimize introduction of oxygen from air, for years iron-oxidizing bacteria were only a minor nuisance. However, in the 1980s, the state environmental agency decreed that oil-lubricated pumps could no longer be used (they often leaked oil into wells), and instead, water-lubricated pumps were to be used. When not in operation, these pumps were lubricated by a constant stream of tap water running down the well. This, of course, injected a constant stream of oxygen into an anaerobic aquifer—the perfect environment for iron-oxidizing bacteria. The predictable result was a series of massive well-clogging problems. These problems were solved only when the city gave up on water-lubricated turbine pumps and installed submersible pumps.

Well clogging is not the most insidious aspect of iron-oxidizing bacteria, however. In pipes containing oxygenated water, iron oxidizers may form colonies. The inside of the colonies are typically anaerobic. The difference of electrical potential between the aerobic and anaerobic zones encourages the flow of electrons, which in turn corrodes the iron pipe. This kind of cathodic corrosion, mediated by iron-oxidizing bacteria, is the source of millions of dollars' worth of damage per year. Well operators go to extraordinary lengths to minimize this damage. Periodic chlorination of well pipes, or other antibacterial measures, are the most common damage-control methods.

High-Iron Concentrations in Ground Water By far the most economically important aspect of iron cycling in ground-water systems is the production of high iron concentrations in ground water. It is often observed that high-iron ground water occurs in distinct zones (Langmuir, 1969; Back and Barnes, 1965). As was the case with other biologically active elements, the accumulation of dissolved iron in ground water represents a truncation of the iron cycle.

When Fe(III) reduction occurs at the interface of an aerobic and anaerobic zone, such as in aquatic sediments, there is the possibility that some Fe(II) produced will be cycled back to Fe(III) oxyhydroxides. In many aquifers, however, Fe(III) re-

duction occurs where molecular oxygen is absent and there is no possibility of reoxidation. In these cases, dissolved Fe(II) may accumulate in solution, causing high-iron concentrations in ground water. The truncation of the iron cycle due to the lack of iron reoxidation is an important mechanism leading to the accumulation of iron in ground water.

9.5 THE SULFUR CYCLE

Sulfur occurs in three common oxidation states in nature. These are the -2 state, in which sulfur occurs as sulfides ($S^{=}$); the 0 state, in which sulfur occurs as elemental sulfur (S^{o}); and the $+4$ state, where sulfur occurs as sulfate ions ($SO_4^{=}$). As in the case of carbon, nitrogen, and iron, this variety of oxidation states allows chemical energy to alternately be stored and released. The series of sulfur-based energy-storage and energy-releasing processes is known as the sulfur cycle.

The major microbially mediated processes involved in the sulfur cycle are shown schematically in Figure 9.10. Sulfur in the -2 state is an integral component of many proteins. Most microorganisms are therefore capable of reducing sulfate to sulfide in order to supply the necessary reduced sulfur. This process is termed assimilatory sulfate reduction. Interestingly, assimilatory sulfate reduction is carried

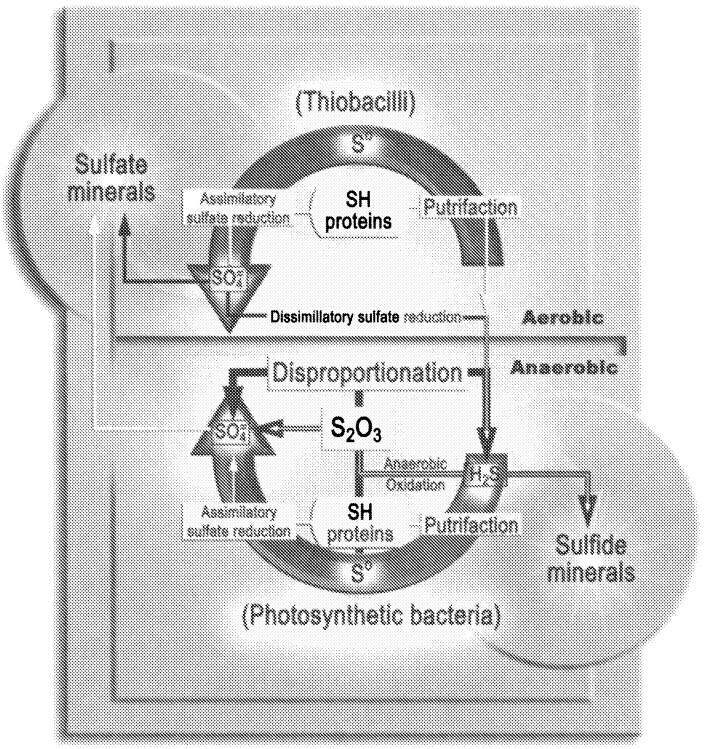


Figure 9.10. The sulfur cycle.

out under both aerobic and anaerobic conditions. The reduced sulfur component of proteins is returned to the sulfur cycle by means of putrefaction, in which sulfur is released from decaying tissue in the form of hydrogen sulfide (H_2S).

Hydrogen sulfide is also produced in anaerobic environments by means of dissimilatory sulfate reduction. In this process, sulfate acts as the terminal electron acceptor in the oxidation of organic carbon or elemental hydrogen. Dissimilatory sulfate reduction is extremely important in the global cycling of carbon. In marine and estuarine environments, sulfate reduction is often the most important process leading to the oxidation of plant debris and the consequent production of carbon dioxide.

The fate of hydrogen sulfides produced by assimilatory and dissimilatory sulfate reduction is extremely complex. Some portion of the H_2S reacts with metals, especially iron, in order to form sulfide minerals. Such mineral formation temporarily removes sulfur from the cycling process. Some of the sulfide is cycled back to sulfate as it diffuses into aerobic zones. The oxidation of H_2S to sulfate under aerobic conditions is mediated by a number of microorganisms, notably *Thiobacillus. Thiobacillus* is capable of using H_2S as its sole energy source during growth and reproduction.

It has recently been shown that much H_2S produced under anaerobic conditions is also reoxidized anaerobically (Jorgensen, 1990). The anaerobic oxidation of sulfide appears to be coupled to the reduction of Fe(III) compounds present in the sediments, and thiosulfate ($S_2O_3^{=}$), a partially oxidized intermediate compound, is produced. Thiosulfate is then disproportioned to either sulfate or sulfide according to the stoichiometry

$$S_2O_3^{=} + H_2O \rightarrow SO_4^{=} + HS^{-} + H^{+}$$

The term "disproportioned" refers to the fact that the thiosulfate anion contains a sulfur atom in the oxidized (+4) state and one in the reduced (−2) state. Thus, the decomposition of thiosulfate to sulfate and sulfide does not involve an oxidation or a reduction step. This "thiosulfate shunt" for the anaerobic oxidation of sulfide appears to be a significant process for the cycling of sulfur in aquatic sediments.

The thiosulfate shunt is not the only pathway by which sulfides may be anaerobically oxidized. Most photosynthetic cyanobacteria and algae use H_2O as an electron donor, with the consequent production of molecular oxygen (O_2). The anaerobic photosynthetic sulfur bacteria, which includes the green and purple sulfur bacteria, use H_2S as an electron donor. The oxidation of H_2S by these microorganisms is another anaerobic mechanism by which reduced sulfur is cycled back to sulfate.

In the same manner that reduced sulfur can be sequestered and temporarily removed from the sulfur cycle by precipitation of sulfide minerals, sulfate may also form mineral deposits. Because of the relatively high solubility of most sulfate minerals, significant sulfate mineralization occurs only in evaporative basins. However, the precipitation of sulfate minerals in these hot, dry environments is responsible for the temporary removal of much sulfate from the sulfur cycle. The exposure and weathering of both sulfate and sulfide minerals during tectonic events eventually returns most of this sequestered material to the sulfur cycle.

9.5.1 Sulfur Cycling in Ground-Water Systems

The behavior of sulfur in ground-water systems depends in large part on the presence or absence of sulfur-bearing minerals. Many clastic aquifers lack sulfate minerals such as gypsum or anhydrite. In these cases, sulfur cycling reflects reactions involving sulfide minerals and pools of dissolved sulfate available in pore water. As was the case with oxygen, carbon, and nitrogen, the behavior of sulfur in ground-water systems often reflects the truncation of the cycle due to lack of energy input. However, in the case of sulfur, the processes leading to these truncations are more varied.

Sulfide Oxidation in an Oxygenated Aquifer An example of sulfur accumulation due to cycle truncation was given by Chapelle and Kean (1985). In the outcrop area of the Patuxent aquifer near Baltimore, Maryland, ground water is oxygenated (Fig. 9.3). This aquifer, however, contains pyrite as a minor constituent, and the oxidation of pyrite has two apparent effects as illustrated by the overall stoichiometry

$$FeS_2 + 15/4\ O_2 + 7/2\ H_2O \rightarrow Fe(OH)_3 + 4H^+ + 2\ SO_4^=$$

First, concentrations of sulfate increase along aquifer flowpaths. Second, the pH of the ground water decreases due to the release of H+ ions. Thus, when the sulfur cycle is truncated after sulfide oxidation, the accumulation of sulfate in solution is observed in some systems.

In the Patuxent aquifer, the sulfur cycle truncates after sulfide oxidation because of interaction with the iron cycle. After dissolved oxygen is consumed, iron reduction becomes the predominant microbial process as indicated by the rapid increase in iron concentrations. Because Fe(III) reduction precludes active sulfate reduction, sulfate is not immediately recycled and sulfate accumulates in solution. One would expect, however, that once Fe(III) reduction ceased, sulfate reduction activity would increase and the sulfur cycle would continue. This interplay between the iron and sulfur cycles occurs widely in ground-water systems and is one more mechanism leading to the (temporary) truncation of the sulfur cycle.

Sulfate Reduction in Sulfate Mineral-Free Aquifers A wide variety of aquifer systems lack significant mineral sources of sulfate. Clastic sediments deposited in humid or temperate fluvial and marine environments generally lack such mineralization. Despite the lack of a mineral source of sulfate, however, sulfur cycling is often important to the water chemistry of these ground-water systems.

The presence or absence of sulfate reduction in clastic coastal plain aquifers is one of the oldest debates in ground-water chemistry. Cedarstrom (1946) proposed that sulfate reduction was the primary oxidation process driving the accumulation of bicarbonate in coastal-plain aquifer ground water. As Foster (1950) correctly pointed out, however, there were no observed changes in sulfate concentrations along aquifer flowpaths. In both recharge and discharge areas, sulfate concentrations remained in the 10–20 mg/L range. There was a brief heated debate between

supporters of each position in the early 1950s, and then, due largely to the lack of any new ideas for resolving the issue, interest in the problem waned.

As often happens in science, however, new data from an unexpected source cast the dilemma in a new light. In 1984, as part of the U.S. Geological Survey's RASA (Regional Aquifer Systems Analysis) program, an effort was made to more accurately locate the salt water–fresh water interface in the coastal plain of the eastern United States. As finishing and developing wells was very expensive, the hydrologist in charge of the project, Harold Meisler, decided to squeeze cored sediments and analyze the pore water for chlorides and other ions. As it turned out, the analytical procedure—ion chromatography—was capable of quantifying sulfate together with chloride, and so sulfate was analyzed as well.

The results (Trapp et al., 1984) were puzzling. While the aquifers contained relatively low concentrations of sulfate (20–30 mg/L), the confining beds contained relatively high concentrations (200–600 mg/L). This suggested that diffusion of sulfate from confining beds to aquifers was possible. Could this be the source of sulfate for sulfate reduction? The data collected by Meisler were intriguing, but by themselves were not conclusive. Beginning in 1987, a more comprehensive study of confining bed pore water chemistry was conducted in the New Jersey Coastal Plain by Pucci and Owens (1989). These authors confirmed the presence of relatively high concentrations of sulfate in some but not all confining beds. Specifically, confining beds of marine origin contained up to 600 mg/L of dissolved sulfate, whereas confining beds of nonmarine origin contained low concentrations (<50 mg/L). Similar results were subsequently observed by Chapelle and Lovley (1990) in coastal plain sediments of South Carolina.

By now it was clear that coastal plain sediments contained a previously unknown pool of sulfate that was present in the fine-grained confining beds. Now the question was how was this sulfate involved in sulfur cycling in these ground-water systems? This question was addressed by Chapelle and McMahon (1991), who repeated the observation that confining beds contained higher concentrations of sulfate than did aquifers. In addition, it was shown that while viable sulfate-reducing bacteria were readily recovered from aquifer sediments using culture techniques, such bacteria were not recovered from confining bed sediments. Evidently, sulfate-reducing activity was depressed in confining beds relative to aquifers, possibly explaining the observed higher concentrations of sulfate. More important, it was shown that the diffusive flux of sulfate into the aquifers implied by the concentration differences balanced the observed rate of microbial CO_2 production in this system. Forty-five years after Cedarstrom (1946) proposed that sulfate reduction accounted for the observed CO_2 production in these coastal plain aquifers, the mechanisms involved in the process were finally worked out.

One unresolved issue is the source of the observed sulfate. Because pore-water concentrations of chloride were relatively low (~20 mg/L), it did not seem to be residual seawater. In any case, if diffusion were ongoing in this system, seawater sulfate would be exhausted within just a few million years. Pucci and Owens (1989) pointed out that the confining beds contained large amounts of sulfide minerals that could be oxidized by Fe(III)-bearing minerals. This process of slow oxidation could maintain sulfate concentrations as long as Fe(III) minerals were present. Interestingly, Jorgensen (1990) suggested that Fe(III) oxidation was an important mechanism in the anaerobic oxidation of sulfides generated by sulfate reduction in aquatic

sediments. While the ultimate source of sulfate in confining beds is presently still unknown, it is possible that it is an important component of the sulfur cycle in some aquifer systems.

The essential features of sulfur cycling in sulfate mineral-free, coastal-plain aquifers involves a complex interplay between aquifers and their confining beds (Chapelle and McMahon, 1991). Confining beds contain relatively high concentrations of sulfate but lack substantial sulfate-reducing activity. In the aquifers, on the other hand, there is active sulfate-reduction. The concentration gradient that develops allows sulfate to diffuse to aquifers. Thus, even though there is ongoing sulfate reduction in the aquifers, there is little overall change in sulfate concentrations along aquifer flowpaths.

Sulfides produced by sulfate reduction are rapidly immobilized by reaction with Fe(III)-bearing minerals, particularly glauconite, and so there is little accumulation of dissolved sulfides. The overall result is the net transport of sulfur from fine-grained confining beds to coarse-grained aquifers and the accumulation of reduced metal sulfides in the aquifers. Concurrently, there is a net accumulation of dissolved inorganic carbon in the aquifers.

Sulfate Reduction in Sulfate Mineral-Bearing Aquifers Many aquifer systems contain a mineral source of sulfate, and this markedly affects observed patterns of sulfur cycling. One of the best-documented examples of sulfur cycling in this type of aquifer is the Floridan aquifer of central Florida (Rightmire et al., 1974; Plummer, 1977).

The Floridan aquifer consists of limestones and dolostones of the Avon Park, Ocala, Suwannee, and Tampa Formations. Some of these limestones were deposited under shallow marine conditions in a hot, arid climate. These conditions resulted in the accumulation of significant amounts of gypsum during carbonate deposition, particularly in the Avon Park Formation. This gypsum, in turn, is a ready source of sulfate to ground water associated with the Floridan aquifer.

This distinctive mineralogy is reflected in the water chemistry of the aquifer. As one would expect, dissolution of dolomite and gypsum results in increasing concentrations of calcium, magnesium, bicarbonate, and sulfate. In the recharge areas of the Floridan, the aquifer is oxygenated and sulfate reduction is not an important process. Downgradient, however, the aquifer becomes anaerobic and sulfate reduction becomes the predominant microbial process. This is evident from the relatively high concentrations of hydrogen sulfide present in the ground water (~1–2 mg/L). It is not evident, however, from any change in sulfate concentration along the flowpath. This occurs because the flux of sulfate to the aquifer via gypsum dissolution (1.970 mmol of gypsum per liter of ground water between Wauchula and Arcadia) is much greater than the rate of sulfate reduction (0.225 mmol sulfate reduced per liter of water).

A better indicator for the presence of active sulfate reduction in this aquifer system is the isotopic composition of dissolved sulfate. Gypsum that has precipitated from seawater typically has a $\delta^{34}S$ of about +22 per mil. Maritime rainfall, on the other hand, has a $\delta^{34}S$ of about +10 per mil. Thus, if the only processes affecting the isotopic composition of sulfate in this system were input of sulfate from rainwater and from dissolution of gypsum, $\delta^{34}S$ values would track along a mixing curve (Rightmire et al., 1974). In the upgradient aerobic portion of the

aquifer where sulfides are absent, observed values of $\delta^{34}S$ of the sulfate did scatter in the vicinity of this mixing curve. In the anaerobic downgradient portion of the aquifer, where the water contains sulfides, the $\delta^{34}S$ values were consistently more positive than predicted by the mixing curve. Rightmire et al. (1974) interpreted this as reflecting isotopic fractionation during sulfate reduction. Sulfate-reducing microorganisms actively discriminate against the heavier stable isotope of sulfur (^{34}S), preferring the more reactive light isotope (^{32}S). Thus, sulfate reduction occurring along the aquifer flowpath will actively enrich the available pool of sulfate in ^{34}S, resulting in more positive $\delta^{34}S$ values.

The essential features of sulfur cycling in the Floridan aquifer therefore include production of dissolved sulfate via dissolution of gypsum. In the aerobic portion of the aquifer, sulfate accumulates without significant sulfate reduction. In the anaerobic portion of the aquifer, sulfate reduction becomes an important process. However, because the rate of gypsum dissolution outpaces the rate of sulfate reduction by at least an order of magnitude, this process is not evident from concentration changes of sulfate. Sulfate reduction is evident, however, from the accumulation of dissolve sulfides and by the observed fractionation of sulfate stable isotopes.

From a water quality standpoint, one important way that sulfur cycling in the carbonate Floridan aquifer differs from sulfur cycling in the clastic Black Creek aquifer is in the availability of iron. In the Black Creek aquifer, iron is present in a number of mineral phases and is available in excess to react with and precipitate pyrite. There is therefore no observed accumulation of dissolved hydrogen sulfide, which imparts the characteristic "rotten egg" smell, in Black Creek aquifer water. In the carbonate Floridan aquifer, where clastic iron-bearing minerals are much less abundant, sulfide production outpaces pyrite formation and significant quantities of sulfide accumulate in the water. Thus, even though sulfate reduction dominates sulfur cycling in both systems, the sulfide water quality problem associated with it is present only in the Floridan aquifer.

9.6 CHAPTER SUMMARY

Biologically active elements, oxygen, hydrogen, carbon, nitrogen, iron, and sulfur, are cycled continuously between oxidized and reduced states in the biosphere. These cycles are driven by the input of energy from the sun. Because subsurface environments are isolated from solar energy, many elemental cycles are truncated. In some cases, such as the oxygen cycle, this truncation results in the depletion of oxygen in ground water. In other cases, such as the carbon cycle, this truncation results in the accumulation of dissolved inorganic carbon species in solution. The truncation of biogeochemical cycling is one of the unique features of microbial processes in subsurface environments and has a significant impact on observed ground-water chemistry.

Consideration of biogeochemical cycling in ground-water geochemistry is useful primarily in that it gives a qualitative framework for understanding of the behavior of biochemically active elements. It is difficult, however, to use this framework in a quantitative manner. As we have seen, these cycles do not act independently of each other. For example, the oxygen and hydrogen cycles are intimately connected

with the carbon cycle, and all three interact with the nitrogen and sulfur cycles. To make things even more difficult, mineral sources of inorganic carbon and sulfur often overshadow biological sources in many systems. Thus, while useful in describing overall behavior, the concepts of elemental cycling are an inadequate basis for a quantitative treatment of ground-water chemistry. Such quantitative treatments, which integrate both biological and nonbiological processes, are considered in the next chapter.

QUESTIONS TO CONSIDER

1. What primary characteristic of subsurface results in the truncation of the oxygen and carbon cycles in ground-water systems?
2. When a biogeochemical cycle is truncated in ground-water systems, what characteristic of that cycle leads to (a) the depletion of some dissolved chemical constituents (such as oxygen), or (b) the accumulation of other dissolved chemical constituents (such as dissolved inorganic carbon)?
3. Fixing organic carbon and organic oxygen requires large inputs of energy. How is this energy supplied to drive the carbon and nitrogen cycles? How does this energy input differ between these cycles?
4. List at least three ways of distinguishing sources of dissolved nitrogen species to ground water.
5. Explain how the microorganisms *Geobacter* and *Thiobacillus* can have a symbiotic ecological association.
6. Assuming that the locations of well screens are entirely within a well-field operator's control, how can judicious placement of these screens prevent iron-clogging problems resulting from iron-oxidizing bacteria?
7. Undesirably high concentrations of iron and sulfide are the most common water-quality problems in the world. How can this be explained by the concept of truncated biogeochemical cycles?

CHAPTER 10

OXIDATION-REDUCTION PROCESSES IN GROUND-WATER SYSTEMS

The electron-transport systems of microorganisms (Chapter 4) are fundamental to their metabolism, growth, and reproduction. In fact, it is possible to represent microbial metabolism with a simple wiring diagram, as shown in Figure 10.1. In many electrical machines, electrons circulate from a battery (electron donor), through a resister (which generates useful work, such as making a lightbulb glow), and are deposited to a ground (electron acceptor). By way of analogy, microorganisms also require an electron donor; for heterotrophic bacteria this source is organic carbon. For autotrophic bacteria, the sources are reduced inorganic chemicals such as Fe^{2+}, H_2S, or H_2 (Fig. 10.1). But in addition to having a source of electrons, a microorganism must also have an electron acceptor—a repository to which the electrons can move freely. Many microorganisms use oxygen as an electron acceptor (Fig. 10.1). But other bacteria have mechanisms for using oxidized compounds such as nitrate, manganese oxides, ferric iron, sulfate, and carbon dioxide as electron acceptors (Fig. 10.1). Once an electron donor and an electron acceptor is available to a microorganism, it is able to move electrons through its "circuitry." These flowing electrons are capable of doing work, and the microorganism uses this work to accomplish all of its life functions, including cellular maintenance, genetic expression, protein synthesis, growth, and reproduction (Fig. 10.1). The only real difference between microbial processes and the wiring diagram shown in Fig. 10.1 is that microorganisms use many different chemical electron carriers, such as cytochromes—not electrical wiring—to conduct electrons. The kinetic energy in these flowing electrons is then captured and transformed into chemical energy by chemiosmosis (Chapter 4).

Microorganisms that inhabit ground-water systems sustain their life functions with electron transfer—that is, reduction/oxidation (redox)—processes. As they carry on these redox processes, microorganisms effect many geochemical changes on solid-phase aquifer materials and on dissolved constituents present in ground water. This chapter presents an overview of microbially mediated redox processes, shows how these processes can be identified in subsurface environments, and pres-

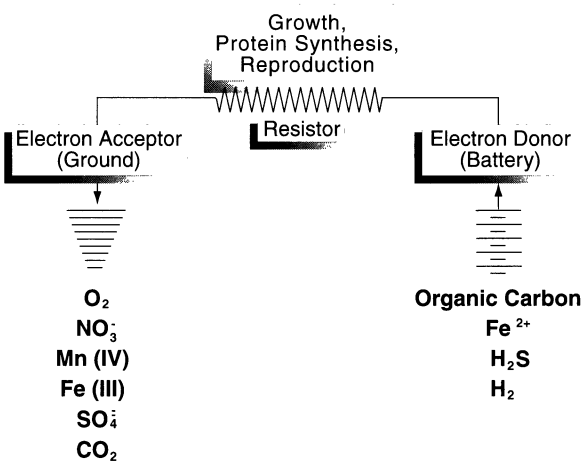


Figure 10.1. Wiring diagram of electron flow in microorganisms from electron donors to electron acceptors with the work used to support life functions.

ents examples of how redox processes affect the chemistry of ground water and aquifer materials in both pristine and contaminated ground-water systems.

10.1 OVERVIEW OF REDOX GEOCHEMISTRY

Quantitative consideration of redox processes has always been important in ground-water geochemistry (Back and Barnes, 1965). Treatment of redox processes in geochemistry has traditionally followed conventions and methods developed from classical physical chemistry (Stumm and Morgan, 1981). It is useful to begin a treatment of redox processes in ground water with a review of these traditional methods.

10.1.1 The Equilibrium Approach

In physical chemistry, the tendency for particular chemicals to donate or accept electrons is defined in terms of the standard hydrogen electrode (Fig. 10.2). The standard hydrogen electrode is constructed from a water-containing vessel, an apparatus for delivering hydrogen gas (H_2) at 1 atmosphere pressure, and platinum in contact with the solution. The purpose of the platinum is to provide a catalytic surface so that the hydrogen species (H_2, H^+, and H_2O) come to chemical equilibrium. There are three important thermodynamic conventions associated with the standard hydrogen electrode:

1. The electrical potential between the platinum and the solution is zero.
2. The standard free energy of formation of H^+ ions in solution is zero.
3. The standard free energy of formation of an electron in solution is zero.

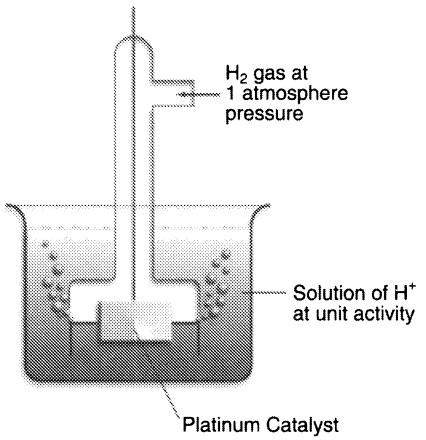


Figure 10.2. The standard hydrogen electrode.

All of these conventions are arbitrary and serve to define a scale to which free energies of other dissolved species can be compared.

Once the conventions surrounding the standard hydrogen electrode have been assigned, it can be used to assess particular electron-transfer reactions that occur in solutions. To do this, the standard hydrogen electrode is connected to a separate solution containing electroactive chemicals by a platinum electrode and a salt bridge (Fig. 10.3). The salt bridge usually consists of a solution of potassium chloride, and its purpose is to maintain electrical contact between the cell containing the standard hydrogen electrode and the cell containing other electroactive chemicals, in this case Fe^{2+} and Fe^{3+}. Since both cells are required to establish an electrical circuit, they are often referred to as *half cells*.

If one half cell has a higher tendency to donate electrons than the other, electrons can be transferred through the wire from one solution to the other, and the current

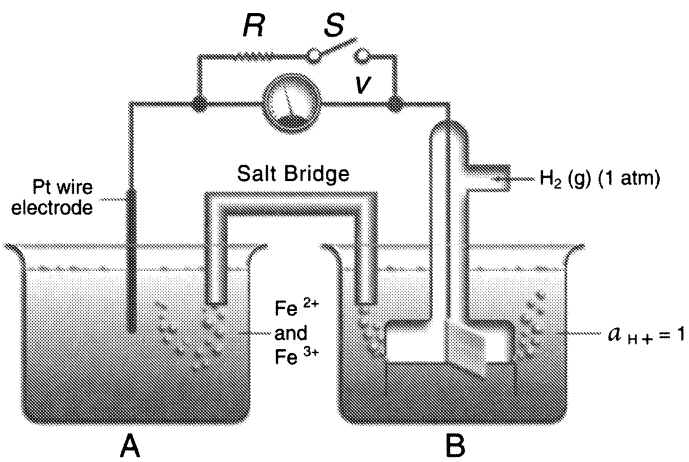


Figure 10.3. Coupling the standard hydrogen electrode to a solution containing other electroactive redox couples such as Fe^{2+} and Fe^{3+}.

(amperage) of this electron transfer can be measured (Fig. 10.3). Alternatively, the flow of electrons can be interrupted by opening a switch, and the electrical potential difference (voltage) between the solutions measured. When the chemical species within each half cell are at thermodynamic equilibrium, the potential difference between the solution in cell A relative to the standard hydrogen electrode of cell B is called the Eh. By convention, a positive Eh indicates that cell A has a tendency to donate electrons to the standard hydrogen electrode, and a negative Eh indicates that the standard hydrogen electrode has a tendency to donate electrons to cell A. Once both cell A and the standard hydrogen electrode have reached equilibrium, the Eh of solution A can be measured with a voltmeter (Fig. 10.3).

In addition, if the equilibrium concentrations of Fe^{2+} and Fe^{3+} are known in solution A, the Eh can be calculated from thermodynamic principles. Given the overall reaction between Cell A and B:

$$Fe^{3+} + \tfrac{1}{2} H_2 \rightleftarrows Fe^{2+} + H^+ \tag{1}$$

The Eh of solution A can be calculated from the measured ratio of Fe^{3+} to Fe^{2+} with the Nernst equation:

$$\text{Eh} = E^0 + \frac{2.303RT}{nF} \log \frac{a\text{Fe}^{3+}}{a\text{Fe}^{2+}} \tag{2}$$

Where E^0 is the electrode potential the cell would have if Fe^{2+} and Fe^{3+} concentrations were at standard state (unit concentration), R is the gas constant (1.987×10^{-3} kcal/deg mol), T is temperature (degrees Kelvin), n is the number of electrons transferred in the reaction, and F is Faraday's constant.

Many modern geochemical texts, following terminology formalized by Stumm and Morgan (1981), treat redox processes in terms of pe instead of Eh. The concept of pe is directly analogous to pH. Since pH is defined as the activity of H^+ ions in solution:

$$\text{pH} = -\log_{10} a\text{H}^+$$

pe is defined as the activity of electrons in solution:

$$\text{pe} = -\log_{10} a\, e^-$$

It can be shown that pe and Eh are linearly related by the equation:

$$\text{pe} = \frac{F}{2.303RT} \text{Eh} \tag{3}$$

This treatment of redox chemistry illustrates an important point. The concept of Eh is meaningful *only when a solution is at chemical equilibrium.* If concentrations of ferrous and ferric iron in solution A are not at equilibrium, the voltage difference measured between cell A and B would be different from the true Eh. Similarly, if concentrations of ferrous and ferric iron are not at equilibrium, an Eh calculated

from the Nernst equation (equation 2) would not be accurate. Thus, the concept of Eh and its measurement *requires* thermodynamic equilibrium in the solution being considered. For this reason, it is referred to as the *equilibrium approach* to redox chemistry.

10.1.2 The Kinetic Approach

Electrical processes can be assessed under electrostatic conditions, in which an electrical potential (voltage) exists between two solutions. This is illustrated in Figure 10.2 when the circuit between the two solutions is not completed. In this case, the electrical potential difference can be measured, and when Fe^{3+} and Fe^{2+} concentrations are at thermodynamic equilibrium, a unique Eh can be either measured (Fig. 10.3), or calculated from the Nernst equation (equation 2).

However, when the circuit between the two solutions is completed by closing the switch between them, electrical potential energy (voltage) is converted into electrical kinetic energy and electrons flow (amperage) between the two cells. As electrons flow, however, ferric ions react with hydrogen gas (equation 1), depleting concentrations of both reactants. Thus, in order for electrons continue to flow, concentrations of ferric iron and hydrogen must be continually replenished. In this case, the electrical processes can be described in terms of the source of electrons (the electron donor), the ultimate sink for electrons (the electron acceptor), and the electrical current generated (the amperage). This way of characterizing electrical processes is referred to as the *kinetic approach* to describing redox chemistry.

10.1.3 Redox Processes in Ground-Water Systems

We have seen that all ground-water systems contain viable microorganisms (Chapter 7). Furthermore, we have seen that these microorganisms use electron-transfer processes to maintain life functions, to grow, and to reproduce (Chapter 4). Thus, all redox reactions carried out by microorganisms in subsurface environments are inherently kinetic processes. This creates an obvious conflict with the traditional Eh approach as it has been applied to ground-water systems. To obtain unique Eh measurements characteristic of a particular redox couple (Fig. 10.3), strict equilibrium (electrostatic) conditions are required. However, in order for microorganisms to remain viable in ground-water systems, electrokinetic conditions are necessary.

There are many examples in the literature of how Eh measurements do not provide a unique description of redox processes. Perhaps the best example, however, was provided by Lindberg and Runnels (1984). In this paper, the authors considered approximately 500 high-quality measurements of ground-water chemistry made throughout the United States by the U.S. Geological Survey. Each of these analyses included platinum electrode Eh measurements as well as measurements of various redox couples, including O_2/H_2O, NO_3/NH_4, Fe^{3+}/Fe^{2+}, SO_4/H_2S, and CO_2/CH_4. When platinum electrode Eh measurements were plotted versus Eh values calculated from the redox couples using the Nernst equation, there was no correlation at all observed (Fig. 10.4). The conclusion made by Lindberg and Runnels was inescapable: The ground-water samples simply could not be at thermodynamic equilibrium.

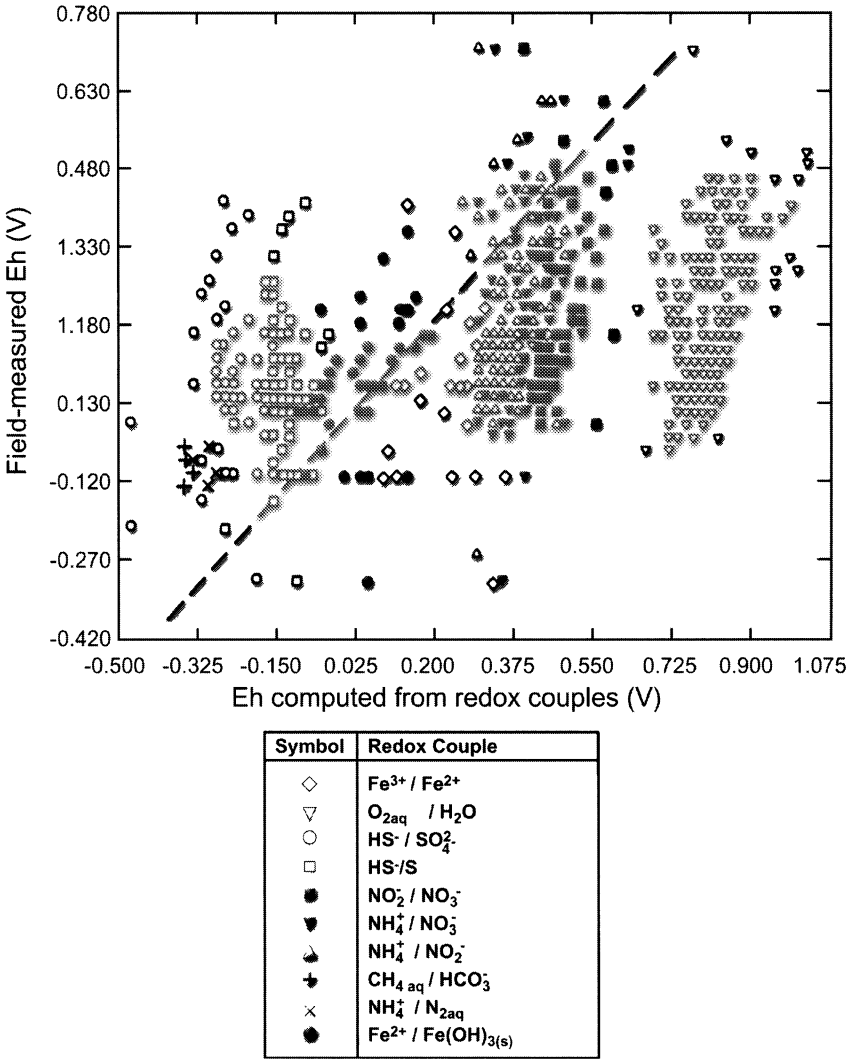


Figure 10.4. The lack of internal consistency between Ehs calculated with different redox couples as observed by Lindberg and Runnels (1984).

In the early 1980s, before many studies on the presence of microorganisms in ground-water systems were available, the reasons for the observed nonequilibrium of ground water were not immediately obvious. At that time, it was not widely accepted that microorganisms could live and reproduce in ground-water systems. However, with benefit of hindsight, the reasons for the observed nonequilibrium are obvious: The respiration, activity, and growth of microorganisms creates electrokinetic conditions in ground-water systems. These electrokinetic conditions invalidate the assumption of thermodynamic equilibrium required by the definition of Eh, and thus no unique Eh can exist in these ground-water systems.

Because of the widespread presence of ongoing microbial processes, Eh cannot be used as a quantitative indicator of redox processes in ground-water systems.

The challenge, therefore, is to identify redox indicators that reflect ambient microbial processes, which can be physically measured, and which can be interpreted in a meaningful manner.

10.2 DESCRIBING KINETIC REDOX PROCESSES IN GROUND-WATER SYSTEMS

Kinetic descriptions of redox processes in ground-water systems can include at least three components (Fig. 10.1). These are (1) documenting the source of electrons (electron donor) that supports microbial metabolism, (2) documenting the final sink for electrons (electron acceptor) that supports microbial metabolism, (3) and documenting rates of electron transfer (metabolism) that occur in a system. In many ground-water systems, identifying electron donors is trivial, since organic carbon is far and away the most common source of electrons for subsurface microorganisms. In other applications, identifying the electron source is more important. An example of this might be investigating the causes of decreasing yields in production wells. If Fe^{2+} is an important electron donor, and oxygen the electron acceptor, iron fouling and clogging may well be the cause of decreased well yields. Finally, documenting rates of electron transfer processes may be important for determining rates of organic contaminant degradation in contaminated ground-water system. In any case, evaluation of redox processes begins with the fact that microorganisms catalyze most of the redox processes that occur in aquatic sediments and ground-water systems (Lovley and Goodwin, 1988). Given this realization, and given what is known about microbial ecology and about microbial cycling of carbon, nitrogen, iron, and sulfur, it is possible to construct a kinetic framework for characterizing these processes.

10.2.1 Identifying Electron Donors

The classic scientific example of why it can be important to identify electron donors in microbial metabolism comes from a combined ground-water hydrology and oceanography problem. In the 1950s and 1960s, there were many aspects of ocean geochemistry that were puzzling. For example, concentrations of magnesium in seawater were much lower than they should have been based on how much magnesium was being delivered from terrestrial sources. Another example was the problem of manganese nodules. Many parts of the seafloor were covered with small nodules with high concentrations of manganese and other metals. Where did they come from and how did they form?

The resolution of these mysteries began in 1977 when two oceanographers in the submarine *Alvin* observed hot springs gushing from the floor of the Pacific Ocean. These springs turned out to be the product of a huge subsurface flow system that underlies many parts of ocean basins. In spreading centers, where the crust is being pulled apart by tectonic forces, basaltic lava erupts from cracks in the earth and spreads out over the ocean floor. Because this basalt is so porous, seawater seeps into it, circulates downward where it is heated at depth, and is forced back to the surface as hot springs. As the water circulates in the basalt, certain components of the seawater—notably dissolved oxygen, dissolved organic carbon, and

magnesium—are stripped out of solution. Other components, such as dissolved Fe^{2+}, Mn^{2+}, and H_2S are added to solution. When the hot water containing these reduced chemicals surges out of the hot springs, it mixes with oxygenated seawater. While there is little or no organic carbon available to act as an electron donor, there is abundant Fe^{2+}, Mn^{2+}, and H_2S. These reduced compounds act as electron donors supporting microbial life at these deep-sea vents, and oxygen acts as the electron acceptor. The metals oxidized in this fashion slowly precipitate out of solution and contribute to the formation of manganese nodules.

This is an example of how identifying the electron donor in microbial redox processes was crucial to solving an important scientific problem. In ground-water hydrology, identifying Fe^{2+} as an electron donor is often the first step in solving clogging problems associated with production wells.

Even when carbon is the primary electron donor for microbial redox processes, identifying the carbon species acting as the principal electron donor can be important. For example, dichloroethene (DCE), a common contaminant associated with chlorinated solvent spills in ground-water systems, can act either as an electron donor (Bradley and Chapelle, 2000) or as an electron acceptor (McCarty and Semprini, 1994) in microbial metabolism. If DCE acts as an electron acceptor (i.e., reductive dechlorination), a chlorine is stripped off of the molecule and vinyl chloride is formed:

DCE Vinyl Chloride

$$C_2H_2Cl_2 + H^+ + e^- \rightarrow C_2H_3Cl + Cl^-$$

$$C_2H_2Cl_2 + H^+ + e^- \rightarrow C_2H_3Cl$$

On the other hand, if DCE acts an electron donor in microbial metabolism, carbon dioxide and chloride are formed:

DCE

$$C_2H_2Cl_2 + 2O_2 \rightarrow 2CO_2 + 2Cl^- + 2H^+$$

Because vinyl chloride is a known carcinogen, knowing whether DCE acts as an electron acceptor or electron donor in any given ground-water system is a matter of environmental significance.

10.2.2 Identifying Electron Acceptors

Many chemical compounds including molecular oxygen, nitrate, Mn(IV), Fe(III), sulfate, and carbon dioxide can serve as electron acceptors in microbial metabolism. Whereas organic carbon is far and away the most common electron donor, there is much more variety in the kinds of electron-accepting processes that occur commonly. For this reason, identifying electron accepting processes in ground-water systems is often much more challenging than identifying electron donors. However, several ecological characteristics of microbial metabolism make identifying electron-accepting processes feasible. The most important of these is the process of microbial competition.

Microbial Ecology and Competition Microorganisms, whether they live in relatively nutrient-rich aquatic sediments or more nutrient deprived deep subsurface environments, actively compete for available resources (Chapter 6). Microorganisms that have physiological characteristics suited to the conditions of a given environment thus have a competitive advantage, and will thrive and dominate that environment. Conversely, those microorganisms whose physiological characteristics are not suited to an environment cannot compete effectively for available resources, and thus tend to be excluded from that environment.

This concept of competitive exclusion can be illustrated quantitatively (Lovley and Goodwin, 1988). Molecular hydrogen (H_2) is the most widely utilized substrate for anaerobic respiration in sediments or aquifer systems. Hydrogen is a waste product of the fermentative bacteria that initiate oxidation of complex organic matter. This hydrogen is then utilized by respiring microorganisms such as nitrate reducers, Fe(III) reducers, sulfate reducers, and methanogens. The uptake of hydrogen by respirative bacteria is described by Michaelis-Menton Kinetics:

$$v = \frac{v_{max}\,[H_2]B}{K_s + [H_2]} \tag{4}$$

where v (moles of hydrogen per unit time) is the rate of hydrogen uptake, v_{max} (moles of hydrogen per unit time per gram of cells) is the maximum rate of hydrogen uptake when hydrogen uptake is not limited by hydrogen availability, K_s (moles of dissolved hydrogen per liter) is the hydrogen concentration at which v equals 0.5 v_{max}, and B is the number of grams of hydrogen-consuming microorganisms. In addition, the growth of microorganisms on hydrogen can be described by the differential equation:

$$\frac{dB}{dt} = (vY) - (bB) \tag{5}$$

Where dB/dt is the rate of microbial growth (grams of cells per unit time), Y is the yield coefficient (grams of cells formed per mole of hydrogen consumed), and b is the cell death coefficient (units of time^{-1}). At steady-state conditions, where the rate of cell growth is exactly balanced by the rate of cell death ($dB/dt = 0$), equation 5 can be solved for v:

$$v = \frac{bB}{Y}$$

When this result is substituted into equation 4 and solved for $[H_2]$, we find:

$$[H_2] = \frac{K_s}{(v_{max}\,Y/b) - 1} \tag{6}$$

Equation 6 indicates that under the steady-state conditions characteristic of aquatic sediments and ground-water systems, the hydrogen concentration depends solely upon the physiological characteristics (K_s, v_{max}, Y, and b) of the microorganisms

consuming the hydrogen. Other factors that might seem to be involved, such as the rate of hydrogen production, do *not* affect the steady-state hydrogen concentration. Of these physiological parameters, the half-saturation constant is observed to decrease as the potential energy available from hydrogen oxidation increases, whereas Y increases. Given the standard free energy available from hydrogen oxidation processes (Table 10.1), equation 10.6 predicts that steady-state concentrations in sediments will follow the order: methanogenic > sulfate-reducing > Fe(III)-reducing > Mn(IV)-reducing > nitrate-reducing.

It follows from this reasoning that hydrogen concentrations should be lowest for nitrate-reducing sediments and will increase in the order nitrate reducing < Mn(IV)-reducing < Fe(III)-reducing < sulfate-reducing < methanogenic.

This predicted behavior can be verified experimentally. Figure 10.5 shows data from one such experiment in which methanogenic aquatic sediment was collected and placed into different vials. The sediment in one treatment was left unamended (methanogenic), another was amended with nitrate, another with $Fe(OH)_3$, and another with sulfate. After a few hours, measured concentrations of hydrogen began to change in the amended vials. Hydrogen concentrations in the nitrate-amended vials dropped to below 0.1 nanomole per liter (nM), to 0.3 nM in the $Fe(OH)_3$-amended vial, to 1.0 nM in the sulfate-amended vial, and remained between 10 and 15 nM in the methanogenic vial (Fig. 10.5). This shows that the differences in hydrogen concentrations predicted by equation 6 are in fact observed (Table 10.1).

These different hydrogen concentrations that develop due to the differing uptake efficiencies of microorganisms have an important ecological effect. If Fe(III) is present in sufficient abundance in a sediment, hydrogen concentrations will be maintained in the 0.2–0.8-nM range characteristic of Fe(III). These hydrogen concentrations are too low for sulfate-reducing or methanogenic microorganisms to effectively respire. Thus, Fe(III) metabolism can be said to outcompete sulfate reduction or methanogenesis. Similarly, if sulfate reduction will tend to dominate over methanogenesis when sulfate is available.

The way that microbial competition manifests itself in aquatic sediments and aquifer systems is shown schematically in Figure 10.6. Complex sedimentary organic matter is initially decomposed by fermentative bacteria that produce molecular hydrogen and organic acids as metabolic by-products. These metabolic

TABLE 10.1 Potential Energy Yield and Steady-State Hydrogen Concentrations Characteristic of Different Anaerobic Oxidation Processes

Oxidation/Reduction Reaction	Potential Standard-Free Energy (kJ per H_2)	Characteristic Hydrogen Concentration (nM)
$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O$	224	0.01–0.05
$MnO_2 + H_2 \rightarrow Mn(OH)_2$	163	0.1–0.3
$2Fe(OH)_3 \rightarrow 2Fe(OH)_2 + 2H_2O$	50	0.2–0.8
$SO_4^{2-} + 4H_2 + H+ \rightarrow HS^- + 4H_2O$	38	1.0–4.0
$HCO_3^- + 4H_2 + H^+ \rightarrow CH_4 + 3H_2O$	34	5.0–15.0

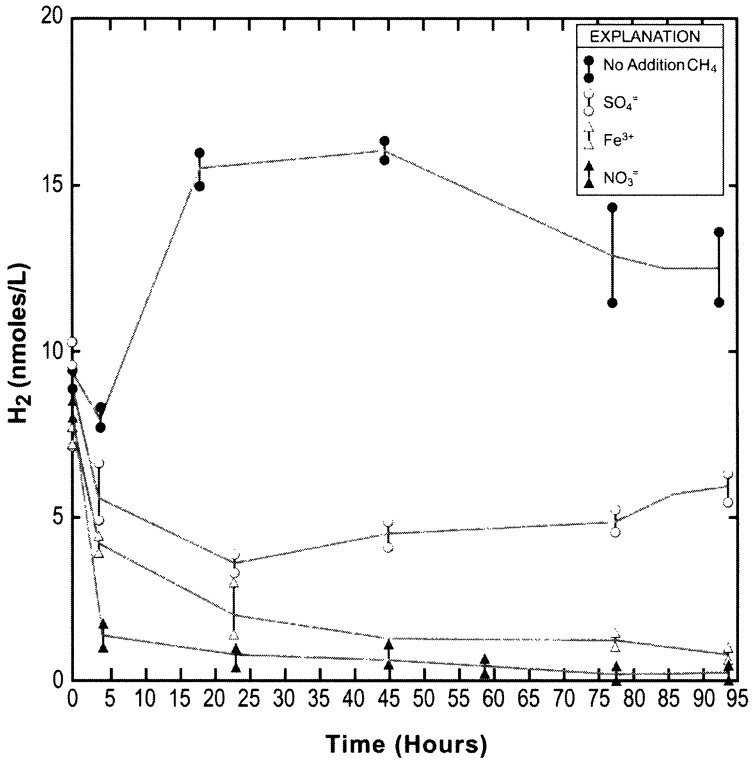


Figure 10.5. Experiment showing how addition of nitrate, Fe(III), and sulfate to initially methanogenic sediments leads to the different hydrogen concentrations characteristic of each terminal electron-accepting process (TEAP).

by-products are then utilized by respirative bacteria such as nitrate-reducers, Fe(III) reducers, sulfate reducers, and methanogens. However, because of the differing efficiency of hydrogen uptake, nitrate reducers will capture most of the electron flow if sufficient nitrate is present in the system. If nitrate is not present but Fe(III) is, Fe(III) reducers will capture the electron flow and so on (Fig. 10.6).

The net effect of these processes is to segregate aquifers into zones dominated by certain electron-accepting processes (Lovley et al., 1994). This is shown schematically in Figure 10.7. In many pristine aquifers that lack large amounts of organic electron donors, recharge areas are dominated by aerobic metabolism. However, once ground water is isolated from the atmosphere, oxygen is consumed, and the aquifer is dominated by a succession of terminal electron-accepting processes (TEAPs) ranging from nitrate reduction to methanogenesis (Fig. 10.7a). Contaminated ground-water systems containing large amounts of organic electron donors are also observed to segregate into TEAP zones, but because organic substrates tend to deplete the most efficient electron acceptors first, the order of zonation is reversed (Fig. 10.7b). This characteristic zonation of TEAPs is a fundamental property of redox processes in aquatic sediments and ground-water systems.

The zonation of redox processes into distinct zones results from competition for the electron donor (hydrogen) that limits microbial growth. If available electron

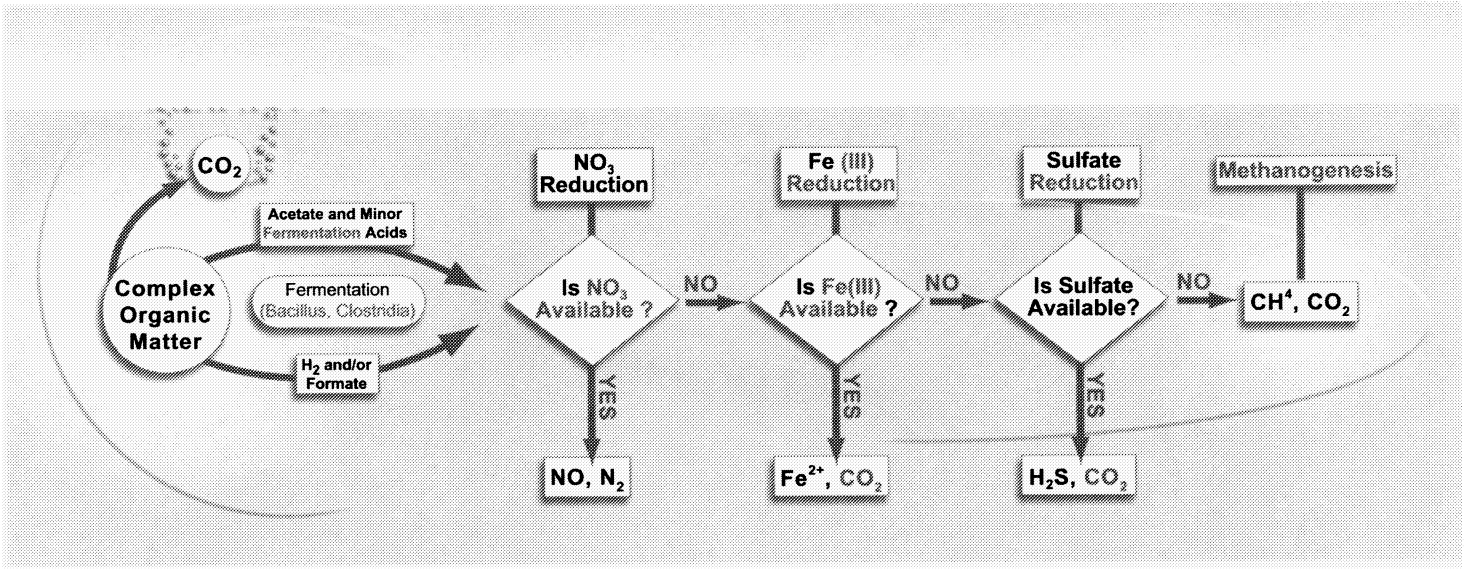


Figure 10.6. How microbial competition is manifested in aquatic sediments and aquifer systems.

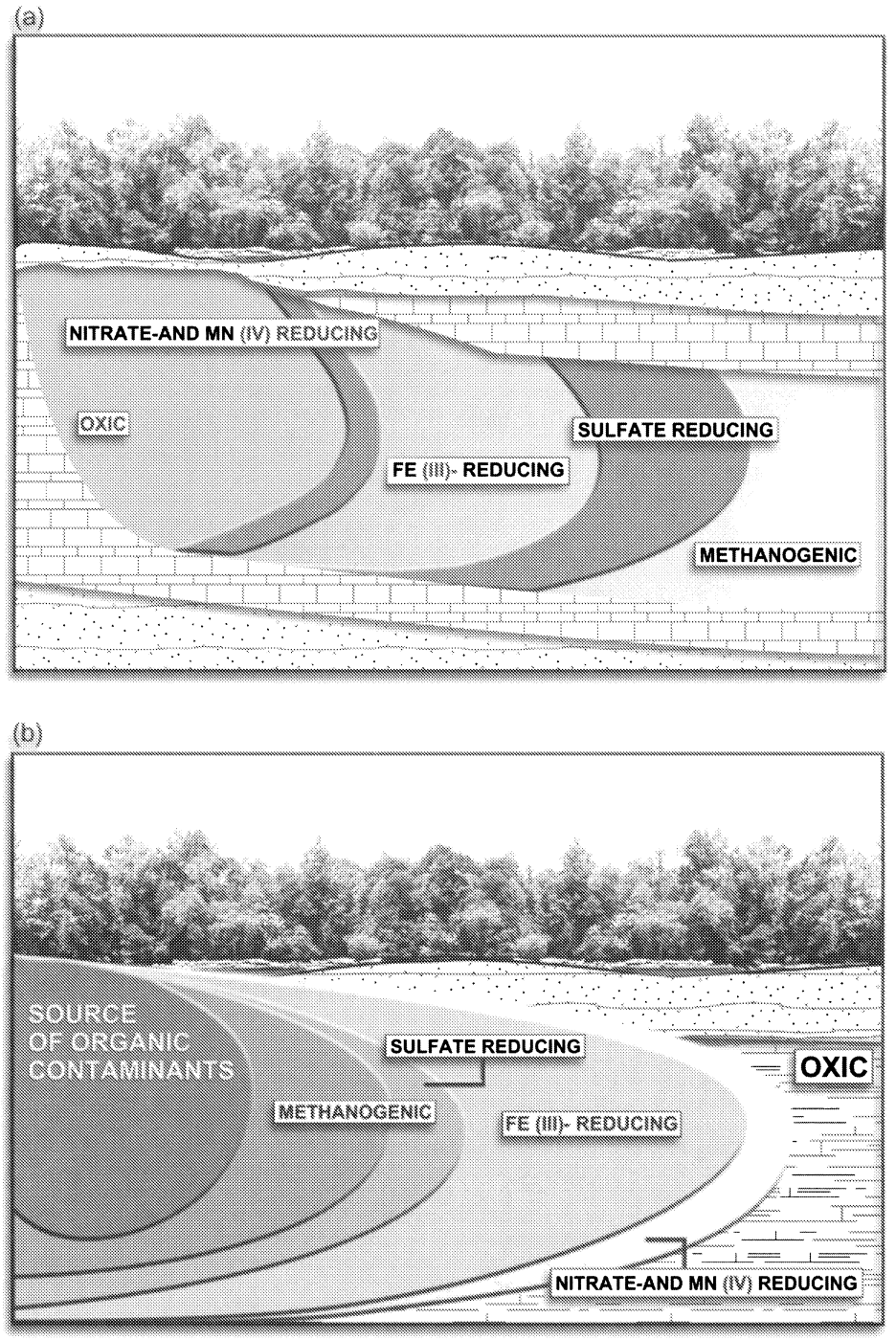


Figure 10.7. Characteristic succession of redox processes (*a*) for an electron donor-poor pristine aquifer, and (*b*) for an electron donor-rich contaminated aquifer.

donors are so plentiful so as not to be limiting, as sometimes happens in petroleum-hydrocarbon aquifers, there is less tendency for redox processes to segregate into distinct zones. For example, if hydrogen concentrations are maintained at high concentrations due to a large supply of organic carbon, competition is diminished and concomitant methanogenesis, sulfate reduction, and Fe(III) reduction are possible if those electron acceptors are available.

10.3 IDENTIFYING TERMINAL ELECTRON-ACCEPTING PROCESSES (TEAPs) IN THE ENVIRONMENT

We have seen that in microbially active aquatic sediments and ground-water systems, redox processes tend to segregate into discrete zones dominated by one terminal electron-accepting process (TEAP). Thus, identifying ambient TEAPs is fundamental to describing redox processes in these natural systems. Historically, aquatic sediment geochemists were the first to take this approach to describe ambient redox processes. More recently, similar approaches have been applied to ground-water systems. In this section, methods for identifying TEAPs in sediments and ground-water systems are considered.

10.3.1 Redox Zonation in Aquatic Sediments

The manner in which aquatic sediments tend to develop discrete redox zones is important to aquatic geochemists interested in global carbon cycling. A classic example of the aquatic sediment approach to this problem was provided by Froelich et al. (1979). These investigators measured concentrations of oxygen, nitrate and nitrite, Mn^{2+}, Fe^{2+}, $SO_4^{=}$, and $S^{=}$ as a function of depth below the sediment–water interface in deep-sea pelagic sediments. The resulting pattern (Fig. 10.8) showed that oxygen concentrations decreased with depth below the sediment–water interface, indicating active oxygen reduction. Once oxygen was depleted, nitrate concentrations began to fall as well, indicating nitrate reduction. Once the sediments were completely anoxic, Mn^{2+} began accumulating in solution, indicating active Mn(IV) reduction. Finally, once nitrate was completely consumed, concentrations of Fe^{2+} began to increase indicating active Fe(III) reduction. In these sediments, concentrations of sulfate were constant throughout the sediments, and dissolved sulfide was absent, indicating the absence of significant sulfate reduction.

These data are a good example of the discrete redox zonation characteristic of aquatic sediments that are not regularly disturbed by sedimentation events (as often happens in stream and river sediments) where redox processes approach steady-state conditions. Furthermore, because solute transport in these sediments is dominated by relatively slow molecular diffusion, the redox zones can easily be deduced from the presence/absence of particular electron acceptors (oxygen and nitrate), and final products of each redox process (Mn^{2+}, Fe^{2+}). These data suggest that similar geochemical methods can be used to deduce redox zonation in ground-water systems as well. However, because solute transport in ground-water systems is dominated by flowing ground water, as opposed to diffusion, interpretation is more complex.

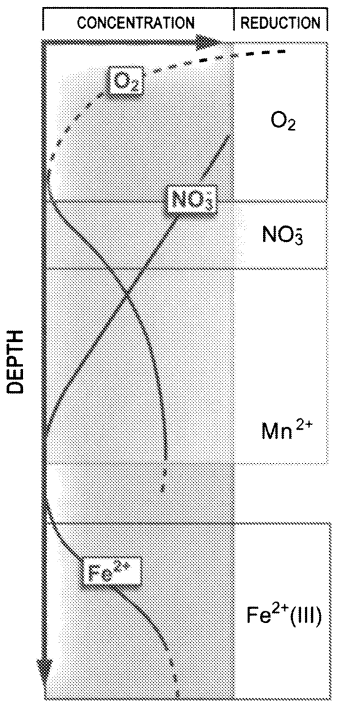


Figure 10.8. Characteristic vertical succession of redox processes in aquatic sediments observed by Froelich et al., 1979.

10.3.2 Redox Zonation in Ground-Water Systems

In principle, the redox zonation of ground-water systems can be deduced in exactly the same way as marine sediments (Froelich et al., 1979) by documenting concentrations of redox-sensitive parameters such as O_2, Mn^{2+}, nitrate, Fe^{2+}, sulfate, sulfide, and methane. For example, studies have delineated denitrifying zones based on the consumption of nitrate (Baedecker and Back, 1979; Bulger et al., 1989), Fe(III)-reducing zones based on the accumulation of dissolved iron (Langmuir, 1969; Chapelle and Lovley, 1992), sulfate-reducing zones based on the consumption of sulfate (Thorstenson et al., 1979) or on the production of sulfide (Jackson and Patterson, 1982), and methanogenic zones based on the accumulation of methane (Thorstenson et al., 1979; Baedecker et al., 1988).

In practice, however, this approach often leads to equivocal results. The most important reason for this is that the transport of dissolved constituents in ground-water systems is driven primarily by advective flow. Because of this, it is common for methane to be produced in an actively methanogenic zone, and carried by flowing ground water to nonmethanogenic zones. Thus, the presence of methane in ground water does not mean that methanogenesis is occurring in the zone screened by the well producing the water. Rather, it simply means that methanogenesis is occurring somewhere upgradient of the well. It is common, for example, for the same sample of anoxic ground water to contain Fe^{2+}, sulfide, and methane. In this case, it is not possible to identify the redox process or processes that dominate in the sampled horizon.

It is in this context that hydrogen (H_2), the most ubiquitous intermediate product of anaerobic microbial metabolism, can help identify TEAPs. Because H_2 is continuously cycled by microorganisms, it is not transported by flowing ground water in the same way as more stable constituents such as methane, sulfide, or Fe^{2+}. Thus, H_2 concentrations in ground water indicate redox processes operative in the interval tapped by the monitoring well.

The most reliable way to identify redox zonation in aquifers is to consider the consumption of electron acceptors (oxygen, nitrate, and sulfate) with the production of reduced products (nitrite, Fe^{2+}, H_2S, and CH_4) along aquifer flowpaths in combination with H_2 concentrations. These patterns of electron acceptor consumption, final product accumulation, and H_2 concentrations can be combined into a hierarchical framework that leads logically to the identification of TEAPs (Chapelle et al., 1995). One such framework is shown schematically in Figure 10.9. This framework refers to electron acceptor or final product concentration changes along a flowpath segment of a hydrologic systems. Because final products such as methane or sulfide are potentially stable in anaerobic water, they may be transported between

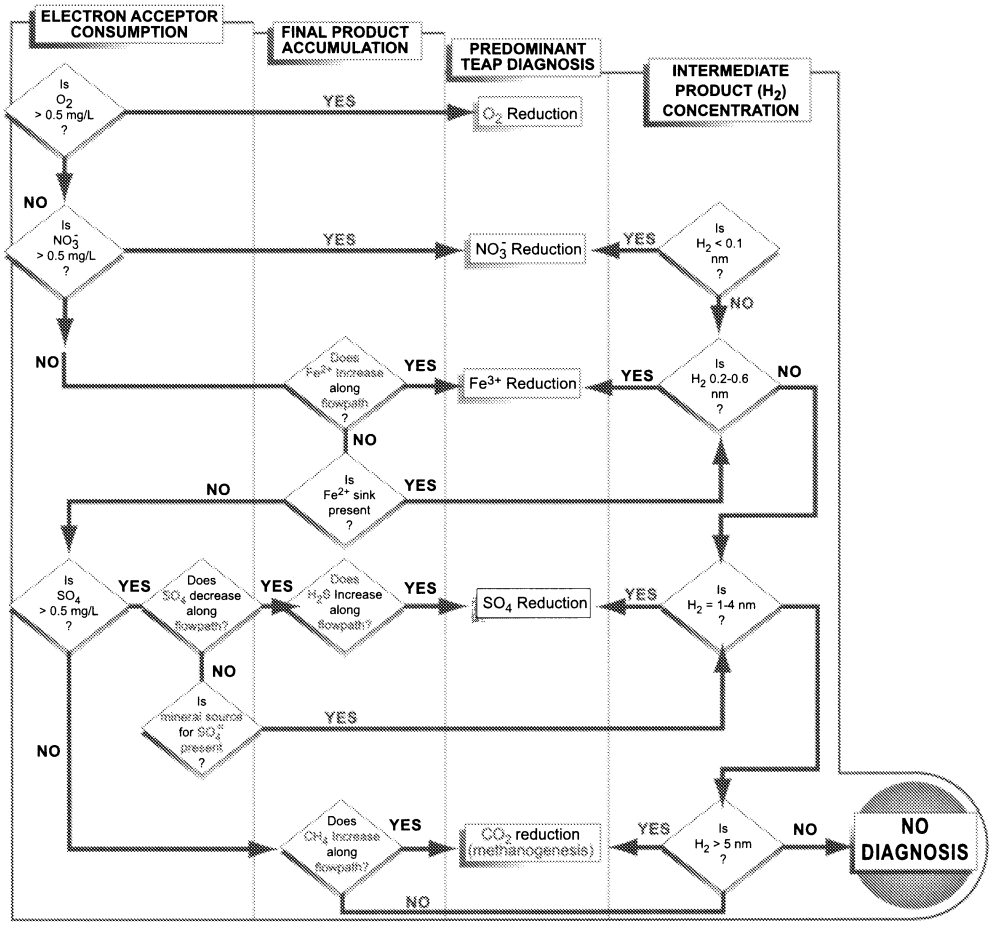


Figure 10.9. Flowchart for deducing the zonation of redox processes in ground-water systems.

zones dominated by different TEAPs. Thus, the presence of methane does not necessarily indicate active methanogenesis in the sampled zone. Rather, active methanogenesis is indicated by *increasing* methane concentrations along a given flowpath segment. Because discrete flowpaths are difficult to define in some hydrologic systems, the use of electron acceptor consumption and final product accumulation is often limited.

In cases where electron acceptor consumption and final product accumulation are equivocal, concentrations of H_2 can aid in determining operative TEAPs (Fig. 10.9). Ideally, all three indicators of TEAPs—electron acceptor consumption, final product accumulation, and H_2 concentration—can be used to come to a determination. When all three potential indicators point to the same TEAP determination, a high degree of confidence in the conclusion is warranted. If two of the three possibilities converge on a TEAP determination, somewhat less confidence is warranted. The least confidence is warranted where only a single line of evidence points to a TEAP determination. Note that for TEAPs in which the electron acceptor is not water soluble (i.e., Fe(III) reduction), only two lines of evidence are available.

Another factor that complicates identification of TEAPs is that of scale. Microbial competition—and thus the development of distinct redox zones—is a general characteristic of microbial ecology in aquatic sediments and aquifer systems. However, this general principle operates on many different scales. It is possible for redox zones in aquifers to be tens of kilometers wide in some intermediate or regional flow systems (Fig. 6.7). It is also possible for this zonation to occur on the scale of centimeters or even milimeters, as often happens in aquatic sediments. Because the scale of ground-water sampling is limited to the scale of the screened or open interval of particular wells, it is not always possible to distinguish individual redox zones. If a well screen intersects two or more discrete redox zones, water chemistry data from that well will be some composite of the different zones. This is another important reason why the logic of Figure 10.9 may lead to equivocal results, and should always be considered in any application. In these cases, equivocal results may be evidence for small-scale redox zonation in particular ground-water systems.

10.4 REDOX PROCESSES IN PRISTINE GROUND-WATER SYSTEMS

Given a framework for identifying redox processes in ground-water systems (Fig. 10.9), it is possible to determine the zonation of redox processes in a variety of hydrologic systems. This methodology is first illustrated for pristine ground-water systems. The first example, the Black Creek aquifer of South Carolina, considers a regional aquifer system. The second, the Floridan aquifer of Georgia, considers a local flow system.

10.4.1 Black Creek Aquifer of South Carolina

The Black Creek aquifer crops out in the upper Coastal Plain of South Carolina and runs parallel to the Fall Line (Fig. 10.10). The aquifer consists of unconsolidated clastic sediments and carbonate shell material. The sediments are upper Cre-

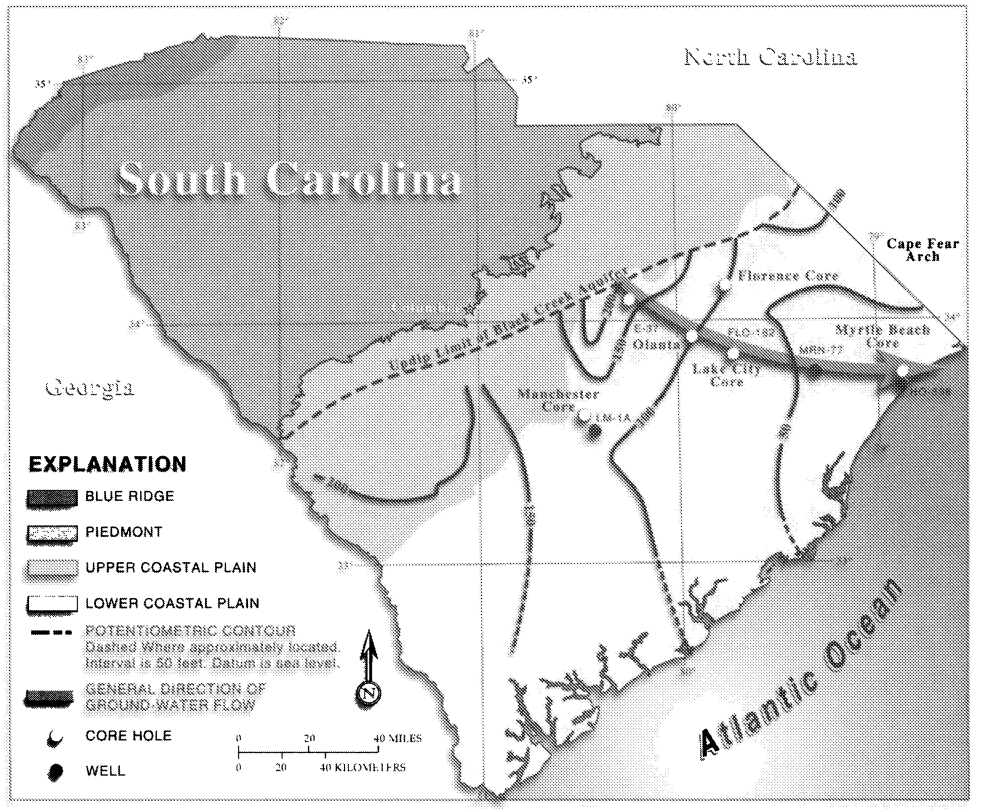


Figure 10.10. Directions of ground-water flow and locations of wells used to delineate redox zonation in the Black Creek aquifer, South Carolina.

taceous in age and dip toward the southeast. Ground-water flow in this regional aquifer system is controlled by recharge in the outcrop area and discharge by diffuse upward leakage near the Atlantic coast (Aucott and Speriran, 1985). Because of this well-defined flow system, it is possible to collect ground-water samples that are oriented along discrete flowpaths (Fig. 10.10).

Concentrations of redox-active dissolved species in this system are plotted versus distance along the flowpath in Fig. 10.11. Concentrations of dissolved oxygen and nitrate are below detectable levels throughout the flow system. Concentrations of dissolved iron are highest near the outcrop area and decrease downgradient (Fig. 10.11a). Concentrations of sulfate are relatively constant for most of the flowpath but decrease between 60 and 80 miles along the flowpath (Fig. 10.11b). Methane concentrations are low throughout the flow system but increase between 60 and 80 miles along the flowpath (Fig. 10.11c). Finally, H_2 concentrations are in the 2–4-nM range for the first 60 miles of flowpath, and increase to 5 nM by 80 miles along the flowpath (Fig. 10.11d).

These water chemistry data illustrate a common dilemma when only electron acceptor consumption and final product production are available for identifying ongoing redox processes. The water is anoxic and lacks measurable nitrate con-

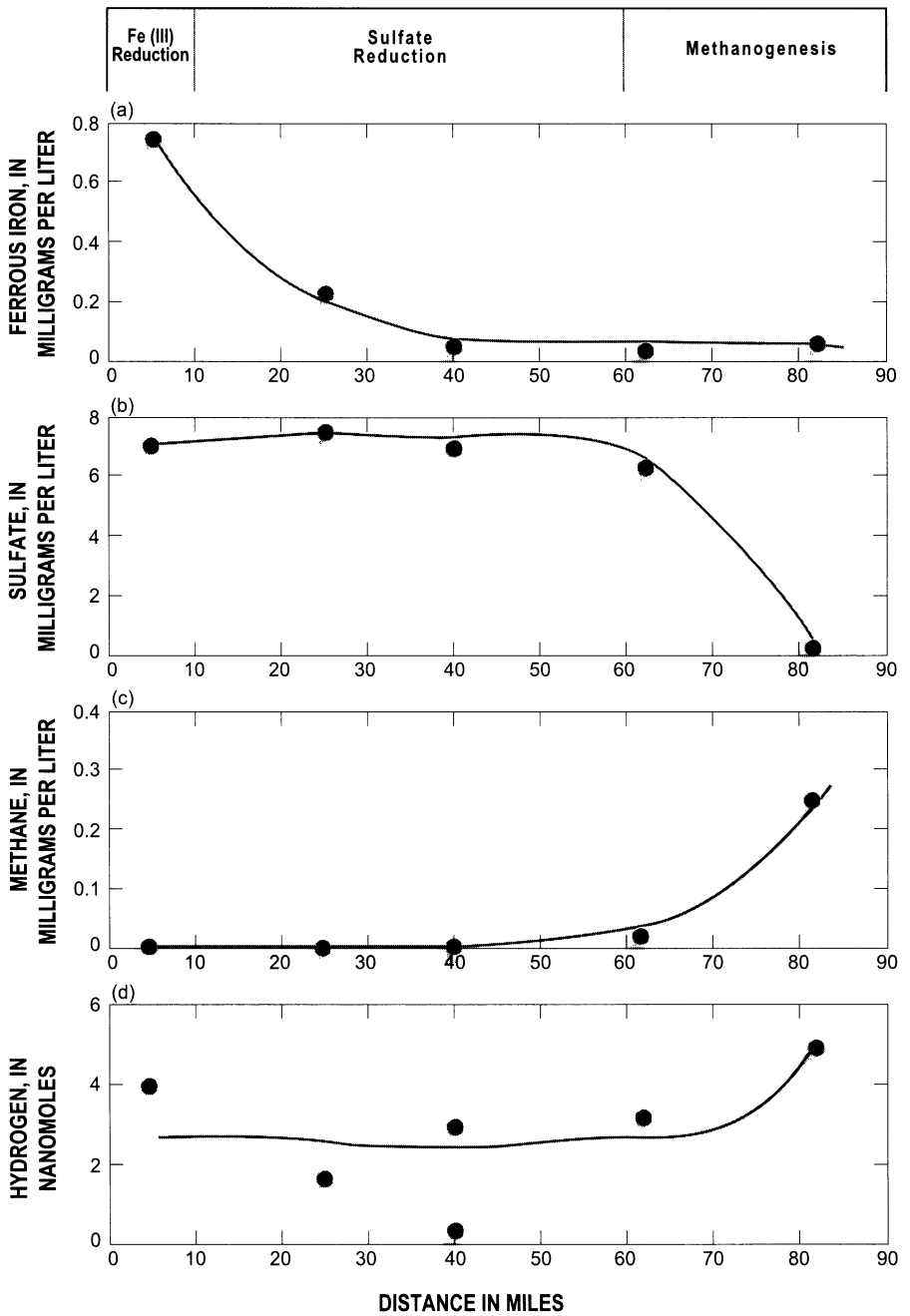


Figure 10.11. Concentration changes of dissolved iron, sulfate, methane, and hydrogen along the flowpath of the Black Creek aquifer, South Carolina.

centrations throughout the flow system which rules out oxygen reduction and nitrate reduction as possible TEAPs. The high concentration of iron at the beginning of the flowpath suggests Fe(III) reduction, but subsequent declines of Fe^{2+} concentrations do not indicate active Fe(III) in downgradient parts of the aquifer. Even more puzzling is the behavior of sulfate, which maintains relatively constant concentrations until between 70 and 80 miles along the flowpath. Only the methane data appear to indicate the initiation of methanogenesis in the most downgradient portion of the aquifer. Thus, throughout most of this flow system, it is not possible to determine the operative TEAP.

However, hydrogen concentrations in the 2–4-nM range indicate sulfate reduction as the operative TEAP. At first, this seemed to be in conflict with the observation that sulfate concentrations are relatively constant in this flow system. However, additional studies have shown that the confining beds overlying and underlying the Black Creek aquifer contain pore water that has relatively high concentrations of sulfate (Chapelle and McMahon, 1991). In this system, it appears that diffusion from confining beds provides a constant flux of sulfate to the Black Creek aquifer, and that this sulfate flux supports sulfate reduction throughout most of the aquifer. Petrographic studies have shown that iron sulfide cements are present at the contact between the confining beds and the Black Creek aquifer, which is consistent with this interpretation (McMahon et al., 1992).

The Black Creek aquifer is an example of how just considering electron acceptor consumption and final product production can lead to erroneous conclusions about the distribution of TEAPs in ground-water systems. In the absence of the H_2 measurements, it would only be possible to postulate Fe(III) reduction at the beginning of the flowpath and methanogenesis at the end of the flowpath. The importance of sulfate reduction, which is the most important redox process in the aquifer, could not be recognized.

10.4.2 Floridan Aquifer of Georgia

Delineation of redox processes in ground-water systems is greatly facilitated by a well-defined and predictable flow system such as the Black Creek aquifer of South Carolina. However, many ground-water systems occur in fractured rocks in which discrete flowpaths are difficult or impossible to define reliably. In aquifers like this, evaluation of redox processes is still possible. An example of such a delineation is the Floridan aquifer of Georgia.

The Floridan aquifer, which underlies much of the southeastern United States, is one of the most productive and economically important hydrologic systems in the world. The aquifer consists of indurated Tertiary limestones and dolomites characterized by the presence of modular gypsum and by locally extensive fracture systems (Miller, 1986). Where this unit crops out at land surface, secondary dissolution features (Karst topography) such as sinkholes and disappearing streams are common. These features contribute to particular water-quality problems, such as is observed near the town of Valdosta, Georgia (Krause, 1976).

Near Valdosta, the channel of the Withlacoochee River intersects a series of sinkholes (Fig. 10.12). These sinkholes capture a portion of the river's flow and the water recharges the Floridan aquifer. Because the Withlacoochee River is a typical South Georgia blackwater stream, characterized by high concentrations of

dissolved and particulate organic carbon, a large amount of metabolizable carbon is delivered to the Floridan aquifer at this location. This metabolizable organic carbon is a potential electron donor for microbial metabolism. The presence of such metabolism is indicated by the rapid decrease of total organic carbon (TOC) along the flowpath of the aquifer (Fig. 10.12) and by the accumulation of final products such as sulfide (~1 mg/L) and methane (~1 mg/L).

Concentrations of dissolved oxygen, sulfate, sulfide, methane, and H_2 plotted versus distance from the recharge sinkhole are shown in Figure 10.13. In the up-

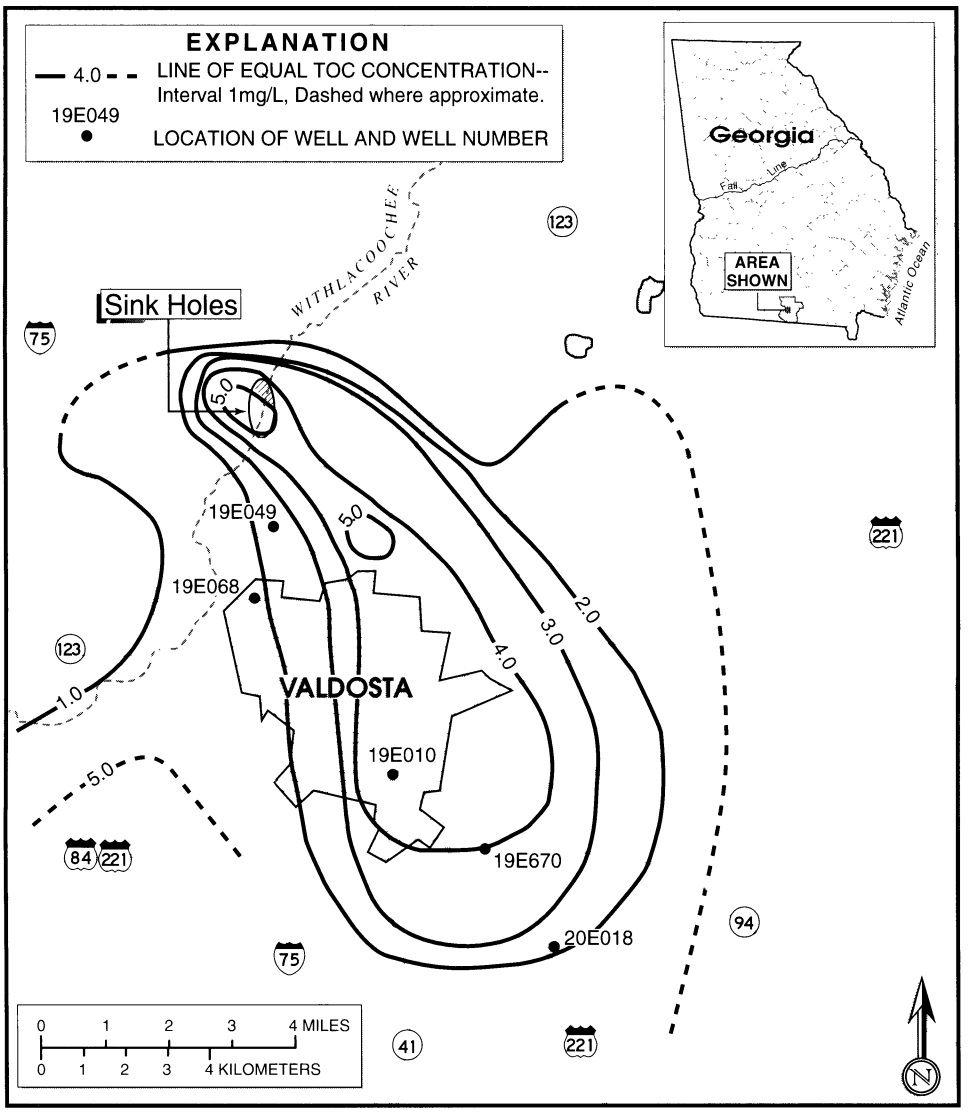


Figure 10.12. Plume of high total organic carbon (TOC) concentrations in water recharging the Floridan aquifer from the Withlacoochee River near Valdosta, Georgia and locations of wells used to delineate redox conditions.

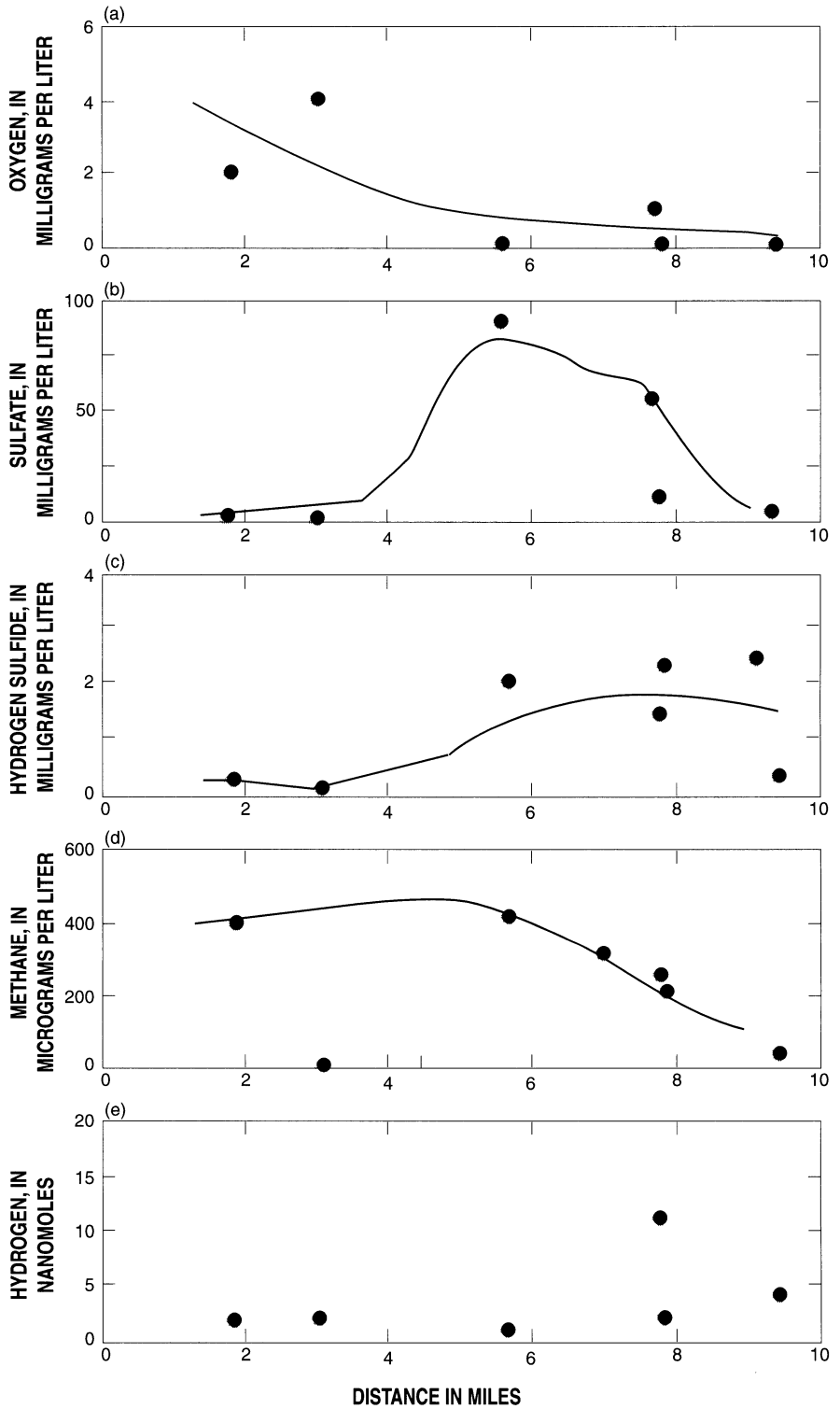


Figure 10.13. Concentrations of oxygen, sulfate, hydrogen sulfide, methane, and hydrogen in ground-water from the Floridan aquifer near Valdosta.

gradient part of the aquifer, dissolved oxygen was present, indicating the presence of aerobic metabolism. Concentrations of sulfate, sulfide, methane, and H_2 were variable. Sulfate was low near the recharge area, increased downgradient, then decreased. This pattern was mirrored by dissolved sulfide concentrations. Methane concentrations show little systematic pattern. Hydrogen concentrations ranged from 1 to 10 nM, with four wells exhibiting concentrations in the 1–4-nM range characteristic of sulfate reduction.

In a ground-water system like this, where ground-water flow occurs along fracture planes and solution conduits, it is difficult to delineate well-defined redox zones. Rather, it appears that different wells, even though placed relatively near each other, often tap different sets of fractures that have much different water chemistry. The unpredictable occurrence of nodular gypsum also contributes to the redox heterogeneity. This heterogeneity is illustrated in Figure 10.14, which shows concentrations of sulfate and H_2 in anaerobic ground water in this system. Where

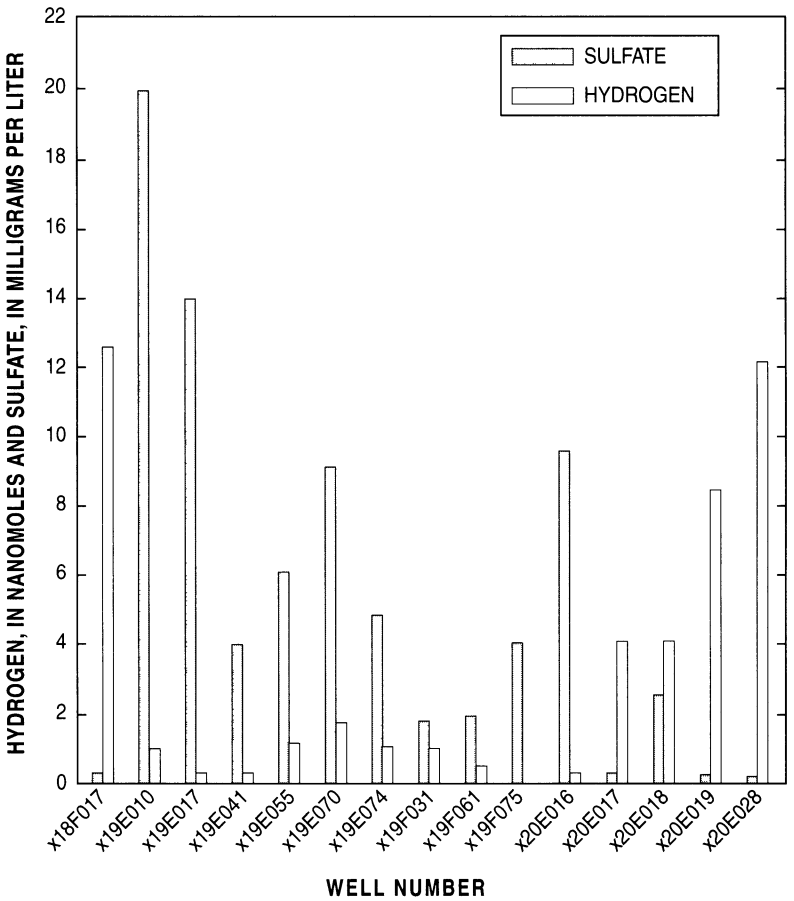


Figure 10.14. Comparison of hydrogen and sulfate concentrations in anaerobic ground water from the Floridan aquifer near Valdosta. Note that where sulfate concentrations are high, hydrogen concentrations are poised in the 1 nM range, whereas when sulfate concentrations are low, hydrogen concentrations are in the 4–20 nM range.

sulfate concentrations are greater than 1 mg/L, H_2 concentrations are generally in the range characteristic of sulfate reduction. However, when sulfate concentrations are less than 1 mg/L, H_2 concentrations are in the 5–20-nM range characteristic of methanogenesis. This suggests that, while the spatial distribution of redox zones is not predictable due to aquifer inhomogeneities, redox processes nevertheless follow the same pattern of microbial competition observed in more homogeneous aquifers.

Microbial physiology, and the interaction of this physiology with ground-water systems, leads to well-defined redox zones in ground-water systems. However, depending on the hydrologic characteristics of these aquifers, the distribution of these redox zones may be more or less regular. The Floridan aquifer near Valdosta is an example of highly irregular redox zones can occur due to caused by aquifer inhomogeneities associated with nodular gypsum and hydrologic complexity associated with fracture flow.

10.5 REDOX PROCESSES IN CONTAMINATED GROUND-WATER SYSTEMS

The framework used for identifying redox processes in pristine ground-water systems (Fig. 10.9) is applicable to contaminated aquifers as well. The principal difference between redox zones in pristine and contaminated ground-water systems generally has to do with the scale of the zones that develop. Because many organic contaminants acts as electron donors in microbial metabolism, they tend to compress the size of redox zonation. Thus, while pristine aquifers commonly have Fe(III)-reducing or methanogenic redox zones tens of miles wide, many contaminated aquifers have redox zones compressed to tens of meters (or less) wide. The methodology for determining redox zonation in contaminated aquifers, therefore, is substantially similar to that of pristine aquifers. This methodology is first illustrated for a petroleum hydrocarbon–contaminated aquifer, and then for a chlorinated ethene–contaminated aquifer.

10.5.1 A Petroleum Hydrocarbon–Contaminated Aquifer—Charleston, South Carolina

The contamination of shallow water table aquifers with petroleum hydrocarbons is a common environmental problem. Because of the large amounts of metabolizable carbon associated with petroleum spills, they are commonly anoxic. At one time it was thought that petroleum hydrocarbons were not biodegraded under anoxic conditions. In more recent years, however, it has been shown that petroleum hydrocarbons degrade under methanogenic (Grbić-Galić and Vogel, 1987), sulfate-reducing (Lovley refs), Fe(III)-reducing (Lovley et al., 1989), and nitrate-reducing (Hutchins et al., 1991) conditions. Furthermore, it has been shown that discrete zones of anaerobic microbial processes develop around petroleum spills that can be identified based on electron acceptor consumption and final product production (Baedecker et al., 1988).

The Defense Fuel Supply Center, located near Charleston, South Carolina (Fig. 10.15a), has experienced a number of accidental fuel spills since the 1940s. The

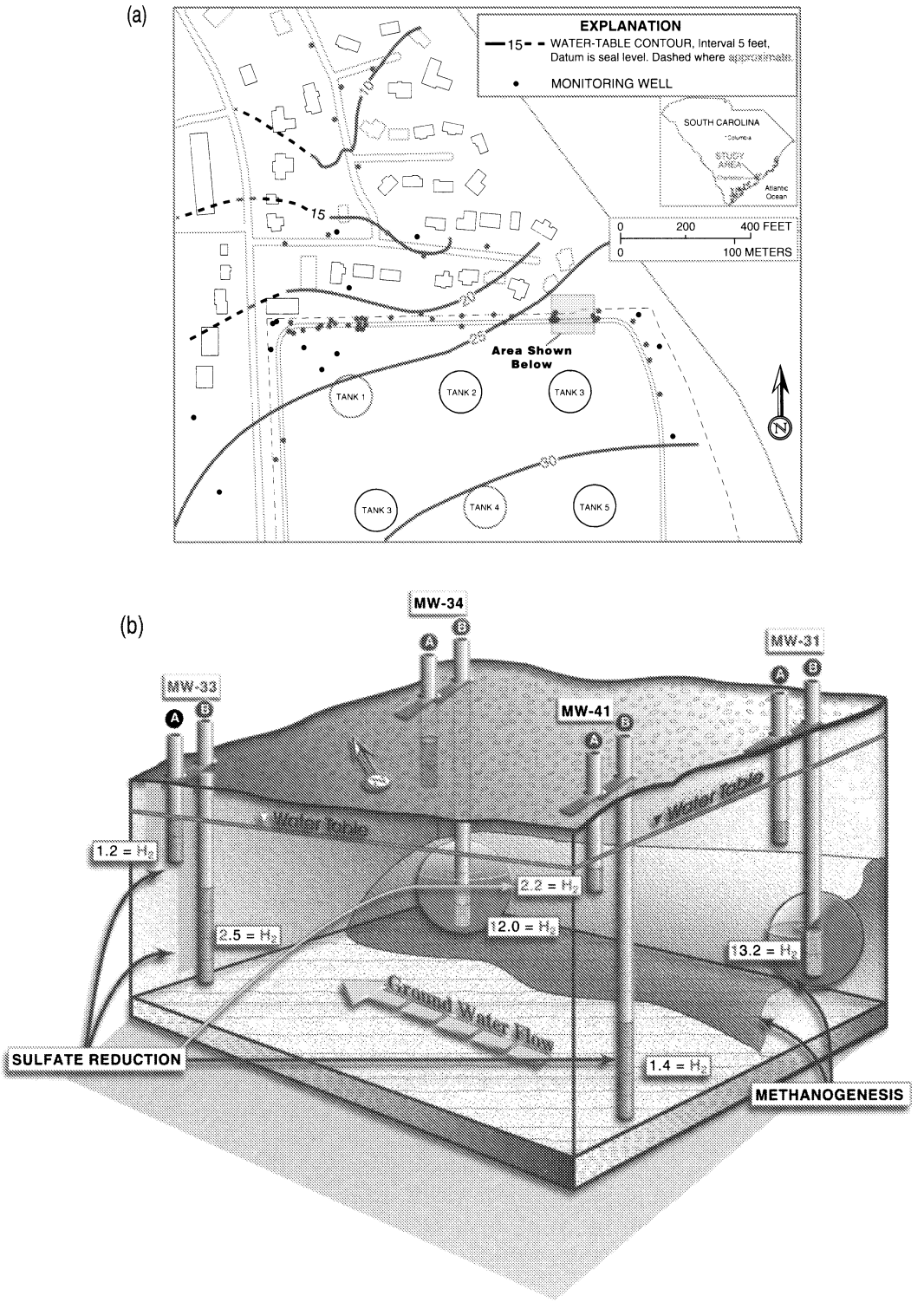


Figure 10.15. Small-scale distribution of redox processes in a portion of a petroleum-hydrocarbon contaminated aquifer in Hanahan, South Carolina.

shallow water table aquifer underlying the site is about 30 feet thick, and consists of unconsolidated marine sands with lenses of clay. A relatively impermeable unit known as the Cooper Marl underlies the aquifer and serves as a lower confining bed. Directions of ground-water flow and the zonation of redox processes in a small portion of the site are shown schematically in Figure 10.15b. Concentrations of oxygen were measurable in two shallow wells at the time of this sampling (Sept. 1992). Concentrations of nitrate and sulfate decreased with depth in the system, whereas concentrations of sulfide and methane increased with depth. Hydrogen concentrations ranged from 13.2 to 1.2 nM, with the highest concentrations found in the deep sampling intervals. In all cases, concentrations of H_2 were in the 1–4-nM range when sulfate concentrations were greater than 0.6 mg/L, and in the 12–23-nM range when sulfate concentrations were less than 0.6 mg/L.

The vertical and lateral zonation of redox processes in this part of the site are shown schematically in Figure 10.15b. The deep zone tapped by wells MW-31B and MW-34B has low-sulfate water with hydrogen concentrations in the 10–20-nM range characteristic of methanogenesis. The shallow zone tapped by wells MW-31A and MW-34A, and both the deep and shallow zones tapped by well clusters MW-41 and MW-33, had sulfate-containing water with H_2 concentrations in the 1–4-nM range characteristic of sulfate reduction. These data show the relatively small-scale redox zones that have developed in this aquifer. Experience at numerous other sites with petroleum hydrocarbon contamination show similar small-scale redox zones that have developed in response to the large supply of electron donors (Chapelle et al., 1996; McMahon and Bruce, 1997).

Data from this site have also demonstrated that the distribution of redox processes is not constant in time, but rather they vary depending on atmospheric recharge events (Vroblesky and Chapelle, 1994). Specifically, one set of H_2 measurements made during June and July 1991, a period of time following long drought (i.e., a lack of recharge), showed that methanogenesis was the predominant microbial process at this site (Fig. 10.16a). In contrast, some months later following a period of rain, the methanogenic zone had shrunk considerably and sulfate reduction was much more areally extensive (Fig. 10.16b). In this system, dry deposition of sulfate on land surface is extensive due to a nearby pulp-paper mill. Vertically percolating recharge carries this sulfate to the water table, initiating sulfate-reducing conditions. Similar behavior has been documented at other sites as well (McMahon and Bruce, 1997). This indicates that, contrary to the widely held notion that redox zonation in aquifers is static, redox zones can grow or shrink significantly, depending on the delivery of alternative electron acceptors by recharge events. This, in turn, suggests that dynamic changes in redox zonation are fairly common in shallow local flow systems.

10.5.2 A Mixed Petroleum Hydrocarbon/Chlorinated Solvent–Contaminated Aquifer, Plattsburgh, New York

Most instances of ground-water contamination involve several kinds of chemical contaminants. In the 1950s and 1960s, for example, the U.S. Air Force used waste petroleum hydrocarbons and solvents as fuels in fire-training exercises. These fuels were sprayed onto simulated aircraft in "fire-training" pits, and the aircraft ignited to give fire crews experience in extinguishing fires. Not surprisingly, large quantities

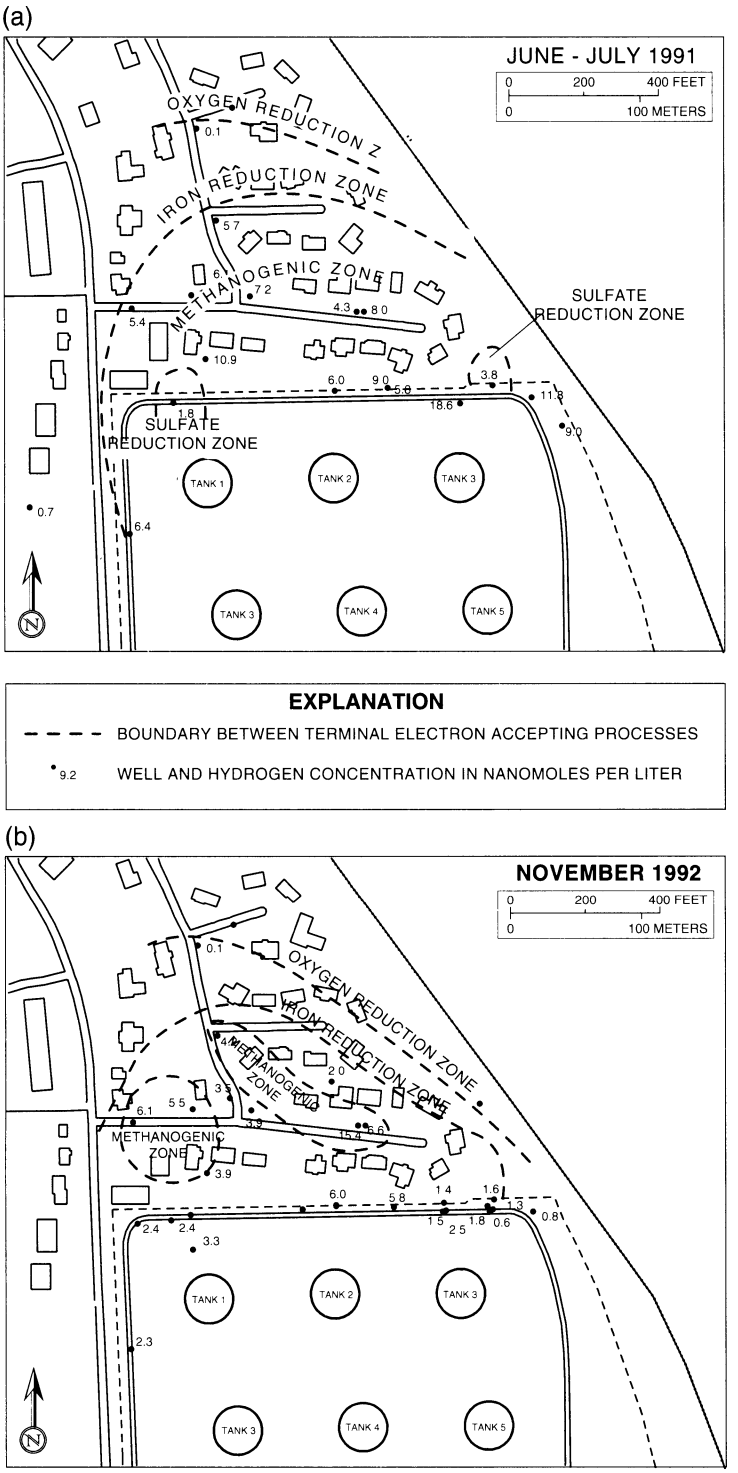


Figure 10.16. Temporal changes shift of redox processes from predominantly methanogenesis to predominantly sulfate reduction in a petroleum hydrocarbon-contaminated aquifer. This shift was due to the lack of recharge to the aquifer in the June–July period in 1991, and abundant sulfate-bearing recharge to the aquifer in November, 1992.

of mixed solvents and fuels seeped to underlying ground-water systems, causing widespread contamination. One example of this kind of mixed petroleum hydrocarbon/chlorinated solvent contamination occurred at the now-closed Plattsburgh Air Force Base in New York.

The fire-training area at Plattsburgh AFB was located on a ridge overlooking the main runway (Fig. 10.17). This ridge is underlain by highly permeable glacial outwash sediments consisting of interbedded sands and gravels. Underneath the fire-training pit, a pool of mixed solvents and jet fuel developed over the years. A plume of benzene-toluene-ethylbenzene-xylene (BTEX) and chlorinated ethene-contaminated ground-water emanated from the free product. Concentrations of dissolved oxygen were depleted in the plume (Fig. 10.17a), concentrations of dissolved iron were elevated (Fig. 10.17b), concentrations of methane were elevated (Fig. 10.17c), and measured Eh values were negative. The measured Eh of ground water, when plotted on a standard eH-pH diagram (Fig. 10.18), suggests that Fe(III) reduction is the predominant electron-accepting process. However, the documented presence of methane (Fig. 10.17c) clearly shows that methanogenesis is ongoing in the system. This illustrates the fact that, while Eh measurements can identify anoxic ground water, they cannot be used to determine which electron-accepting processes predominate in an aquifer. The most probable reason why measured Eh values plot near the Fe^{3+}/Fe^{2+} join on the Eh-pH diagram probably reflects the relative sensitivity of the platinum electrode to the Fe^{3+}/Fe^{2+} redox couple, and the relative insensitivity of the probe to the CO_2/CH_4 redox couple.

Hydrogen concentrations in this system (Fig. 10.17d) indicate a well-defined methanogenic zone near the source area surrounded by largely Fe(III)-reducing conditions away from the source area. It is interesting to note that the highest concentrations of methane (Fig. 10.17c) do *not* coincide with the methanogenic zone indicated by hydrogen. This is commonly observed in aquifer systems, and reflects the fact that methane is progressively produced as ground-water flows through the methanogenic area. The highest methane concentrations, therefore, occur *downgradient* of the methanogenic zone, not in the methanogenic zone itself.

10.5.3 Complications Associated with Small-Scale Redox Zones

The microbial ecology of ground-water systems tends to cause a segregation of redox processes into distinct zones. However, the scale of these redox processes varies widely. In pristine ground-water systems, it is common for redox zones to be miles or tens of miles wide. In contaminated ground-water systems, these redox zones tend to be somewhat smaller. An example of small-scale redox zonation was given by Chapelle et al. (1996) for a contaminated glacial outwash aquifer near the town of Oscoda, Michigan.

The study site was, like Plattsburgh AFB, a fire-training pit used during the 1950s and 1960s. A plume of contaminated ground-water emanated from the source area and moved downgradient. In order to document the vertical distribution of contaminants in the aquifer, the National Center for Integrated Bioremediation Research and Development (NCIBRD) installed a series of multilevel sampling wells at the site. The wells were constructed of 1-inch PVC casing equipped with 1-foot screens. The vertical spacing of the screens varied from about 1 foot to 6 feet. The multilevel wells were oriented in a line perpendicular to the direction of ground-

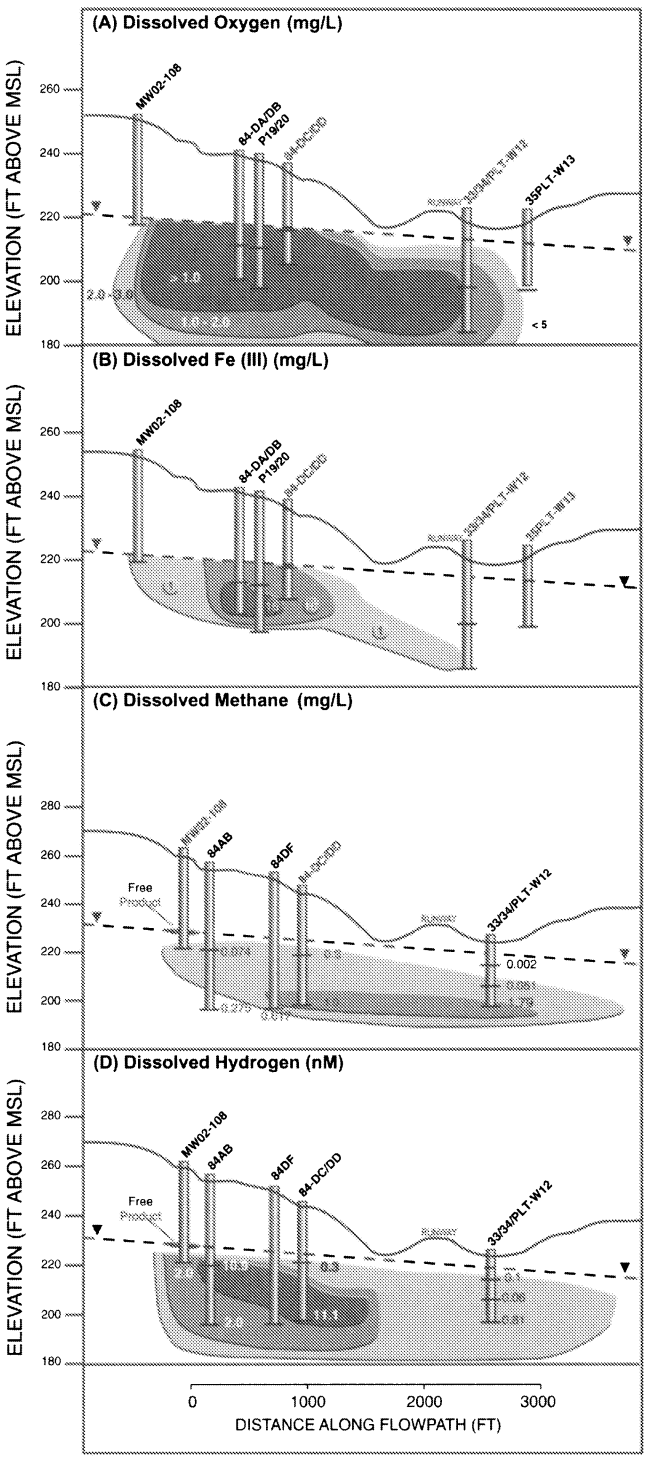


Figure 10.17. Concentrations of oxygen, sulfate, iron, methane, and hydrogen in shallow ground water underlying a burn-pit at the former Plattsburgh Air Force Base, New York.

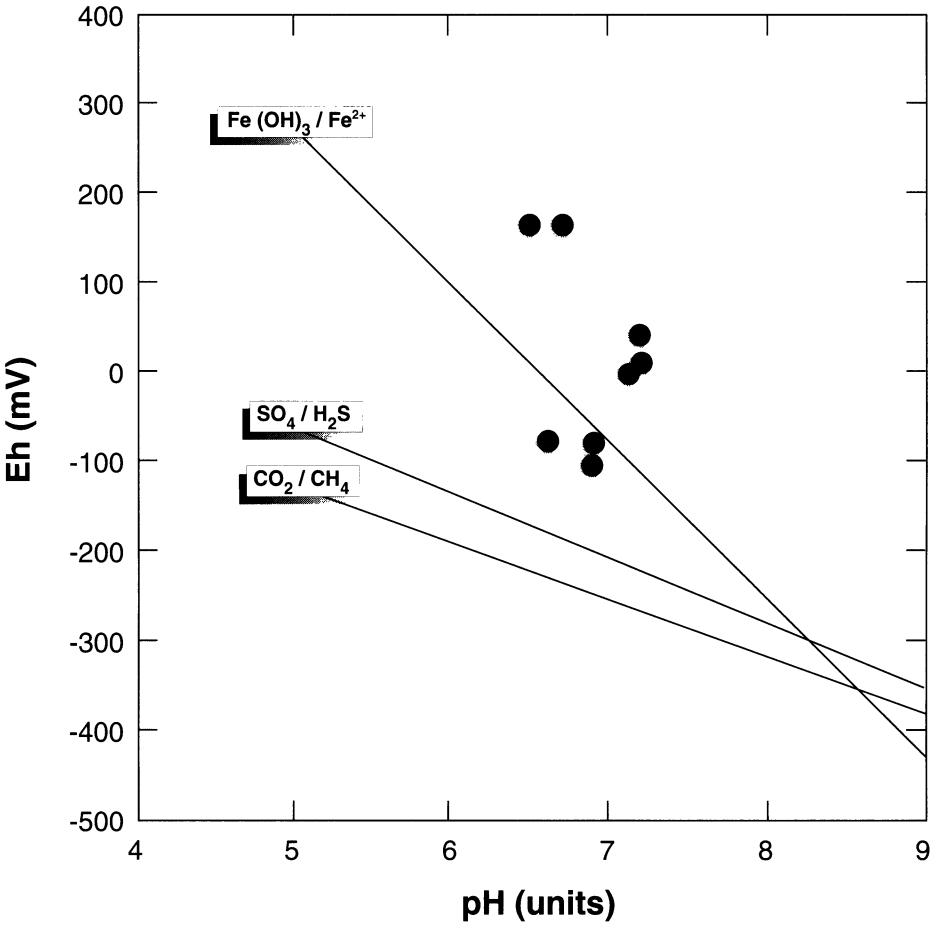


Figure 10.18. Measurements of Eh in shallow ground water underlying a burn-pit at the former Plattsburgh Air Force Base, New York.

water flow, and were used to delineate the small-scale distribution of redox processes at this site.

Concentrations of dissolved oxygen, ferrous iron, sulfate, and methane in the cross section of the plume are shown in Figure 10.19a–d. In the core of the plume, dissolved oxygen and sulfate is absent, while concentrations of dissolved iron and methane are elevated. Hydrogen concentrations in the plume indicate a discrete zone of methanogenesis near the water table surrounded by a discrete zone of sulfate reduction. These zones, in turn, were surrounded by Fe(III)-reducing and oxygen-reducing zones (Fig. 10.20). These data indicate that the different redox zones in this aquifer are relatively small and extend only a matter of meters. Furthermore, it is entirely possible, for TEAP zones to be on the order of centimeters. The small-scale nature of redox zones, particularly in shallow water table aquifers, must be carefully considered.

The small-scale nature of some redox zones raises the possibility that sampling wells, particularly wells with long screened intervals, may screen more then one

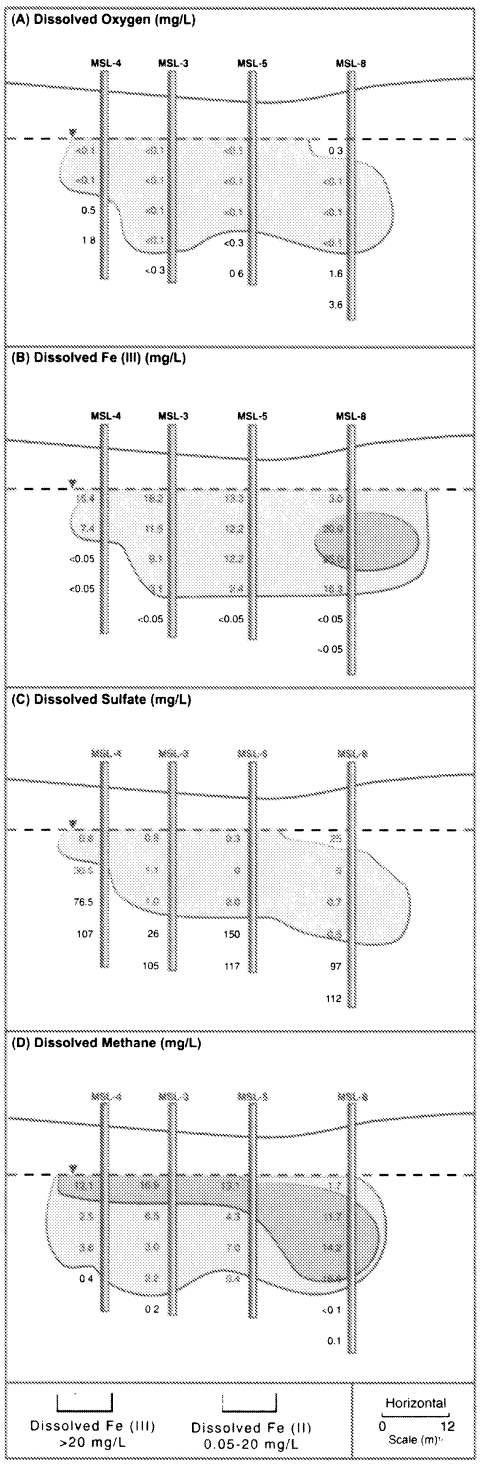


Figure 10.19. Concentrations of oxygen, iron, sulfate, and methane in a cross section of an aquifer downgradient of a burn-pit at the former Wurtsmith Air Force Base, Oscoda, Michigan.

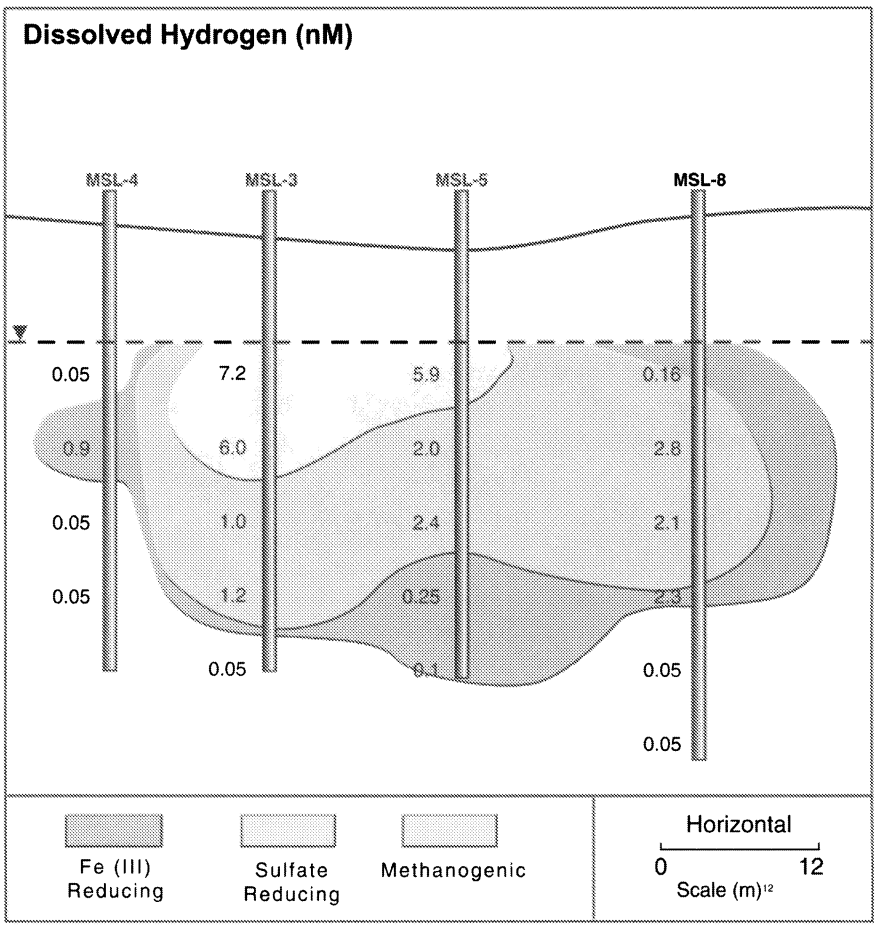


Figure 10.20. Hydrogen concentrations in a cross section of an aquifer downgradient of a burn-pit at the former Wurtsmith Air Force Base, Oscoda, Michigan.

redox zone. In this aquifer, for example, even though the screened intervals were small, there was evidence of such mixing of redox zones. Specifically, H_2 measurements indicated the predominance of sulfate reduction in several screened intervals that lacked significant (<1 mg/L) concentrations of sulfate. This may reflect the fact that a pumping well may draw water from small-scale zones dominated by different redox processes. If a well draws equal volumes of water from closely spaced Fe(III)-reducing ($H_2 \sim 0.2$ nM) and methanogenic ($H_2 \sim 7$ nM) zones, the resulting H_2 measurement (3.6 nM) would inaccurately indicate the predominance of sulfate reduction. Such interzonal mixing may complicate the interpretation of H_2 measurements in systems characterized by small-scale redox zones. For this reason, using H_2 as a sole indicator of redox processes is not appropriate. A more reliable procedure is to always interpret H_2 concentrations in the context of electron-acceptor availability and final product production (Fig. 10.9). In any case, it is important to realize that sampling artifacts caused by small-scale redox zones interacting with sampling wells can affect H_2 measurements, and these potential artifacts must always be considered during data interpretation.

10.6 KINETIC MODELING OF REDOX PROCESSES

In a kinetic approach to redox processes in ground-water systems, we have seen how microbial ecology leads to segregation of redox processes in space, how spatial redox zones can vary with time, and how it is possible to deduce the distribution of redox processes. In order to complete this treatment, it is also important to show how such dynamic redox processes can be modeled. Models describing the equilibrium approach to redox processes are well known (Wolery, 1979; Parkhurst et al., 1980; Kharaka et al., 1988; Allison et al., 1991; Plummer et al., 1991). However, models describing a fully kinetic approach to redox processes have only recently been developed (Wang and Van Cappellen, 1996; Hunter et al., 1998). While kinetic modeling of redox processes is a relatively recent development, these models show much promise for more accurately simulating complex redox behavior in both pristine and contaminated ground-water systems.

10.6.1 Electron Flow in Ground-Water Systems

We have seen that an equilibrium description of redox processes emphasizes the electrostatic (voltage) condition, whereas a kinetic description emphasizes an electrodynamic (amperage) condition. It is conceptually possible to calculate overall electron flow in ground-water systems. For example, if it is assumed that the most important electron donor for microbial metabolism is organic carbon of valence zero (on average), the number of electrons mobilized by complete oxidation of the carbon to valence +4 is accompanied by release of four electrons:

$$C(0) \rightarrow C(+4) + 4e^-$$

For each mole of organic carbon oxidized, therefore, $4 \times 6.02 \times 10^{23}$ electrons (where 6.02×10^{23} is Avogadro's number) are available to flow through a microorganism's electron transport chain. Furthermore, because 1 Coulomb of electrical charge consists of 6.3×10^{18} electrons, it is possible to directly convert organic carbon oxidation per unit time into electrical charge circulated per unit time. If it is assumed that dissolved inorganic carbon derived from organic carbon oxidation is the sole source of electron flow in a ground-water system, it is possible to calculate the total electron flow per unit time (amperage) in that system.

For example, in the pristine Black Creek aquifer of South Carolina (see section 10.4.1), the rate of organic carbon oxidation is approximately 10^{-4} mmoles per liter of water per year. Thus, we have

$$10^{-4}\ \text{mM/L/yr C} \frac{4(6.02 \times 10^{23})e^-}{1\ \text{mM C}} = 24 \times 10^{10}\ e^-/\text{L/yr}$$

or

$$24 \times 10^{19}\ e^-/\text{L/yr} \times \frac{\text{Coulombs}}{6.3 \times 10^{18}\ e^-} = 4 \times 10^1\ \text{Coulombs/L/yr}$$

Converting coulombs per year to coulombs per second, and remembering that the definition of amperage is coulombs per second, gives:

$$1.3 \times 10^{-7} \text{ Amperes/L}$$

In other words, it is possible to estimate the total electron flow in this system per liter of ground water flowing along a given flowpath segment. By way of comparison, one can calculate the approximate electron flow in a petroleum hydrocarbon–contaminated aquifer as well. The contaminated aquifer underlying the Defense Fuel Supply Center near Charleston, South Carolina (section 10.5.1), for example, shows a dissolved inorganic carbon production rate of about 1.3×10^{1} mM/L/yr, which converts to about 0.24 amperes per liter. In other words, there is about 6 orders of magnitude more electron flow in the organic-rich petroleum contaminated aquifer as there is in the organic-poor pristine aquifer system.

This analysis shows that it is indeed possible to describe redox processes in ground-water systems in terms of standard electrodynamic nomenclature (i.e., electrical current). However, because these electron transfer processes are mediated by the individual electron-transport systems of many different microorganisms, and because the electrons are transferred to many different electron acceptors, there is little practical benefit to calculating the overall amperage characteristic of a ground-water system. Rather, a useful kinetic description of electron flow in ground-water systems must describe electron-transfer processes to each different terminal electron-accepting process, and describe the chemical transformations that accompany these processes. Such kinetic models that couple electron-transfer processes to solid-phase and dissolved constituents in ground-water systems have recently been developed and are described below.

10.6.2 Developing Kinetic Models of Microbial Redox Processes

Hunter et al. (1997) described the development of a fully kinetic model, called BIORXNTRN, for microbially mediated redox processes in ground-water systems. In this model, the oxidation of dissolved organic carbon (DOC) and particulate organic carbon (POC) is described by first-order kinetics:

$$R_i^{\text{DOC}} = k_i^{\text{DOC}}[\text{DOC}]_i \tag{6}$$

$$R_i^{\text{DOC}} = k_i^{\text{DOC}}[\text{DOC}]_i \tag{7}$$

where R represents the rate of organic matter degradation, k is the first-order rate constant, DOC is dissolved organic carbon, and POC is solid carbon present in the aquifer matrix. The subscript i indicates the ith fraction of the dissolved or particulate organic matter. This is designed to reproduce the observation that microorganisms preferentially utilize more readily bioavailable carbon first, and turn to more refractory carbon compounds only after readily bioavailable carbon has been exhausted (Jürgenson, 1978). BIORXNTRN allows up to four carbon fractions to be specified, each with its own degradation rate constant, for both DOC and POC. As we saw in section 10.5.1, the rate that electrons are released by organic matter oxidation is easily calculated from reaction stoichiometery. However, the electrons produced are transferred to the terminal electron acceptors O_2, NO_3^-, Mn(IV), Fe(III), SO_4^{2-}, or CO_2, according to the stoichiometry of each half-reaction. The assignment of electrons to each potential electron-accepting process assumes that if O_2 is present above 20 μM (0.64 mg/L), O_2 is the sole electron acceptor. Once

O_2 concentrations drop below this limit, NO_3^- becomes the primary electron acceptor. Once NO_3^- concentrations drop below 5 μM, Mn(IV) becomes the electron acceptor and so on. Because both Mn(IV) and Fe(III) are present as solid-phase minerals, their availability is expressed in terms of μmol/dm^3. The limiting concentration for both Mn(IV) and Fe(III) was set at 60 μmol/dm^3. Finally, the limiting concentration for dissolved sulfate is 0.03 μM (0.003 mg/L). This procedure reproduces the sequential utilization of electron acceptors that is observed in aquatic sediments and aquifers. However, it also allows the coexistence of processes when electron acceptor concentrations are lower. Thus, when O_2 concentrations drop below 0.64 mg/L, oxygen reduction cooccurs with nitrate reduction.

The primary reduction processes considered by BIORXNTRN produce chemically active reduced species such as Mn^{2+}, Fe^{2+}, NH_4^+, HS^-, and CH_4. These species interact in various ways that are represented in the model by bimolecular rate expressions and apparent rate coefficients. Also considered in the model are non-redox mineral precipitation and dissolution reactions for carbonates, sulfides, and sulfate phases.

The dissolved species considered by BIORXNTRN are transported by advective ground-water flow and dispersion. For each dissolved species, the mass balance equation

$$\left(1 + \frac{K_i^{\text{ads}}}{\phi}\right) \frac{\partial C_i}{\partial t} = \nabla \cdot (D \cdot \nabla C_i) - \nabla \cdot (VC_i) + \frac{1}{\phi} R_i^{\text{aq}} \tag{8}$$

is solved, where C_i is the concentration of the ith species, D is the dispersion tensor, V the velocity of ground-water flow, K_i^{ads} the adsorption constant of component i, and R_i^{aq} the net rate of production of dissolved component i. The aquifer matrix is assumed to be immobile, and thus the mass balance for a solid species is simply

$$\frac{\partial \overline{C}_i}{\partial t} = R_i^s \tag{9}$$

where concentrations of the solid species, $\overline{C}_i$, are expressed per unit total volume. Equations 8 and 9 are solved by specifying initial concentrations for all chemical species and appropriate boundary conditions.

In the paper describing the development of BIORXNTRN, Hunter et al. (1998) provide examples of its application. The first example is a simulation of redox processes in a regional aquifer that have developed over 10,000 years (Fig. 10.21). Concentrations of oxygen are initially high at the recharge area and decrease rapidly. Once oxygen has been consumed, NO_3^- concentrations decrease. Once NO_3^- has been consumed, Fe^{2+} concentrations increase. Once Fe(III) has been consumed, $SO_4^=$ concentrations decrease and CH_4 production begins. These patterns are identical to what is actually observed in regional aquifers (Fig. 10.7a; Fig. 10.11).

A second example provided by Hunter et al. (1998) simulates redox processes in a leachate-contaminated aquifer (Fig. 10.22) over 20 years. In this case, the order of redox processes is reversed, with methanogenesis predominated near the leachate source grading to Fe(III) reduction downgradient. This pattern is also identical to what is observed in aquifers characterized by organic loading at the recharge area (Fig. 10.7b; Fig. 10.13).

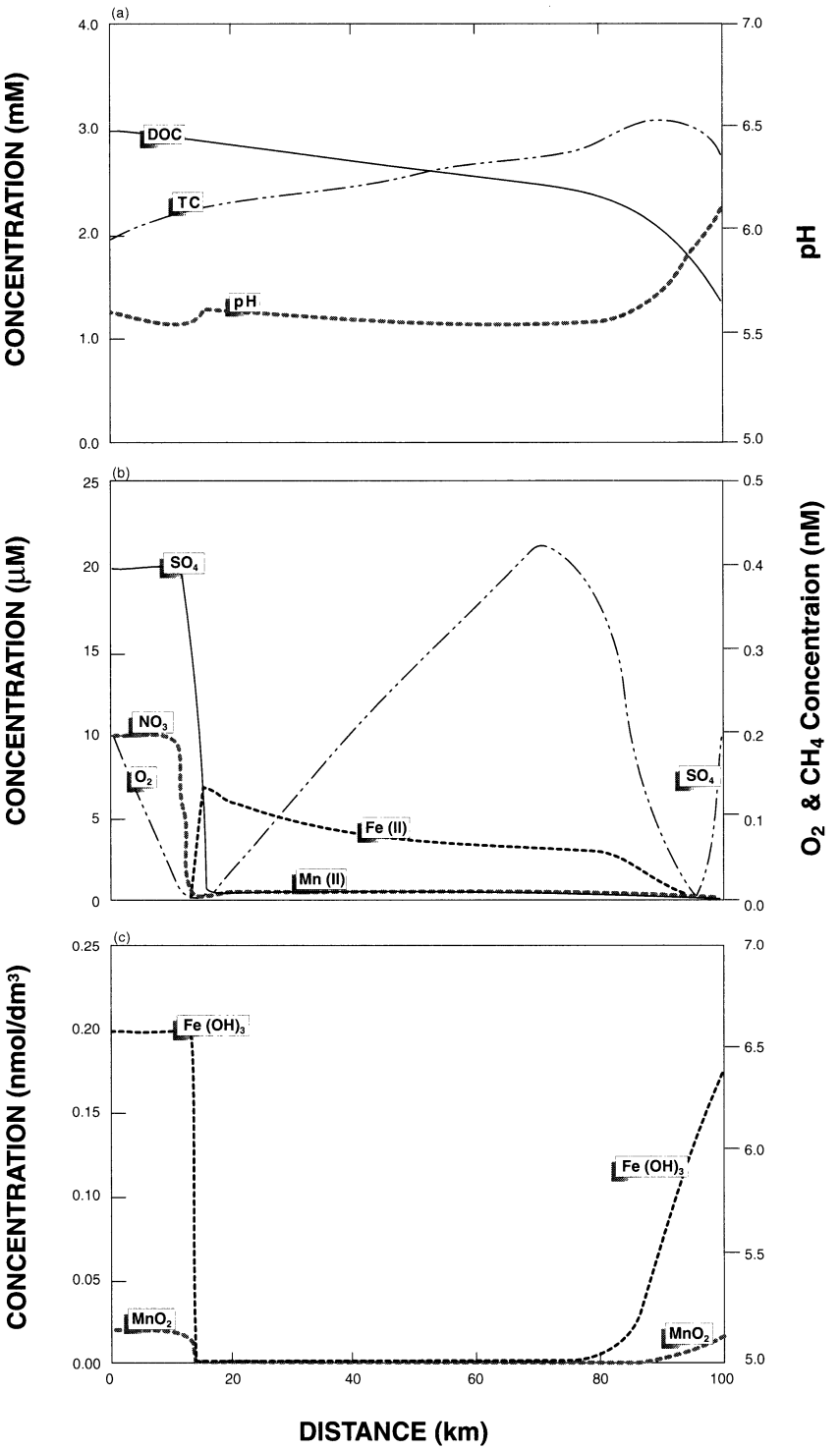


Figure 10.21. Simulated redox conditions in an uncontaminated regional aquifer (Hunter et al., 1998).

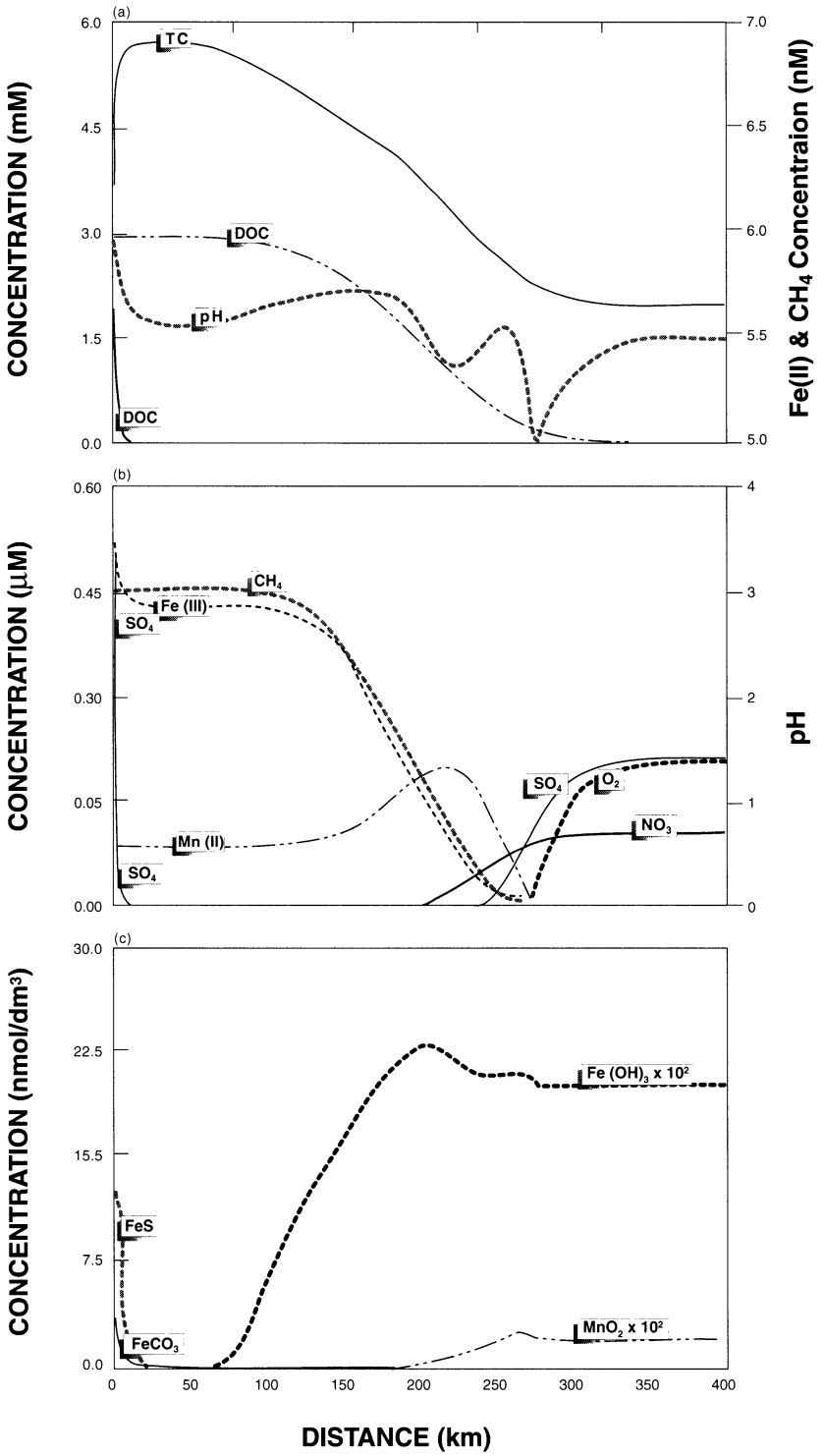


Figure 10.22. Simulated redox conditions in a landfill leachate-contaminated aquifer (Hunter et al., 1998).

These examples show that a fully kinetic treatment of redox processes in ground-water systems is technically feasible. Furthermore, this kinetic approach makes it possible to simulate the complex interactions of microbial and inorganic chemical processes. This approach provides a more realistic and complete representation of physical, chemical, and microbial processes in subsurface environments then has been available in the past.

10.7 CHAPTER SUMMARY

Traditional ground-water geochemistry has emphasized the equilibrium approach for describing redox processes. However, because most redox processes in ground-water systems are driven by kinetic microbial processes, this approach is not proved satisfactory for many applications. By understanding the physiological and ecological properties of microbial processes, it is possible to construct a kinetic description of redox processes. For example, the physiological properties of different electron accepting processes—such as Fe(III) reduction, sulfate reduction, and methanogenesis—lead to differing efficiencies of substrate (hydrogen) utilization. Given these physiological differences, ecological factors such as competitive exclusion lead to the development of discrete redox zones in aquatic sediments and aquifer systems. The scale of these redox zones can range from centimeters to kilometers, depending on a number of hydrologic and geologic factors. Nevertheless, the principles leading to this observed behavior are observed to be consistent between hydrologic systems. The distribution of redox zones in ground-water systems can be deduced by observing patterns of electron acceptor consumption, final product production, and concentrations of dissolved hydrogen. Because of recent advances in biogeochemical modeling, it is now possible to simulate microbial redox processes using a fully kinetic—as opposed to an equilibrium—approach.

QUESTIONS TO CONSIDER

1. Name the assumptions used to define the standard hydrogen electrode.
2. Under what conditions is an eh measurement in effect a hydrogen measurement?
3. Explain how concentrations of dissolved Fe^{2+} and Fe^{3+} can be used to define the eh of an aqueous system. What assumption about concentrations of each dissolved iron species must be made?
4. Assuming that Fe(III)-reducing microorganisms are responsible for observed concentrations of Fe^{2+} in a ground-water system, will calculating an eh based on dissolved Fe^{2+} and Fe^{3+} concentrations (a) underestimate, (b) underestimate, or (c) exactly determine the appropriate value?
5. It is observed that steady-state hydrogen concentrations in ground-water systems reflect the free energy of reaction of ongoing redox processes. Does this observation indicate that these redox processes are at thermodynamic equilibrium?
6. Do hydrogen concentrations in ground water reflect the rate of hydrogen production by fermentative bacteria? Why or why not?

7. Are redox processes in ground-water systems always segregated into distinct zones? Under what conditions is the answer yes, and under what conditions is the answer no?
8. If a ground water lacks dissolved oxygen but contains dissolved methane, does this mean that the well tapping the aquifer is screened in an actively methanogenic zone? What does this observation indicate about the location of methanogenic conditions in the aquifer?
9. Under what conditions can a 2-nM concentration of dissolved hydrogen inaccurately indicate the predominance of sulfate reduction as the ambient TEAP?

PART III

MICROBIAL PROCESSES IN CONTAMINATED GROUND-WATER SYSTEMS

CHAPTER 11

MICROBIAL ACCLIMATION TO GROUND-WATER CONTAMINATION

A topic that never fails to attract interest at professional meetings of microbiologists is the presence of microorganisms in what are called "extreme environments." A handy definition of an extreme environment is one that is either so cold or so hot or so saline or so oligotrophic or so chemically contaminated that it strains human credulity that life can exist at all. Microorganisms have been found living in boiling water emanating from volcanic terrains, on the snowpack of Arctic and Antarctic wastelands, in brines three or four times more concentrated than seawater, in the bottom waters of deep ocean basins, and in sediments contaminated with the most noxious, poisonous, xenobiotic chemicals imaginable. As a general rule, most microbiologists have ceased expressing amazement at the ability of microbes to adapt themselves to difficult circumstances. Rather, understanding the biochemistry of microbial acclimation to stressed environments has become an active field of investigation.

Understanding the mechanisms of microbial acclimation to various stresses is interesting in its own right, but it also has very practical applications to pollution-control technology. Numerous studies of soils, sediments, and aquifer systems have shown that indigenous microorganisms are capable of acclimating to a variety of chemical stresses imposed by human activity. In the process of acclimating themselves to these stresses, microorganisms often accelerate the natural degradation of the chemicals involved. Thus, understanding the acclimation response is basic to the utilization of microorganisms in bioremediation technology.

In this chapter, aspects of microbial acclimation to chemical stresses in ground-water systems are considered. The first step in this discussion is to consider different manifestations of microbial acclimation to chemical stresses. The second step is to discuss the various mechanisms that lead to microbial acclimation. As we shall see, there is a wide variety of biochemical and genetic mechanisms that enable microorganisms to acclimate themselves to various conditions. The final step is to give some examples of microbial acclimation in aquifer systems, and to consider how such acclimation may affect the efficiency of bioremediation efforts.

11.1 MICROBIAL RESPONSE TO ENVIRONMENTAL CHANGES

When microorganisms collected from just about any natural environment are returned to the laboratory and placed in enrichment media, there is often a characteristic lag time before they begin to grow and reproduce actively. The generally accepted reason for this phenomenon is that the conditions encountered in the enrichment media are considerably different from those in the environment to which the microorganisms are accustomed. Even if a microbiologist were careful to provide a carbon source, electron acceptor, trace nutrients, and a physical environment similar to that of the natural environment, there would inevitably be some differences. For example, the enrichment media may contain higher concentrations of carbon sources than are present in the natural environment. The temperature, ionic strength, pH, or one of a dozen other environmental factors may be different from what the microorganisms are used to. Presented with these differences, microorganisms typically require an adjustment period to acclimate themselves to the new conditions. This period of acclimation is the reason for the observed lag time before active growth commences.

The extent of the lag time is dependent upon a number of factors. If the conditions offered are reasonably similar to those in the environment, the lag time generally is fairly short. On the other hand, if there are significant differences between the media and the environment, the lag time generally is more extended. If the differences are too extreme, the microorganisms will be unable to acclimate themselves and will become inactive or eventually die.

Once acclimation to laboratory conditions has been achieved, however, microbial growth becomes exponential and continues until nutrients are exhausted or a buildup of toxic by-products limits growth. When acclimated microorganisms are reinoculated onto fresh media, growth continues at an exponential rate with no lag time. Thus, one indication that microorganisms have acclimated themselves to particular conditions is the absence of a lag time.

In many ways, ground-water contamination is analogous to the case of microorganisms acclimating themselves to a particular growth media under laboratory conditions. In both cases, conditions for growth are substantially changed and microorganisms must adapt themselves to the new conditions. Also, many kinds of contamination offer potential substrates for microorganisms to grow on, although these substrates are often much different from those encountered in pristine aquifers. If this analogy held, one would predict that microorganisms subjected to contamination in subsurface environments would behave in at least two ways. First, when the contaminant was introduced, there should be a lag time as microbial populations adjust themselves to the new conditions. Second, one would expect that microorganisms that were acclimated to the contamination would utilize the contaminants more efficiently than microorganisms that are were not acclimated to the contamination.

This kind of behavior is widely observed in studies of aquatic sediments and contaminated ground-water systems. An example of this observed behavior was given by Madsen et al. (1991) in a study of a shallow water table aquifer contaminated by coal tar derivatives. In sediments cored at the water table (WT) and in the shallow saturated (SS), zones of the pristine part of the aquifer, there was a pronounced lag of ten or twelve days before mineralization of radiolabled

p-hydroxybenzoate was apparent (Fig. 11.1). In sediments cored from within the contaminated zone, on the other hand, mineralization of p-hydroxybenzoate occurred with no lag at all (Fig.11.1).

This behavior, which has been observed in a number of studies, has important implications for bioremediation of contamination in ground-water systems. First of all, it is evident that rates of biodegradation will be a function of the acclimation of indigenous microorganisms to the contaminated conditions. In turn, this implies that biodegradation rates will change over time as acclimation proceeds. Finally, it is evident that acclimation has the potential for substantially increasing biodegradation rates. Thus, technologies for improving the acclimation of indigenous microorganisms to specific compounds are potentially powerful tools for remediating contaminated environments. For these reasons, understanding the metabolic, physiologic, and genetic mechanisms involved in microbial acclimation is especially important.

11.2 MECHANISMS OF ACCLIMATION

Microbial cells are capable of adapting themselves to a wide variety of environmental conditions. For example, bacteria regulate cellular water content by adjusting the properties of their cell walls. Within limits, bacteria can reduce the osmotic flux of water into or out of the cell by adjusting the membrane properties of the cell wall. In similar fashion, bacterial cells can adjust to different pH and temperature conditions. In addition to adapting to various environmental conditions, microorganisms are capable of adjusting their metabolism to new substrates. From

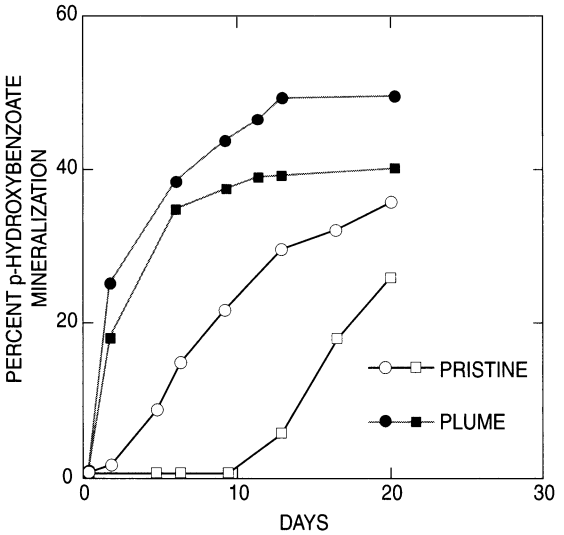


Figure 11.1. Evidence for a lag time in the mineralization PHB by subsurface bacteria in pristine sediments and the lack of a lagtime in PHB-contaminated sediments (Modified from Madsen et al.,1991, with permission courtesy of the American Association forthe Advancement of Science).

the viewpoint of microorganisms present in ground-water systems, contamination events often introduce previously unavailable substrates. It is generally recognized that microbial populations adapt to new substrates by means of three distinct mechanisms: (1) induction of specific enzymes not present (or present at low levels) before exposure, (2) selection of new metabolic capabilities produced by genetic changes, and (3) an increase in the number of organisms able to metabolize newly available substrates.

11.2.1 Induction

The process of induction has been discussed previously in the context of bacterial metabolism (Chapter 4). Induction refers to the series of processes by which microbial cells produce enzymes specific to a particular substrate only when that substrate is actually present. One of the best-studied examples of induction in bacteria is that of the lactose operon in *E. coli*. Metabolism of lactose requires the presence of three enzymes: beta-galactosidase, galactoside permease, and thiogalactoside transacetylase. Beta-galactosidase cleaves the disaccharide lactose into the monosaccharides galactose and glucose, and galactoside permease is required to transport lactose across the cell membrane. The role of thiogalactoside transacetylase is still unclear.

The induction of the enzymes needed to metabolize lactose involves the activation of specific regions of the bacterial genome (Fig. 11.2). The lactose operon consists of a promoter region (P), a regulatory gene (R) that codes for a repressor protein, and an operator region (O) that occurs between the promoter and the genes coding for the lactose enzymes. When lactose is not present in the cell (Fig. 11.2a),

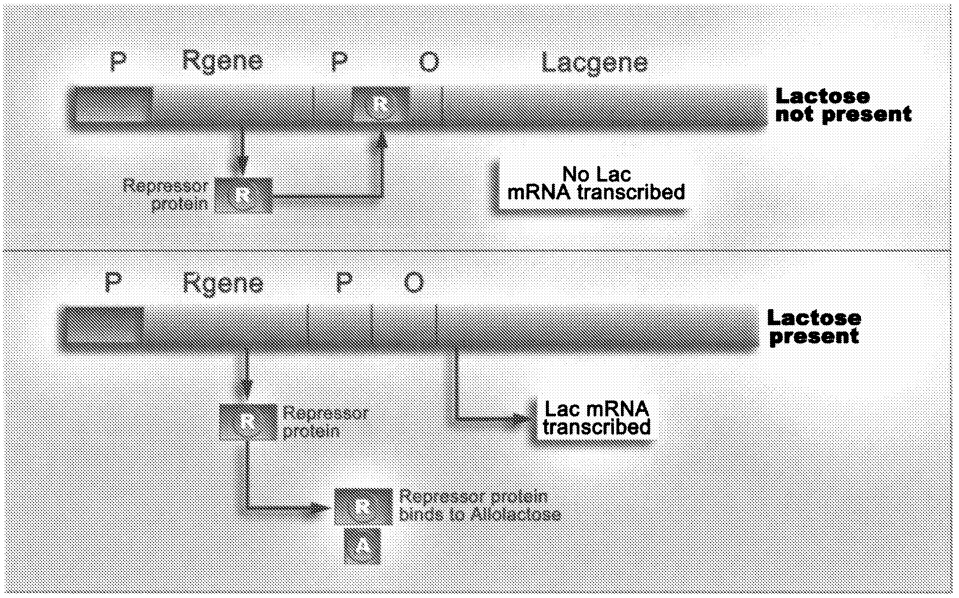


Figure 11.2. (*a*) Respression of LAC genes in the absence of lactose, and (*b*) expression of LAC genes in the presence of lactose.

the regulatory gene is expressed and a repressor protein binds to the promoter region of the lactose operon. This effectively blocks transcription of the Lac genes that code for lactose-metabolizing enzymes. However, when lactose is present (Fig. 11.2b), a derivative of lactose, allolactose, acts as an inducer by binding to the repressor protein. This inactivates the repressor and allows the synthesis of the three enzymes needed to metabolize lactose.

Induction of Hydrocarbon-Degrading Enzymes The lactose operon is a particularly clear example of how induction can lead to the acclimation of microorganisms to specific substrates. These concepts are important in the subsurface microbiology of contaminated sites, since many contaminant-degrading enzyme systems are inducible. There are numerous examples of this in the literature, but one particularly good example is the induction of aliphatic hydrocarbon-degrading enzyme systems.

The lactose operon is present on the chromosomal DNA of *E. coli*. However, it is commonly observed that enzyme systems for degrading xenobiotic compounds are found in extrachromosomal DNA (plasmids). Well-known examples of this are the OCT and TOL plasmids of *Pseudomonas,* which code for the degradation of n-octane and toluene, respectively.

The OCT plasmid of *Pseudomonas putida* has been shown to be inducible by a wide range of straight-chained compounds. Induction promotes the synthesis of two enzymes, alkane hydroxylase and alcohol dehydrogenase, which are involved in alkane degradation. In the case of the lactose operon, only one compound (allolactose) served as the inducer. In contrast, alkane hydroxylase and alcohol dehydrogenase are induced by a wide variety of straight-chained compounds. Any straight-chained compound in the C_6–CI_O range will induce the synthesis of these enzymes. Synthesis occurs even if carbonyl, hydroxyl, or methyl groups are present on the terminal carbon of one side of the chain. Interestingly, these enzymes may be induced by compounds against which the enzymes have no activity. For example, dicyclopropyl ketone and dicyclopropyl methanol will induce alkane hydroxylase activity, even though they are not degraded by the enzyme. These compounds are referred to as *gratuitous inducers*. Conversely, some compounds, such as undecane, will not induce enzyme production but are degraded if the enzymes are produced gratuitously.

11.2.2 Catabolite Repression

Induction allows microorganisms to synthesize degradative enzymes if a particular substrate becomes available. However, the mere availability of a substrate does not necessarily mean that its utilization is in the microorganism's best interest. For example, if two usable substrates, A and B, are present but more energy is available from substrate B, clearly the microorganism is better off suppressing A-degrading enzymes and producing B-degrading enyzmes. In fact, microorganisms have evolved mechanisms for distinguishing between available substrates. One of these mechanisms is termed catabolite regression.

In 1942 Jacques Monod discovered the phenomenon called diauxie, or the ability of certain microorganisms to utilize one substrate present in growth media preferentially to another substrate. The classic example of this is the sequential utilization

of glucose and lactose. When a glucose-lactose growth medium is inoculated with *E. coli,* there is a brief lag time as the cells acclimate themselves to the new conditions. After growth commences, growth is supported only by glucose oxidation with lactose not being used at all. After the glucose is exhausted, there is another brief tag as the cells acclimate themselves to lactose utilization, followed by growth at the expense of lactose. The phenomenon of diauxie typically produces a biphasic growth curve.

It took many years to elucidate the mechanism by which microorganisms accomplish diauxie. In the case of glucose-lactose utilization, it turns out that glucose utilization greatly decreases the intracellular concentration of cyclic adenosine monophosphate (AMP). When glucose is used up, intracellular concentrations of cyclic AMP increase, allowing the formation of a complex called the cyclic AMP repressor protein (CRP). The CRP complex binds to the promoter region of the lac operon, which cannot be activated in the absence of the CRP complex. Once the CRP complex is in place, allolactose is able to induce production of lactose-degrading enzymes (Fig. 11.2) with subsequent utilization of lactose as a substrate. Because low levels of cyclic AMP result from glucose catabolism, this mechanism has come to be called catabolite repression.

Catabolite Repression of Organic Compound Oxidation There is some evidence to suggest that catabolite repression of organic contaminants may occur under some conditions. As is the case with glucose-lactose, microorganisms can obtain more energy from glucose oxidation than from oxidation of petroleum hydrocarbons. It is therefore in their interest to oxidize hydrocarbons only in the absence of glucose as a substrate.

Van Eyk and Bartels (1968) showed that n-alkane oxidation in *Pseudomonas aeruginosa,* presumably conferred by the OCT plasmid, could be suppressed by addition of glucose or malate to the growth media. There is also some evidence that the mechanism of the catabolite repression is similar to that of glucose repression of lactose oxidation. Dalhoff and Rehm (1976) showed that the repression could be reversed by adding cyclic AMP plus an inducer substrate to the growth media.

Catabolite repression probably explains the observation that certain petroleum hydrocarbons, such as toluene, are observed to be preferentially utilized relative to compounds like benzene or MTBE in contaminated ground-water systems (section 12.7.2). Thus, this phenomenon is important to consider when considering bioremediation strategies. Although it is important to stimulate growth of natural populations in order to facilitate degradation, it is equally important not to add nutrients or carbon sources that might repress degradation of organic contaminants.

11.2.3 Genetic Mutations

The dual mechanisms of induction and catabolic repression provide microorganisms with powerful tools for acclimating their metabolism to different conditions. Clearly, however, microorganisms have evolved these pathways because they were advantageous in helping them deal with the environmental conditions they encountered. Since enzymatic mechanisms for degrading particular substrates have developed in the past, it reasonable to expect that microorganisms might evolve new mechanisms for degrading xenobiotic compounds.

There is an interesting literature on the genetic mechanisms involved in producing new metabolic capacity in microorganisms (Clarke, 1984). For example, Betz and Clarke (1972) showed that point mutations produced changes in substrate specificity of *Pseudomonas aeruginosa* amidase. In this case, these changes enabled the mutant strains to grow on amides that the wild-type strain was unable to use. Furthermore, sequential mutations were shown to convert what had been solely an acetamidase to a phenylacetamidase. These and other experiments have shown that the specificity of an enzyme can be changed by point mutation of the genome, and that these point mutations can sometimes improve the ability of an enzyme to degrade particular substances.

In addition to point mutations, the metabolic capabilities of bacteria can be significantly altered by deletions and additions of DNA via transposable genetic sequences (transposons). For example, in the process of constructing a 4-chlorobenzoate degrading strain, Jeenes et al. (1982) found that by inserting the TOL plasmid into a particular *Pseudomonas* strain, the plasmid lost a sequence of 39 kilobases. This particular strain gained the ability to degrade 4-chlorobenzoate but lost the ability to degrade m-toluate. These authors suggest that transposable genetic elements are an important source of variation in the kinds of substrates that microorganisms can degrade.

These are just a couple of examples of the tremendous capacity for change inherent in the bacterial genome. It is reasonable to assume that point mutations, transposon deletions, and transposon insertions occur as a matter of course in microorganisms. Most of the time, such genetic changes result in a decrease in metabolic efficiency. However, if a microorganism is subjected to novel substrates, there is a finite chance that a fortuitous genetic change will increase its efficiency. Thus, it is entirely possible for microorganisms to develop the ability to degrade even the most xenobiotic compounds.

11.2.4 Acclimation to Available Electron Acceptors

Pristine water table aquifers are often aerobic systems. This is because, under pristine conditions, the input of dissolved oxygen from percolating recharge typically exceeds the input of organic carbon substrates. Thus, microorganisms are more carbon-limited than oxygen-limited. With the advent of organic chemical contamination, however, the situation often rapidly changes. With the sudden influx of potential carbon substrates, carbon limitations disappear and oxygen is rapidly consumed. Where aerobic microbial metabolism might have dominated before contamination, anaerobic metabolism often dominates after contamination. Thus, in addition to acclimating to new carbon substrates, indigenous microorganisms must acclimate to electron acceptors other than oxygen.

One well-documented example of changing electron acceptor availability and its influence on the degradation of organic chemicals is provided by studies of a crude oil spill near Bemidji, Minnesota, conducted by the U.S. Geological Survey (Baedecker et al., 1988; Cozzarelli et al., 1988). In August 1979, a pipeline burst and spilled about 100,000 gallons of crude oil onto a glacial-outwash aquifer. This aquifer was under water table conditions and, due to a lack of natural organic matter, contained ground water saturated with dissolved oxygen (~10 mg/L 02). However, with the introduction of hydrocarbons, the aquifer in the vicinity of the spill rapidly became anaerobic. Because of the density difference between the crude

oil and ground water, the oil formed a lens that floated on the water table. However, soluble components of the oil, such as volatile aromatic hydrocarbons (benzene, toluene, xylene) readily leached into the ground water, forming a plume. Microbial oxidation of these hydrocarbons consumed oxygen near the oil lens, creating anaerobic conditions. Downgradient of the oil lens, mixing with ambient ground water gradually reestablished aerobic conditions.

The change in the availability of dissolved oxygen near the oil lens imposed a new set of environmental conditions on ambient microbial populations. Clearly, in the absence of dissolved oxygen, oxygen-based respiration was no longer an advantageous metabolic strategy. However, the glacial outwash sediments were characterized by the presence of ferric oxyhydroxides that occurred as coatings on mineral grain sediments. This Fe(III) represented a pool of potential electron acceptor that was available to microorganisms with the appropriate enzymatic capacity. Measurements of dissolved ferrous iron, the soluble product of microbial Fe(III) reduction, increased over time at the site (Fig. 11.3), indicating the initiation of microbial Fe(III) reduction. This interpretation was confirmed by later studies (Lovley et al., 1989) of Fe(III) respiration at the site.

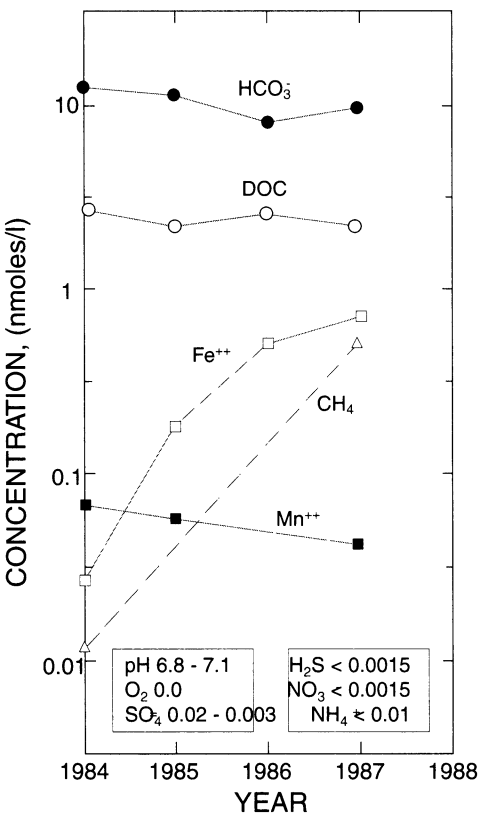


Figure 11.3. Increase of dissolved iron and methane over time in ground water near a crudeoil spill, indicating the inition of Fe(III) reduction and methanogenesis in a previously aerobic aquifer. (Modified from Baedecker et al., 1988.)

It is of historical interest that these studies produced the first documented evidence that a particular family of anaerobic bacteria (the *Geobacteraceae*) were capable of completely oxidizing aromatic compounds to carbon dioxide and water (Lovley and Lonergan, 1990). Although such mineralization had been indicated by numerous field and microcosm studies, it had not previously been shown to occur pure cultures of strict anaerobic microorganisms.

Like oxygen, the availability of Fe(III) oxyhydroxides to support microbial respiration near the oil lens was limited. As Fe(III) became depleted in the sediments, methanogenic bacteria were able to establish themselves. This is indicated by the increase of methane concentrations in ground water near the oil lens (Fig. 11.3).

This case study is important for a number of reasons. First of all, it is one of the few cases in which studies were undertaken soon enough after the spill occurred to document the transition from an oxygen-reducing to an Fe(III)-reducing and methanogenic environment. Similar transitions occur at many spills or leaks of petroleum hydrocarbons. Secondly, it gives some indication of the time scales involved in such transitions. With the initial spill occurring in 1979, evidence of Fe(III)-reducing and methanogenic activity were apparent within a few years. Finally, it shows that even strongly aerobic aquifers harbor the potential for developing active populations of anaerobic bacteria. Studies of pristine aerobic aquifers have shown the presence of a small and relatively inactive population of anaerobic bacteria (Balkwill and Ghiorse, 1985; Jones et al., 1989). It is thought that these microorganisms maintain minimal activity by inhabiting anaerobic microenvironments in the sediments. Clearly, once anaerobic habitats and suitable substrates become more available due to organic chemical contamination, anaerobic microorganisms are capable of rapid growth and of establishing themselves much more widely.

11.2.5 Production of Proteins in Response to Chemical Stresses

We have touched briefly on the genetic basis of microbial acclimation to changing environmental conditions such as available electron donors and electron acceptors. It's important to keep in mind, however, that the expression of genetic capabilities results ultimately in the production of proteins that enable microorganisms to carry out useful functions. If the "genome" of a microorganism is the sum of all of its genetic capabilities, the "proteome" can be defined as the sum of those proteins that have been actually produced to allow a microorganism to adjust to certain environmental conditions (review by Anderson and Anderson, 1998). The study of proteins expressed by microorganisms subjected to particular chemical and environmental stresses is one way to evaluate the acclimation response of microbial populations to particular stresses.

The technical problem associated with examining proteins present in microorganisms is, of course, that there are so many of them. Even the simplest bacterial cell produces thousands and thousands of proteins, and so evaluating what proteins are present under certain conditions is not a trivial undertaking. It is possible to separate proteins extracted from cells using gel electrophoresis. However, because of the large numbers of proteins present, and because many proteins have similar isoelectrical properties, this does not provide sufficient separation of the proteins. The breakthough in proteomics came in 1975 when several researchers developed

"2-D" gels (review by Anderson and Anderson, 1998). The basis of this technique was to first separate proteins on electrophoresis gels on the basis of isoelectric point, and then to turn the gel 90° and further separate the protein on the basis of molecular weight. With this 2-D gel technology, it was possible to resolve thousands of individual proteins and it became feasible to identify proteins produced by particular physical and chemical stresses.

For example, Blom et al. (1992) examined proteins produced by *E. coli* in response to chemicals such as petroleum hydrocarbons, solvents, and metals. As might be expected, addition of benzene (2,670 mg/L), trichloroethene (0.009 mg/L), and cadmium chloride (80 mg/L) to actively growing cultures of *E. coli* resulted in a depression of the rate of growth. Using 2-D gel technology, these researchers were able to show that different proteins were produced in response to particular chemical stresses. Furthermore, many of these proteins were shown to be unique to particular chemical stresses. While the function of these proteins remains unknown, they presumably assist microorganisms in detoxifying, metabolizing, or transforming these pollutants. These results indicate that the study of the acclimation response need not be confined to macroscale observations. Rather, it may also be possible to study acclimation based on the actual proteins that are generated during the acclimation response to chemical stress.

11.3 FACTORS AFFECTING MICROBIAL ACCLIMATION

The ability of microorganisms to adapt to new conditions imposed by chemical contamination is influenced by a number of factors. Because induction and catabolite repression are important mechanisms in the acclimation response, it can be anticipated that the kinds of organic compounds available and their relative concentrations will be important factors. In addition, because the characteristic lag time associated with microbial acclimation varies widely with the kinds of organic compounds available, the time of exposure to contamination will likely influence the degree of adaptation attained. Finally, the similarity of introduced contaminants to substrates normally utilized by microorganisms will influence microbial acclimation. If they are very similar, then acclimation may entail only the induction of appropriate degradative enzymes. On the other hand, if there is little similarity, than acclimation may involve serendipitous genetic mutations. This, in turn, probably would vastly increase the length of time involved in microbial acclimation. Evaluating the factors involved in microbial acclimation to chemical stress is an important component of designing bioremediation strategies.

11.3.1 Rates of Acclimation

Rates of microbial acclimation to xenobiotic compounds typically are extremely variable. In many cases, it is difficult to determine the precise reasons for the rate at which a particular microbial community adapts to a xenobiotic compound. Nevertheless, it is possible to make some generalizations.

Spain and van Veld (1983) conducted a study of microbial acclimation of aquatic sediment microbial communities to a number of xenobiotic compounds, including p-nitrophenol (PNP). In this study, sediment and water samples were taken from a

number of sites along the Escambia River near Pensacola, Florida. They prepared three treatments of samples for comparison. First, initial cores were taken from five sites along the river and spiked with 100 nmol/L of [^{14}C]PNP and incubated for eight days. Secondly, control cores were incubated for the same length of time with no addition. Finally, a set of cores were spiked with 100 nmol of unlabeled PNP and incubated for eight days.

The results of this study are shown in Figure 11.4. Of the five sampling sites, the lag time before significant mineralization of [^{14}C]PNP varied between 40 and 120 hours. After the lag time, PNP was rapidly mineralized (Fig. 11.4a). The control samples, which had been incubated without PNP for eight days prior to [^{14}C]PNP addition, showed virtually identical lag periods at the same sampling sites. This showed that the acclimation response was due to exposure to PNP rather than being an artifact of being handled in the laboratory. Finally, the sediments that had been preexposed to PNP showed no tag before mineralizing PNP (Fig. 11.4b).

The general pattern of acclimation response shown by Spain and van Veld (1983) has been observed by a number of investigators. This pattern includes a wide range of acclimation times to a particular xenobiotic that cannot be predicted a priori. Sediment samples from the different locations on the same river exhibited acclimation times that varied as much as fourfold. This variability is even more pronounced in sediments from ground-water systems (Aelion et al., 1987).

For acclimation times on the order of days, such as those shown by Spain and van Veld (1983), enzyme induction is probably the sole acclimation mechanism. If this is the case, it would be expected that acclimation to a particular xenobiotic would persist only as long as that chemical was available for microbial utilization. Once the chemical was no longer available, inducible enzymes would no longer be synthesized and acclimation would disappear. This pattern was in fact observed by Spain and van Veld (1983). Acclimation was defined as the initial rate of $^{14}CO_2$ production from [^{14}C]PNP. If the initial $^{14}CO_2$ rate was low, as with unacclimated sediments, the degree of acclimation was considered to be correspondingly low. By this measure, the sediments reached maximal acclimation at about 200 hours (5 days) following exposure, after which there was a steady decrease in acclimation. After 50 days of nonexposure, there was no remaining acclimation.

These results illustrate that microbial acclimation to xenobiotics does not necessarily confer a permanent capacity for degradation. This finding is of more importance to aquatic surface sediments, where pulses of contamination tend to be much more transient than in subsurface environments. Nevertheless, where enzymatic induction is a principal acclimation process, similar behavior will probably be observed.

11.3.2 Concentration Effects

Concentration of xenobiotic compounds has been shown to have important effects on achievement of acclimation and on the length of the lag time. For example, in a study of sediments from Wintergreen Lake, Michigan, Linkfield et al. (1989) showed that the length of acclimation prior to dehalogenation of 3-chlorobenzoate (3-Cl-BZ) was directly related to the concentrations added to the sediments (Fig. 11.5). At low initial concentrations of 3-Cl-BZ (20 M), acclimation periods of about 60 days were observed before significant dehalogenation occurred. However, at

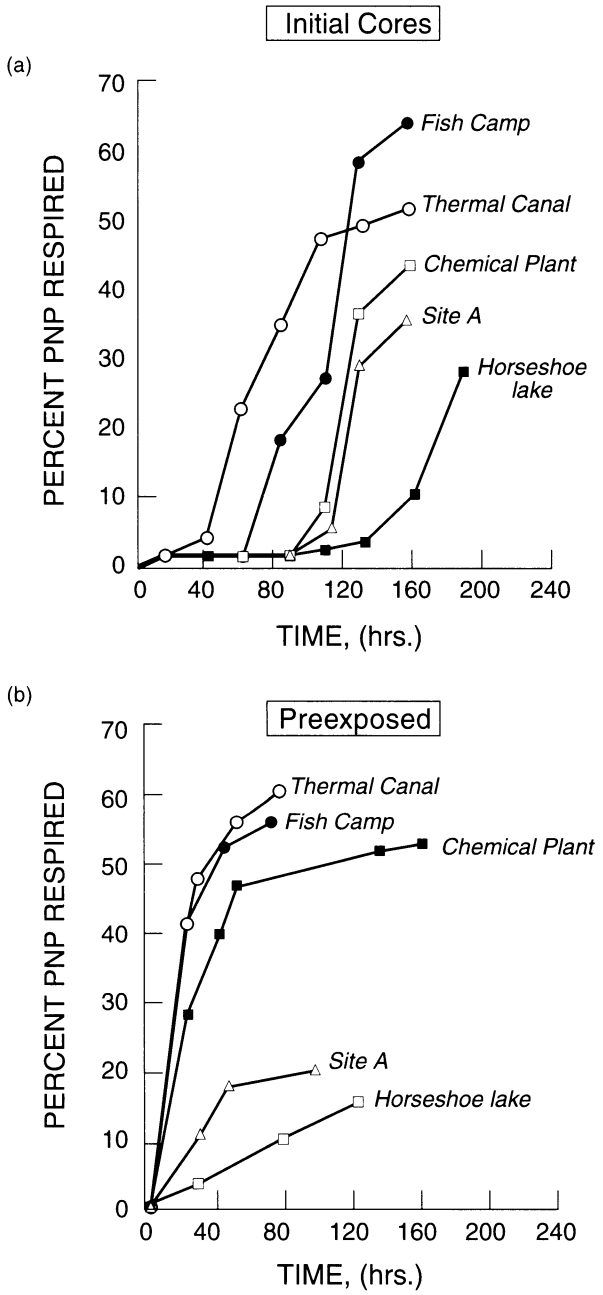


Figure 11.4. (*a*) The presence of a lag prior to PNP mineralization in pristine aquatics ediments and (*b*) the absence of a lag in preexposed sediments. (Modified from Spain and van Veld, 1983, with permission courtesyof the American Society for Microbiology.)

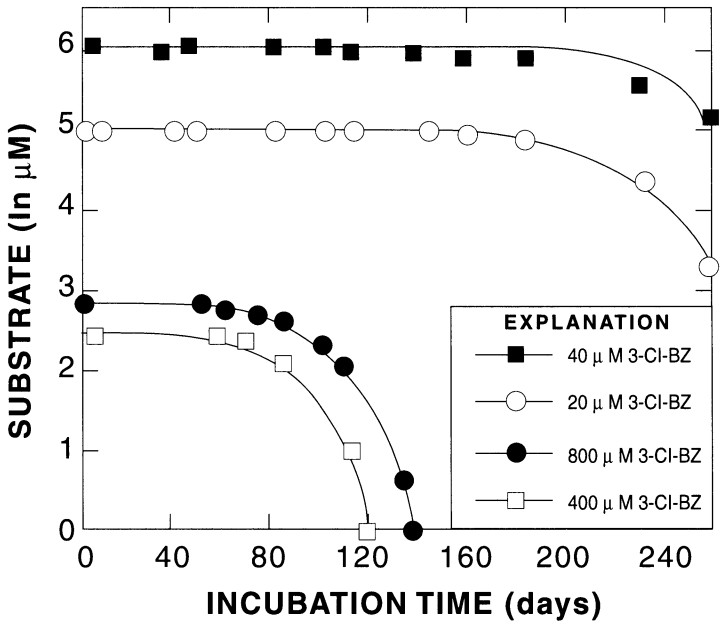


Figure 11.5. Effects of substrate concentration on the length of the acclimation period prior to reductive dehalogenation. (Modified from Linkfield, Suflita, and Tiedje, 1989, with permission courtesy of the American Society for Microbiology.)

progressively higher initial concentrations of 3-Cl-BZ acclimation periods increased, reaching a maximum on 200 days at 800 μM. This pattern apparently reflects the toxicity of chlorinated benzoates. Thus, acclimation times not only reflect the time required for microorganisms to induce appropriate degradative enzyme systems but also may reflect adjustment to unfavorable chemical conditions.

Lower concentrations of xenobiotics are not always favorable to degradation. For example, Linkfield et al. (1989) observed that acclimation to relatively high concentrations (400 μM) of 4-NH_2-3-5-diCl-BZ was evident with subsequent mineralization occurring. However, if the initial concentration of 4-NH2-3-5-diCi-BZ were relatively low (20 uM), no acclimation response or mineralization was evident. This suggests that a minimum threshold exists below which induction of the necessary enzyme systems does not occur.

The mechanism of this minimum threshold effect was not systematically investigated by Linkfield et al. (1989). However, they suggest that a concentration-dependent receptor mechanism may explain the observed effect. According to this hypothesis, a xenobiotic compound must be present in sufficient concentration before binding to inducer proteins regulating gene expression. Alternatively, this effect may simply reflect competitive utilization (i.e., diauxie) of alternative substrates in the sediment. If the xenobiotic compound is present below a particular threshold, then alternative carbon sources may be more attractive to indigenous microorganisms and preclude induction of degradative enzyme systems.

The minimum threshold effect of acclimation has been observed in sediments cored from ground-water systems. In a study of acclimation of pristine aquifer

sediments to xenobiotic compounds, Aelion et al. (1987) showed that relatively low concentrations (14 ng/g sediment) of p-nitrophenol induced little or no acclimation and mineralization. In contrast, sediments exposed to higher concentrations (529 ng/g sediment) exhibited rapid mineralization of p-nitrophenol following a 40-day acclimation period.

11.3.3 Cross-Acclimation of Xenobiotic Compounds

If induction of enzymatic systems is a principal mechanism in the acclimation of microbial populations to xenobiotic compounds, it might be expected that acclimation to one particular xenobiotic compound would confer acclimation to related compounds. This would be the case if several xenobiotics were degraded via the same enzymatic pathway. This behavior has, in fact, been reported in the case of reductive dehalogenation of halobenzoates (Horowitz et al., 1983). In a series of experiments, these investigators showed that acclimation to reductive dehalogenation of one halobenzoates reduced acclimation times to other halobenzoates. For example, the acclimation time of lake sediments to 3-iodobenzoate was on the order of 2–3 weeks. Once acclimation was achieved, however, these sediments were able to degrade other halobenzoates completely in less than one week. This effect is important to bioremediation technology since it implies that acclimation to one class of xenobiotic may confer enhanced degradation rates to a number of related compounds.

11.3.4 Chemical Structure of Xenobiotics

The chemical properties of organic compounds depend largely on their chemical structures. Because of this, it is reasonable to expect that the acclimation of microbial communities to xenobiotic compounds will be affected by chemical structure.

There is evidence that this is the case for microbial communities in aquatic sediments adapting to reductive dehalogenation of halobenzoates (Linkfield et al., 1989). In this study, it was shown that acclimation periods depended largely on the kinds of halogens substituting on benzoate and on their position on the molecule. Figure 11.6a shows the chemical structure of 3-chlorobenzoate, 3,5-dichlorobenzoate, and 4-NH_2-3-5-dichlorobenzoate. As might be expected, the rates of microbial acclimation to these compounds is significantly different. For example, at a substrate concentration of 800 μM (ln 800 μM = 6.7 in Fig 11.6b) 3,5-diCl-BZ exhibited a consistent acclimation period of 29–35 days (Fig. 11.6b). 4-NH_2-3,5-diCl-BZ exhibited a slightly longer acclimation period of about 37 days and 3-Cl-BZ a much longer acclimation period of 125–148 days.

It is interesting to note the differences in acclimation periods imposed by different halogen substitutions on the benzoate molecule. For example, if bromine substituted for chlorine, forming 3-bromobenzoate, the acclimation period would be shorter, on the order of 30 days (Fig. 11.6b). On the other hand, if fluorine substituted into the same position, forming 3-fluorobenzoate, no acclimation at all to the substrate was evident (Fig. 11.6b).

In addition to different halogen substitutions, Linkfield et al. (1989) investigated changes in acclimation period depending on the position of halogen substitution.

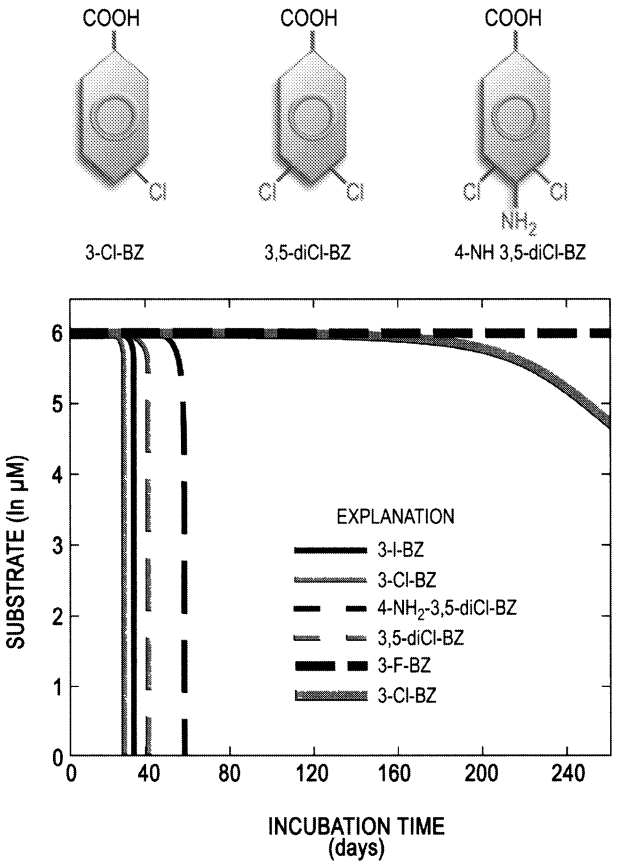


Figure 11.6. Relationship of chemical structure to acclimation times. (Modified from Linkfield, Suflita, and Tiedje, 1989, with permission courtesy ofthe American Society for Microbiology.)

In the case of bromobenzoates, the effect of ring position was particularly clear. The para isomer exhibited greater acclimation periods than did either the ortho or meta forms. For an initial concentration of 900 M, the acclimation periods for the ortho, meta, and para isomers were 20, 23, and 39 days, respectively. Clearly, differences in chemical structure imposed significant differences in the biochemistry of dehalogenation and corresponding differences in acclimation rates.

11.4 ACCLIMATION TO XENOBIOTICS IN GROUND-WATER SYSTEMS

Much of the research concerning microbial acclimation to xenobiotic chemicals has been focused on aquatic surface sediments or soils. However, there is a small but significant literature concerning microbial acclimation in ground-water systems. Two general approaches have been used in these studies. One approach has been to study sites that exhibit contamination by particular classes of compounds (Wilson

et al., 1985). Differences of xenobiotic utilization between contaminated and pristine zones of the same aquifer are then used to document the presence or absence of acclimation effects. Another approach, which is similar to studies of aquatic sediments, is to obtain sediments from pristine subsurface environments, amend them with xenobiotics, and observe the lag period before mineralization commences (Aelion et al., 1987).

11.4.1 Acclimation Response in a Contaminated Aquifer

Wilson et al. (1985) described a case study of microbial acclimation to polyaromatic hydrocarbons (PAH) in a shallow water-table aquifer. The aquifer had been contaminated from creosote wastes over a 23-year period beginning in 1952 and ending in 1975. Creosote is a complex mixture of organic chemicals composed of about 85% PAH such as naphthalene, anthracene, and phenanthrene. In addition, creosote contains about 15% phenolic compounds. As the contamination had been in place for a considerable length of time when the study took place, it was reasonable to hypothesize that microorganisms in the plume of contamination would have become acclimated to PAH mineralization.

In order to test this hypothesis, sediments were cored from within the contamination plume and in nearby pristine sediments. Two sites within the contaminated area and a site outside the contaminated area were examined for differences in mineralizing various PAH compounds.

The results showed that sediments from the contaminated site exhibited significantly more mineralization activity than sediments from the pristine site. Even though sediments from one contaminated site contained no measurable PAH initially, amending these samples with PAH and oxygen showed rapid consumption relative to autoclaved controls. In contrast, sediments from the pristine site showed little or no consumption of PAH. These results are important because they document the importance of the acclimation process to degradation rates of pollutants in aquifer systems.

While these experiments provided documentation of acclimation in the contaminated sediments, it is apparent that, if in situ degradation rates were as high as those measured in the laboratory, the contamination would have been entirely degraded since operations at the creosote plant closed (approximately ten years). Since contamination was still evident, it was clear that the degradation experiments overestimated actual in situ rates. Wilson et al. (1985) attributed this effect to the addition of oxygen to the experimental vials and suggested that the delivery of oxygen to the contaminated zone was the limiting factor for degradation rates under in situ conditions. The tendency for laboratory incubation experiments that do not accurately mimic the electron-accepting processes operative in situ to overestimate actual biodegradation rates has been widely observed and needs to be carefully considered when determining biodegradation rates (see section 12.8.4).

11.4.2 Acclimation Response in Pristine Aquifer Sediments

The study of Wilson et at. (1985) showed that acclimation occurred in response to chemical contamination of a shallow aquifer system. The next logical question to be addressed was at what rate does such acclimation occur in pristine sediments

exposed to various kinds of contamination? These questions were addressed by Aelion et al. (1987) and Swindoll et al. (1988).

The aquifer sediments studied by these investigators were recovered from the pristine Lula, Oklahoma, site that previously had been described by Wilson et al. (1983) and Balkwill and Ghiorse (1985). As this aquifer is aerobic under ambient conditions, all experiments were conducted under aerobic conditions. A number of compounds were selected for study, including chlorobenzene, 1,2,4-trichlorobenzene, m-cresol, phenol, and p-nitrophenol.

Not surprisingly, several different patterns of response were observed (Fig. 11.7). Some compounds, notably m-cresol (Fig. 11.7a), exhibited a linear increase in percent mineralization over time. Other compounds, such as phenol, showed rapid initial mineralization that slowed over time (Fig. 11.7b). The only compound tested that showed a tag time followed by rapid mineralization, the response most often

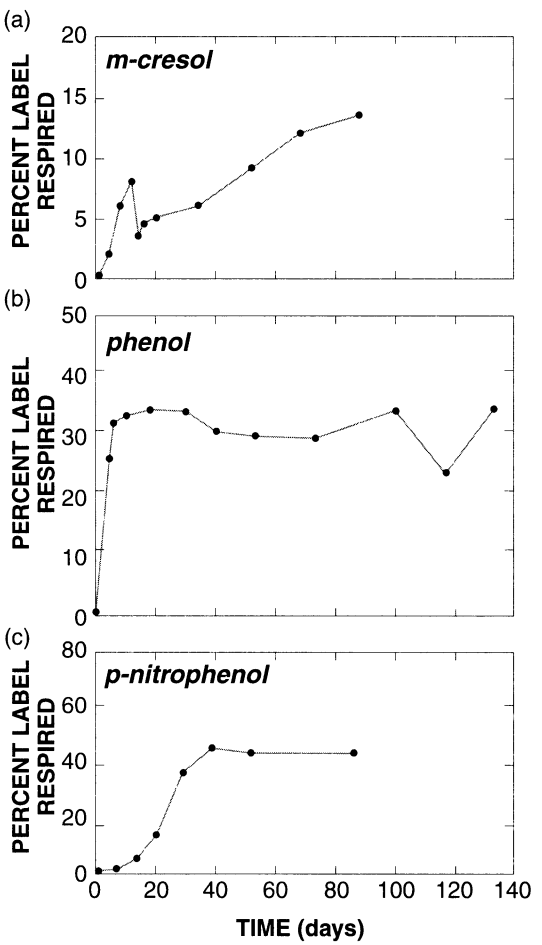


Figure 11.7. Different patterns of acclimation response to particular contaminants. (*a*) linear mineralization of m-cresol, (*b*) rapid initial mineralization of phenol, and (*c*) a lag followed by rapidmineralization of p-nitrophenol. (Modified from Aelion, Swindoll, and Pfaender, 1987, with permission courtesy of the American Society for Microbiology.)

associated with acclimation, was p-nitrophenol (PNP) (Fig. 11.7c). Interestingly, PNP showed a similar classic acclimation response in the studies of Spain and van Veld (1983) using aquatic surface sediments. Other compounds, notably chlorobenzene and 1,2,4-trichlorobenzene, showed no mineralization over time.

These results are important because they suggest that the response of microbial communities to a pulse input of xenobiotic compounds will not be uniform. In the case of compounds such as m-cresol and phenol, the indigenous microfora appear to maintain the ability for degradation without induction of specific enzyme systems. In the case of p-nitrophenol, induction of enzyme systems appears necessary before significant degradation can occur. As was shown to be the case in aquatic surface sediments (Spain and van Veld, 1983), this induction response was concentration-dependent. If sediments were treated with low concentrations (14–31 ng/g sediment) of p-nitrophenol, no acclimation response was observed. However, for higher concentrations (452–529 ng/g sediment) the acclimation response was evident. This effect was reproducible between different sample depths.

In real instances of chemical contamination, it is rare that one compound or even one class of compounds is involved; rather, a complex mixture of xenobiotics, as well as nonxenobiotic chemical compounds, typically are delivered simultaneously. The nature of the mixture would be expected to affect acclimation of indigenous microorganisms. In addition, if it were possible to increase rates of the acclimation response by means of chemical addition, this would have important implications for bioremediation technology.

These questions were addressed by Swindoll et al. (1988), which also utilized water table aquifer sediments recovered from the Lula, Oklahoma, site. Microbial communities in the Lula aquifer sediments exhibited a classic acclimation response to p-nitrophenol. Could this acclimation response be altered by means of additions of nutrients? In one experiment, sediments amended with p-nitrophenol received either no other amendment or were amended with nitrogen, or phosphorus, or nitrogen and phosphorus. The results of this experiment showed no increase in the acclimation response for any of the amendments. Clearly, the enzyme induction response for microorganisms in these sediments was not either nitrogen- or phosphorus-limited.

Other experiments did show some impact of particular nutrients on the acclimation of microbial communities to p-nitrophenol. For example, addition of vitamins to the sediments did appear to increase acclimation rates, whereas addition of glucose did not. The most dramatic increase in acclimation rate was obtained by adding a mixture of nitrogen, phosphorus, mineral salts, and amino acids. In the case of toluene mineralization, nutrient addition actually decreased mineralization. This may be attributed to preferential utilization (diauxie) of glucose and amino acids by indigenous microorganisms.

11.4.3 Acclimation of Eucaryotic Microorganisms

Eucaryotic microorganisms such as fungi and protozoa are present in low numbers in many ground-water systems (Sinclair and Ghiorse, 1987; Sinclair et al., 1990). Fungi, which are usually aerobic heterotrophs, compete directly with bacteria in many environments. Thus, oligotrophic conditions that limit the population density of bacteria in ground-water systems also limit populations of fungi. The low pop-

ulation density of protozoa in pristine aquifer systems is also related to low populations of bacteria. Because protozoa prey upon bacteria, substrate limitations for bacterial populations translate to substrate limitations for protozoa.

It has been observed that one manifestation of microbial acclimation to polluted ground water is an increase in the number of protozoa (Madsen et al., 1991). This implies that microbial acclimation to xenobiotics is not limited to the bacteria but includes acclimation of higher eucaryotic microorganisms as well.

The pattern of microbial distributions, including bacteria, actinomycetes, fungi, and protozoa, shown by Madsen et al. (1991) at a coal tar disposal site documents patterns of eucaryotic acclimation to ground-water contamination. The coal tar leached large amounts of polyaromatic hydrocarbons (PAHS) into the ground water at this site. In response to this chemical stress, counts of viable bacteria were higher than (Fig. 11.8a) were areas outside the zone of contamination. There was, however, no measurable difference in total counts of bacteria (Fig. 11.8b). Interestingly, numbers of actinomycetes were higher in the unsaturated zone (UN) and at the water table (WT) in the contaminated zone but not in the shallow saturated (SS) or deep saturated (SD) zones (Fig. 11.8c). There was no measurable increase in numbers of fungi in the contaminated zone relative to pristine areas (Fig. 11.8d), but numbers of protozoa were significantly higher in the contaminated zone (Fig. 11.8e).

These results suggest that bacterial acclimation and utilization of PAH leads to greater viability, and presumably greater reproduction rates, in the zone of contam-

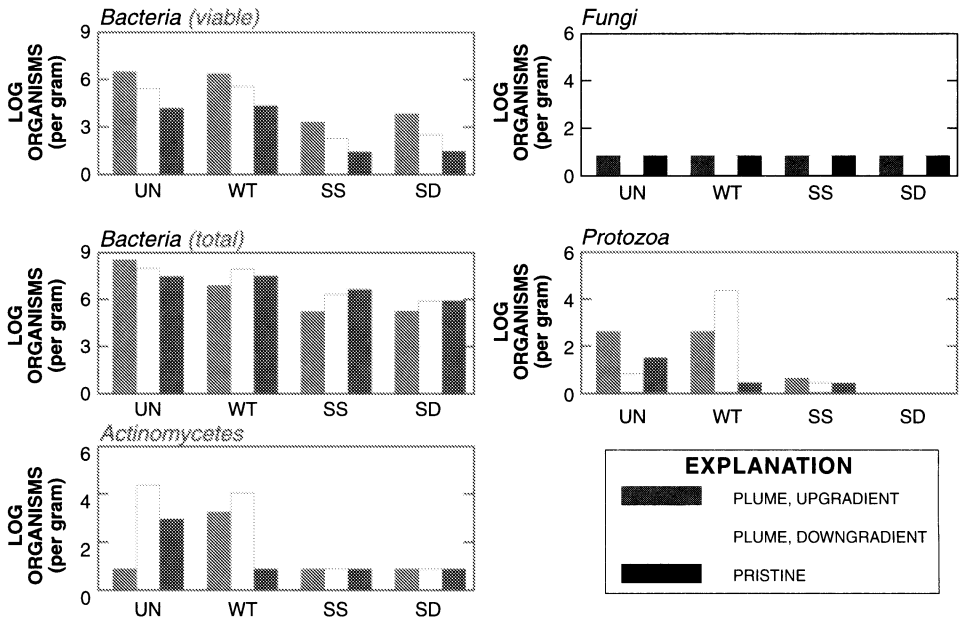


Figure 11.8. Comparison of microbial abundance in the unsaturated (UN), water table (WT), shallow saturated (SS), and deep saturate (SD) zones. Each cluster of bars represents numbers of microorganism in the upgradient (*left*) and downgradient (*center*) parts of thecontaminated plume, as well as in pristine (*right*) sediments. (Modified from Madsen, Sinclair, and Ghiorse, 1991, withpermission courtesy of the American Society for the Advancement of Science.)

ination. Fungi, which may be limited by oxygen availability, are not evidently able to increase their population. Protozoa, on the other hand, which often feed upon bacterial cells, show a noticeable population increase. This, in turn, suggests that these microorganisms are capable of acclimating to the chemical stress, as are the bacteria. The grazing effects of the protozoa are apparently the principal reason that total numbers of bacteria do not increase.

These results are important for two reasons. First of all, they suggest that the ability to acclimate to chemical stress is present in microorganisms other than just the bacteria. The mechanisms by which protozoa acclimate to xenobiotic chemical stress are presently unknown, but are presumably similar to those of the bacteria. Secondly, these results suggest that grazing by protozoa limits bacterial populations in contaminated aquifer systems. This, in turn, may place limits on the ability of bacterial populations to degrade xenobiotics in contaminated environments. The extent or importance of these limits may presently only be guessed at. This is, however, an important topic that warrants future research.

11.4.4 Acclimation in Bioremediation Technology

Available evidence clearly shows that microbial populations exposed to organic chemicals exhibit an acclimation response that, in general, results in increased rates of biodegradation. This is shown by field studies in which microorganisms in sediments exposed to contamination show greater biodegradation than unexposed sediments (Wilson et at., 1985) and by laboratory studies (Aelion et al., 1987; Swindoll et al., 1988) showing that degradation rates increase over time.

The extent to which the acclimation response can be utilized in bioremediation technology is much less clear. Numerous practical considerations intervene. Available evidence shows that significant microbial acclimation occurs in time periods of less than a year. Considering that many instances of ground-water contamination are discovered tens of years after the fact, implementing measures to increase acclimation rates does not seem particularly advantageous. Furthermore, other factors, such as electron-acceptor availability, often are much more limiting to degradation rates than the extent of microbial acclimation (Wilson et al., 1985). Thus, while the acclimation response certainly affects degradation of organic compounds in ground-water environments, its application to bioremediation technology is often impractical.

11.5 ACCLIMATION TO METAL TOXICITY

Metals play important roles in the activity of many enzymatic systems in procaryotic and eucaryotic cells. Obvious examples include the utilization of iron in electron transport mechanisms, the presence of molybdenum in nitrate reductase, or the role of zinc in carbonic anhydrase. However, when one particular metal interferes with enzyme function by competing with or replacing another particular metal, toxicity results. Metal toxicity in this context simply refers to the slowing or complete cessation of cellular growth as a direct result of the presence or absence of a particular metal in the cell's immediate environment.

It is reasonably common for microorganisms, particularly those inhabiting soils or aquatic sediments, to encounter potentially toxic metals. For example, micro-

organisms inhabiting soils developed on metal ore bodies would be exposed to fairly high metal concentrations. Microorganisms inhabiting aquatic sediments or aquifer systems developed from erosion of ore bodies would suffer similar metal exposure with potentially toxic effects. As a result of such exposure, some microorganisms have developed elaborate biochemical mechanisms for dealing with metal toxicity. Because many of these mechanisms involve induction of enzymatic systems, microbial communities having such biochemical capabilities often exhibit an acclimation response to metal exposure.

The acclimation of microorganisms to metal toxicity and mechanisms of metal resistance have been widely studied (Trevors et al., 1985). Much of this work has been motivated by the observation that microbial transformations of metals greatly affect metal mobility in the environment. For this reason, microbial acclimation to metal toxicity is relevant to the microbiology of ground-water systems as well.

11.5.1 Metal Detoxification Mechanisms

Microorganisms employ several different biochemical mechanisms in detoxifying metals in their immediate surroundings. These include (1) binding metals to the cell surface in order to prevent transport into the cell, (2) biotransformation of the metal to a less toxic form, and (3) depositing the metal in an insoluble form. Some of these mechanisms, such as metal deposition as sulfides, are gratuitous in that they result from other metabolic functions, such as sulfide production. Other mechanisms, such as biotransformations, are highly specific and are determined at the genetic level. The ability to transform metals to less toxic or more volatile forms often are coded on plasmids or transposons.

Metal Binding The outer layers of bacterial cells are characterized by numerous anionic sites that are available to bind positively charged metal ions of metabolic importance, such as Mg^{2+}, Fe^{3+}, Cu^{2+}, Na^{+}, and K^{+}. This binding, which often is the first step in active transport into the cell, results largely from interactions with anionic sites on the cell wall such as phophodiesters, carboxyl groups of peptidoglycan, and hydroxyl groups of carbohydrates. Peptidoglycan is especially efficient at metal binding. Such heavy metals as Hg^{2+} and Pb^{2+} are also gratuitously bound by anionic sites on a cell wall. However, the cell wall typically shows less affinity for the heavy metals than for lighter metals such as Mg^{2+}. Once bound to the cell's surface, a potentially toxic metal is immobilized and is effectively prevented from entering the cell, where it might interfere with metabolic functions. Thus, metal binding confers some level of metal resistance to all microorganisms.

Biotransformations An important class of metal resistance mechanisms available to microorganisms is that of biotransformation. These biotransformations include oxidation-reduction reactions and organic-inorganic conversions. In both cases, the mobility or volatility of the metal, or both, may be greatly affected. For example, reduction of Hg^{2+} to HgO^{o} greatly increases the volatility of mercury and may contribute to its transport away from the microorganism's immediate environment. Similarly, the attachment of alkyl groups to metals may greatly change its solubility and volatility. For example, some methylated metals are highly volatile and virtually insoluble in aqueous solution. Thus, they may more easily escape from an aquatic environment. There is some evidence that alkylation of metals

serves as a primary metal-resistance mechanism. Conversely, alkyl substitutions greatly increase the affinity of the metal complex for lipids. This increased lipophilicity is an important factor in metal toxicity in microorganisms and in higher organisms. It has been shown that some microorganisms have the inducible ability to dealkylate metals. In the case of methyl mercury compounds, the first step in this process is demethylation by means of organomercurial lyases followed by the reduction of Hg^{2+} to Hg^{o}. The genetic basis of mercuric reduction is one of the best-documented of all metal-resistance mechanisms and will be discussed in detail in section 11.5.2.

Metal Deposition The ability to precipitate metals in an insoluble form is an attractive mechanism for metal resistance. Most metal sulfide compounds are highly insoluble, and deposition of a metal in this form effectively prevents its transport into the cell. In some cases, as in *Desulfovibrio,* sulfide production and metal immobilization is gratuitous and is simply a by-product of the microorganism's normal metabolism. In other cases, as in the case of some *Clostridia,* sulfide production is inducible and is used as a metal resistance mechanism. The ability of some microorganisms to precipitate metals has applications in biotechnology. Metal-precipitating microorganisms have been used to remove metals from industrial waste solutions. Conversely, metal-solubilizing microbial mechanisms have been used to leach and recover metals from ores.

11.5.2 Plasmid-Encoded Metal Resistance Mechanisms

Metal resistance mechanisms present in procaryotic microorganisms are often plasmid-encoded. This is to say that the genetic material coding for the relevant enzyme systems are present on extrachromosomal DNA. However, as many of these genetic sequences are transposable, they may also be found in chromosomal DNA.

Plasmid-encoded resistance mechanisms have been documented for a wide range of metals, including cobalt and zinc (Duxbury and Bicknell, 1983), cadmium (El Solh and Ehrlich, 1982), silver (Haefeli et al., 1984), and mercury (Silver and Misra, 1984). Of these, the mechanism for mercury resistance in *Staphylococcus aureus,* an opportunistic human pathogen, has been particularly well documented. As such, it serves as a convenient model for illustrating the genetic basis for metal resistance in other microorganisms as well.

A number of different plasmids and transposons, each exhibiting slightly different details, have been shown to confer mercury resistance in *Staphylococcus aureus.* These functions are located on plasmid DNA and are referred to collectively as the "mer operon." The basic components of the mer operon and their functions in conferring mercury resistance are shown in Figure 11.9. The regulatory gene mer R is inducible and codes for a protein that, when bound to the promoter- operator (OP) region, initiates synthesis of the mercury resistance enzyme system. This enzyme system is composed of several different functions. The mer T and mer P regions code for a system that traps extracellular Hg^{2+} and transports it through the cell wall and cell membrane to the cytoplasm. The mer A region codes for mercuric reductase, which is present in the cytoplasm. Hg^{2+} delivered to the cytoplasm by the transport mechanism is then reduced to the volatile and less toxic Hg^{o} form. The mer B region codes for an organomercurial lyase system that serves

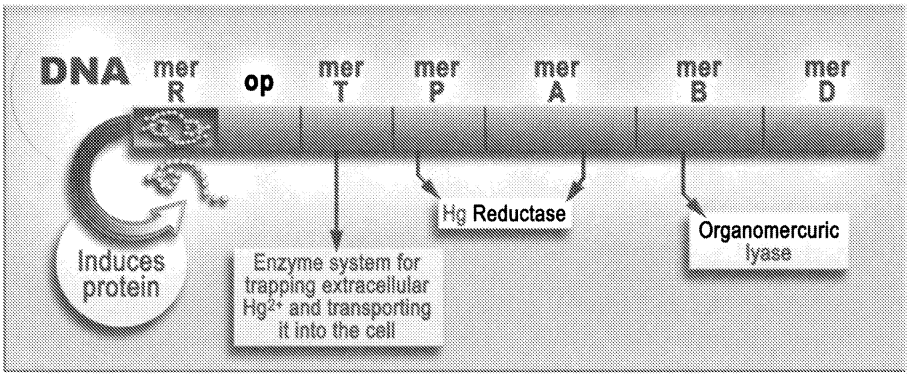


Figure 11.9. Basic components of the mer operon.

to split Hg^{2+} from organic complexes so that it can be subsequently reduced by mercuric reductase.

Depending on the components of the mer operon actually present in plasmid DNA, a wide variety of responses to mercury are possible. For example, if the mer A region, which codes for mercuric reductase, is removed from the operon, the resulting cells have the ability to trap Hg^{2+} but not to detoxify it. Such cells are actually hypersensitive to mercury stress. Cells that have broad spectrum resistance to mercury contain both the mer A and mer B genes. Alternatively, cells containing mer A but not mer B exhibit resistance to Hg^{2+}, but less resistance to organomercuric compounds such as methylmercury.

While the mer operon is certainly the most studied of the mercury-resistance mechanisms, others have been documented. For example, *Clostridium cochlearium* has an entirely different plasmid-encoded mercury resistance mechanism. This mechanism does not include mercuric reduction but, rather, the precipitation of mercuric sulfide. Organomercuric compounds are split, presumably by means of a lyase, and sulfide is generated. Both of these functions appear to be plasmid mediated in the case of *C. cochlearium*. This serves to emphasize the wide range of resistance mechanisms present in microorganisms and illustrates their flexibility in dealing with metal stress.

11.5.3 Acclimation to Mercury Toxicity

The fact that the mer operon, and other plasmid-mediated metal resistance mechanisms, are inducible enzymatic systems suggests that an acclimation period would be expected in response to mercuric stress. Furthermore, this suggests that microbial populations acclimated Hg^{2+} should be much more efficient at Hg^{2+} reduction than nonacclimated populations. It has been widely observed that aquatic sediments exposed to mercuric stress exhibit a higher incidence of mercury-resistant bacteria than nonexposed sediments. In one such study, Barkay and Olson (1986) showed that lake sediments exposed to high concentrations of mercury, up to 40 $\mu g/g$, showed higher counts of mercury resistant bacteria and a higher incidence of the mer operon than did sediments that had not been exposed. Subsequent research has demonstrated the inducible nature of mercury resistance in aquatic-sediment mi-

crobial communities. For example, Barkay (1987) showed that loss of Hg^{2+} from water samples of estuarine showed a distinct lag time. However, samples preexposed to Hg^{2+} showed no such lag time. Furthermore, the half-life of Hg^{2+} in preexposed samples was between two- and fourfold shorter than in unexposed samples. Most of the scientific literature on microbial acclimation to metal stress resides in the aquatic sediment, microbial genetics, or industrial microbiology literature. From that literature, it can be reasonably surmised that microorganisms in ground-water systems behave in a similar fashion.

One example of this similarity was reported by Landmeyer et al. (1996). This study considered the acclimation to metals toxicity in soils and water-table sediments at a small-arms firing range. Bullets from small arms contain many metals, including lead, arsenic, and cadmium. This study showed, using plate counts and activity measurements, that in soils contaminated by metals, lead-resistant bacteria were more numerous then in uncontaminated soils. Thus, the pattern of acclimation to metal toxicity found in aquatic sediments is also found in soils and aquifer sediments.

11.6 SUMMARY

In this chapter, we have seen that microorganisms employ a wide variety of mechanisms for acclimating themselves to changing conditions. In many cases, acclimation consists of inducing appropriate enzyme systems or, alternatively, repressing systems that are inappropriate. These changes are effected at the genetic level but do not involve fundamental changes of the microorganism's genome. It is also possible that genetic changes, initiated by point mutations or wholesale deletions of genetic material, may result in advantageous metabolic capabilities. However, these changes are unpredictable and have an uncertain impact on xenobiotic degradation in natural systems.

The hallmark of the acclimation response is the presence of a characteristic lag time in the microbial growth curve that follows exposure to new substrates. These acclimation periods have been observed in microbial communities from numerous environments responding to a broad spectrum of contaminants, including xenobiotic organic compounds and metals. Microbial communities in shallow ground-water systems appear to acclimate to a variety of chemical stresses in time periods as short as several months. The acclimation time for particular compounds can be reduced somewhat by judicious addition of trace elements or vitamins. However, it is not always practical to apply this to bioremediation strategies.

Microorganisms possess a number of different mechanism for acclimating to the toxic affects of metals. These include metal binding to the cell wall, biotransformations of metal species, and metal precipitation processes. Metal-resistance mechanisms are often plasmid encoded and are induced by metal exposure. Metals resistance has been most extensively studied in aquatic sediments. But studies indicate that ground-water systems contain similar metals-resistance mechanisms as well.

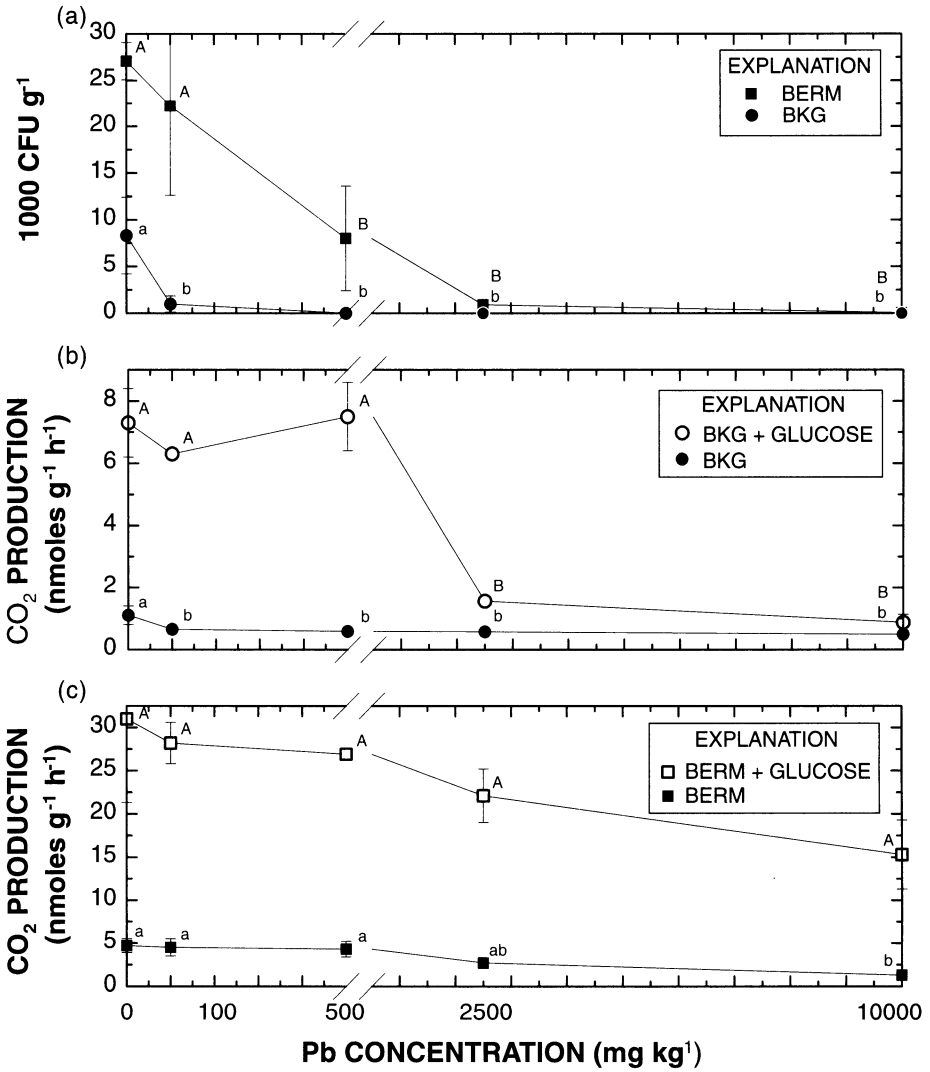


Figure 11.10. Figure showing acclimation of bacteria to Pb contamination in contaminated soils (berm) relative to background (bkg) soils bymeans of (*a*) high numbers of bacteria growing on Pb-bearingmedia, and inhibition of glucose metabolism in (*b*) background soils relative to (*c*) berm soils.

QUESTIONS TO CONSIDER

1. Name at least three mechanisms by which microorganisms can adapt to pollutants introduced to ground-water systems.
2. How might catabolite repression contribute to the observation that toluene is usually degraded preferentially to benzene in gasoline-contaminated groundwater systems?

3. When aerobic aquifers become anoxic following the introduction of petroleum hydrocarbon contaminants, why is so little time required for the predominant metabolism to shift from oxygen reduction to Fe(III) or methanogenic metabolism?
4. Pristine aquatic sediments have been shown to exhibit about a 100-hour lag time for biodegrading paranitrophenol (PNP) relative to preexposed sediments. Assuming ground-water sediments exhibit a similar lag time, how would it affect the mobility of PNP in the subsurface?
5. Name three different mechanisms microorganisms employ to detoxify metals in their environment.
6. What kind of metal-detoxifying process might assist the bioremediation of metal contaminants?
7. What kind of metal-detoxifying process might hinder the bioremediation of metal contaminants.

CHAPTER 12

BIODEGRADATION AND BIOREMEDIATION OF PETROLEUM HYDROCARBONS IN GROUND-WATER SYSTEMS

The uncontrolled release of petroleum hydrocarbons to ground-water systems is a serious and ongoing environmental problem throughout the world. Fuel tanks, of either the aboveground or underground variety, are prone to corrosion, which often causes leaks. Human error, such as not opening or closing the right valves at the right times, frequently results in spills. Finally, transporting petroleum products in ships, trucks, and pipelines regularly leads to accidents of chance and human judgment. The inevitable result of handling the huge volumes of these materials consumed by industrialized societies—about 900 billion gallons per year—is the frequent contamination of shallow ground-water systems by petroleum hydrocarbons.

Once petroleum hydrocarbons are delivered to ground-water systems, they are acted on by a variety of physical, chemical, and biological processes that tend to disperse the contamination. Ground-water flow moves water-soluble hydrocarbons away from the spill site, and the configuration of the water table influences the movement of the nonsoluble free-phase hydrocarbons. Chemical processes, such as sorption and volatilization, alternately immobilize and mobilize particular components. Finally, microbiological processes immediately begin degrading some hydrocarbons to carbon dioxide. These biodegradation processes are varied and depend on the chemistry and hydrology of a particular ground-water system. Because they act to transform potential contaminants from relatively toxic compounds (cyclic hydrocarbons) to relatively nontoxic compounds (carbon dioxide or methane), these microbial processes are important to the eventual fate of petroleum hydrocarbons in ground-water systems. This chapter describes particular aspects of microbial degradation of petroleum hydrocarbons in ground-water systems.

12.1 COMPOSITION OF CRUDE OIL

The term "crude oil" is strictly one of convenience and refers only to what is actually produced from an oil well. It carries no meaning at all with regard to its

actual chemical composition. In fact, crude oils produced from different oil fields differ radically in their organic chemistry. Pennsylvania crude oil, for example, consists almost exclusively (90–95%) of *n*-alkanes. Crude oils of California and western Mexico, on the other hand, consist of less than 50% *n*-alkanes. The relatively uniform composition of gasoline, kerosene, and jet fuels used worldwide owes much to refinery technology and little to a uniform composition of "crude" oil.

Crude oil consists of *n*-alkanes—often termed "paraffins" because of their low aqueous solubility—branched alkanes, cyclopentanes, cyclohexanes, aromatic hydrocarbons (Fig. 12.1). The number of carbon atoms in organic molecules of crude oil range in length from 1 carbon (methane) to over 400 carbons. In spite of the enormous number of possible isomers generated by this variety. Crude oils actually contain surprisingly few compounds. Other than the *n*-alkanes and branched alkanes, most of the cycloalkanes consist of alkyl-cyclopentanes and alkyl-cyclohexanes. The aromatic fraction of most crude oils consists of alkylbenzenes and alkyl naphthalenes. In addition to hydrocarbons, crude oils may contain small amounts of phenols, alcohols, ketones, and fatty acids. The amount of sulfur-bearing asphaltic components, such as the mercaptans, varies widely between crude oils. Pennsylvania-grade crude oils contain less than 0.1% sulfur, whereas some California crude oils contain as much as 5% sulfur. These sulfur-bearing compounds are used in preparing asphaltic materials for surfacing roads.

As one might expect, crude oils are classified on the basis of the relative amounts of paraffins, aromatics, and asphaltic components present in them. Thus, Pennsylvania-grade crude is referred to as "paraffinic," whereas the high-sulfur crude oil of California is referred to as "asphaltic." Various descriptive combinations, such as "paraffinic-aromatic," are also commonly used.

12.2 PETROLEUM REFINING AND FUEL BLENDING

Crude oil can be separated into carbon compounds of particular length by means of fractional distillation. By progressively heating the crude oil from room temperature up to 400°C, carbon compounds of progressively greater boiling points are volatilized. These various fractions can then be condensed for utilization as gasoline, heating oils, or lubricating oils.

Gasoline, for example, consists of hydrocarbons, which boil from about 30° Celsius to 200°C. The amount of gasoline, or any other component, available by means of fractional distillation is limited and depends on the composition of the crude oil. Many crude oils, for example, contain less than 20% of the gasoline fraction.

Because gasoline is one of the most desirable products of crude oils, elaborate chemical processes have been developed for increasing the amount of gasoline that can be recovered from crude oil. Hydrocarbons are very stable molecules and can be distilled without decomposition. However, if they are heated well beyond their boiling points in the absence of oxygen, the molecules can be caused to disintegrate or "crack" into smaller molecules. Often these hydrocarbon-cracking reactions are assisted by the addition of catalysts that enable the reactions to occur at lower

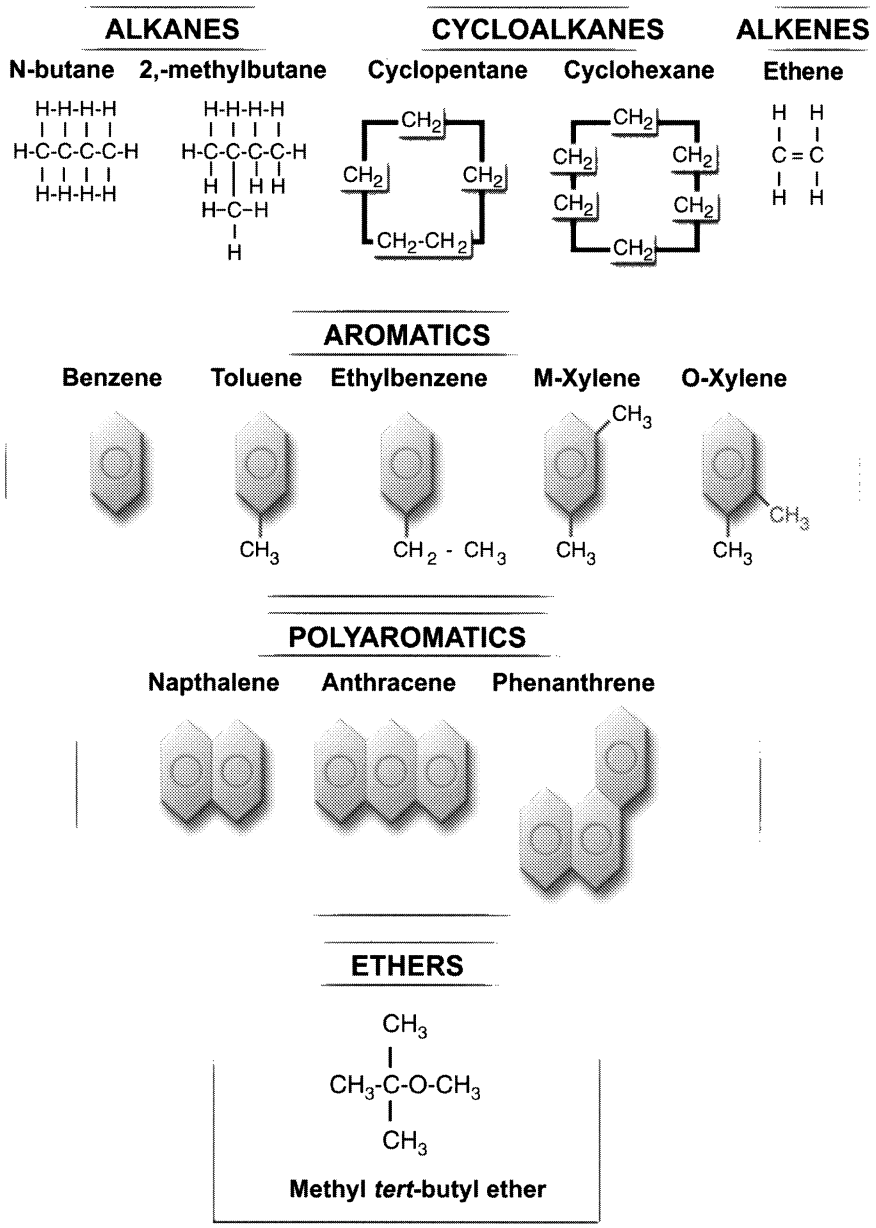


Figure 12.1. Some organic compounds present in petroleum products.

temperatures. These procedures greatly increase the amount of gasoline and other fuels that can be recovered from crude oil.

A consideration that is just as important as the quantity of fuel that can be recovered from a crude oil is the quality of that fuel. It was discovered very early on that some petroleum hydrocarbons make much better fuels than other compounds. In order to compare various mixtures of hydrocarbons in terms of their

quality as gasoline, an arbitrary "knock-rating" scale has been devised. Gasoline engines burning a poor-quality fuel will exhibit a characteristic "knocking" noise that is absent with good-quality fuels.

In order to construct the knock-rating scale, a pure hydrocarbon with excellent knock properties, isooctane (2,2,4 trimethylpentane), was assigned an octane number of 100. Furthermore, a pure hydrocarbon with very poor knock properties, normal heptane, was assigned an octane number of 0. The octane number of a particular gasoline, therefore, refers to its knock properties compared to a mixture of isooctane and *n*-heptane.

The burning properties of fuels can be improved by increasing the percentage of efficiently burning hydrocarbons that are present. A number of techniques are employed to this end. It is generally observed, for example, that branch-chained hydrocarbons burn more efficiently than straight-chained hydrocarbons. *Isomerization* reactions can be used to accomplish this process. In the presence of aluminum chloride and at a temperature of 100°Celsius, *n*-pentane isomerizes to isopentane. This process converts a poor-burning hydrocarbon to a much better one. Another reaction that is used to improve the burning properties of fuels is aromatization. When heated over special catalyst, alkanes can be cyclized and dehydrogenated to aromatic hydrocarbons. For example, heptane reacts with a platinum catalyst and heat to form toluene. Toluene, in turn, has much better burning characteristics than heptane.

The blending of gasolines in the United States changed dramatically with the passage of the Clean Air Act of 1990. This legislation mandated the blending of "fuel oxygenates" in gasoline in metropolitan areas characterized by poor air quality. Carbon monoxide, an important air pollutant, is generated by the incomplete oxidation of hydrocarbons in gasoline. One way to achieve more complete oxidation of gasoline is to increase the amount of oxygen in the air-fuel mixture. This can be done by adding oxygen-containing compounds directly to gasoline. Some commonly used fuel oxygenates that are added to gasoline to improve complete combustion are ethanol, methanol, and methyl *tert*-butyl ether (MTBE). MTBE, the structure of which is shown in Figure 12.1, was first blended with gasoline in the late 1970s as a replacement for tetraethyl lead. With the adoption of amendments to the 1990 Clean Air Act that mandated the use of fuel oxygenates in metropolitan areas characterized by severe air pollution, however, MTBE quickly became the most commonly used fuel oxygenate. In some gasolines, MTBE comprises as much as 15% of the fuel by volume. In 1993, 24 billion pounds of MTBE was produced and consumed in the United States (Squillace et al., 1997).

Gasoline, and other specialized fuels, are a complex mixture of natural and manufactured hydrocarbons derived from crude oil. The composition of these fuels varies with the use for which they were designed. Diesel and jet fuels, for example, can use large percentages of straight-chained hydrocarbons. High-octane gasolines, however, require a higher percentage of branched alkanes, aromatic hydrocarbons, and fuel oxygenates. These differences in fuel composition not only affect their properties as fuels, but they also affect the manner in which microorganisms can use them as carbon substrates. Thus, it is important to be aware of the different compositions of different fuels when one is addressing ground-water contamination by petroleum hydrocarbons.

12.3 MOVEMENT AND SEPARATION OF PETROLEUM HYDROCARBONS IN GROUND-WATER SYSTEMS

Crude oil and refined hydrocarbon products have much different physical and chemical properties than does water. The mean density of gasoline, for example, is 0.9 gm/cm^3, as opposed to 1.0 for fresh water. In addition, water is a highly polar molecule, whereas most components of refined hydrocarbon products are nonpolar molecules. Both of these differences serve to separate water from petroleum hydrocarbons in ground-water systems. The density difference ensures that undissolved hydrocarbons will float on the water table, and the different polarity greatly reduces the solubility of hydrocarbons in water. These physical and chemical properties largely determine the movement and separation of petroleum hydrocarbons in subsurface environments.

12.3.1 Density-Driven Migration of Hydrocarbons

An uncontaminated shallow ground-water system may be viewed as being composed of two immiscible fluids (air and water) that are separated by an interface. The water is present in the saturated zone, the air is present in the overlying unsaturated zone, and the interface between the two fluids is, of course, the water table. When a hydrocarbon is spilled at land surface, a third immiscible fluid is added to the system.

Once a spill has occurred at land surface, the movement of the hydrocarbon is determined by the sum of the forces acting on it. Gravity tends to pull it downward, whereas the air in the unsaturated zone and capillary forces tend to buoy it up. In general, if the pressure at the leading edge of the hydrocarbon is greater than air pressure, the hydrocarbon phase will move downward, displacing air as it goes. If the pressure of the hydrocarbon drops below the displacement pressure of the air, as often happens with small spills, movement of the hydrocarbon will cease and the hydrocarbon will be immobilized in the unsaturated zone. If the pressure is sufficient, the hydrocarbon fluid will continue displacing air and moving downward until it encounters the water table.

Once it encounters the water table, the pressure relationships moving the hydrocarbon fluid change. In order to continue moving downward, there must be sufficient pressure to displace water. On the other hand, hydrocarbon that is still above the water table can continue to displace air laterally. The net effect of this is that a lens of hydrocarbon develops on the surface of the water table and spreads laterally over time (Fig. 12.2). Lateral spreading continues until the hydrocarbon is at or just below the displacement pressure of hydrocarbon into air and water at all points.

The illustration shown in Figure 12.2 represents an aquifer that is isotropic, homogeneous, and lacks a hydrologic gradient. Such idealized conditions do not, for all intents and purposes, exist in nature. In real systems, aquifer heterogeneities impose considerable complexity on the distribution of free-phase petroleum hydrocarbons in ground-water systems.

From the point of view of microbial habitats, Figure 12.2 does illustrate the different environments created by a petroleum hydrocarbon spill. Perhaps the most

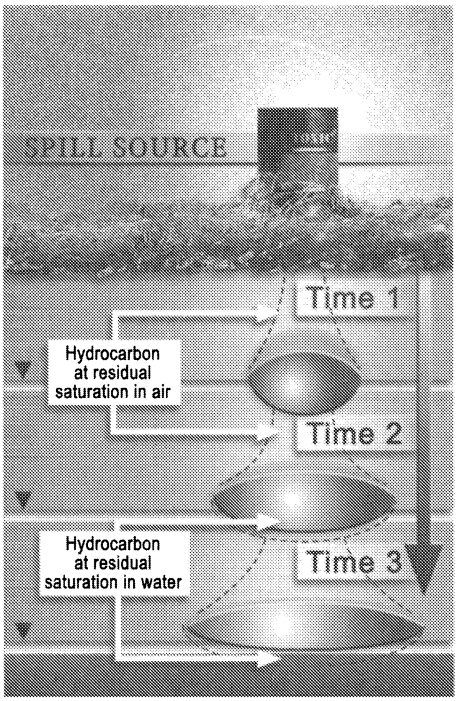


Figure 12.2. Diagram showing the spread of a hydrocarbon lens at the water table and the development of hydrocarbon-saturated air and water.

stressed environment, from a microorganism's point of view, is in the hydrocarbon-saturated sediment. Water is not abundant, which slows delivery of dissolved nutrients and electron acceptors needed for microbial growth. There may also be toxicity stresses associated with particular hydrocarbons. For these reasons, microbial metabolism and growth within the free-product lens of most hydrocarbon spills is often limited.

Above the lens, however, the air tends to be at or near saturation, with the more volatile components present in the hydrocarbon. In Figure 12.2, this is referred to as the zone where the air is at "residual saturation" with respect to the hydrocarbon. This is an environment that is much more conducive to microbial growth. The volatile components of hydrocarbons, short-chained alkanes, cyclic alkanes, and aromatic components are readily oxidized by many kinds of soil bacteria. The presence of air allows oxygen to diffuse into the system with the consequent potential for aerobic metabolism. Because this is occurring in the unsaturated zone, water may be a limiting factor under some conditions. In general, however, the unsaturated zone above a hydrocarbon lens exhibits considerable microbial activity.

The water below the lens is the third general type of habitat created by a hydrocarbon spill. This zone is referred to in Figure 12.2 as being where the hydrocarbon is "at residual saturation in water." In other words, the more soluble components of the hydrocarbon dissolve and reach aqueous saturation near the lens. In fact, actual saturation probably occurs only very near the lens, with a hydrocarbon gradient extending away from it. This also is a favorable habitat for microbial

growth. Water is abundant, electron acceptors may be present, and the toxic effects of the hydrocarbons are diminished by decreasing hydrocarbon concentrations away from the lens.

Each of these three environments is exploited to some degree by microorganisms. However, the kinds of microbial degradation processes that occur are often much different between each of these particular habitats.

12.3.2 Solubility and Hydrocarbon Separation in Ground-Water Systems

Many hydrocarbon compounds present in gasoline, particularly the *n*-alkanes and branched alkanes, have very limited solubility in water. Other compounds, such as the benzene, toluene, ethylbenzene, and xylene (universally referred to as "BTEX" compounds), and fuel oxygenates such as MTBE, are highly soluble. BTEX compounds typically comprise only 2% or 3% of the fuel as a whole. Fuel oxygenates in gasolines formulated after 1990 may comprise up to 15% of the fuel. However, even in fuels containing high percentages of fuel oxygenates, about 80 percent of the compounds present are composed of C_9 and larger hydrocarbons with fairly limited solubility. Thus, only a fairly small portion of the gasoline as a whole is available to dissolve in water. Nevertheless, this "fairly small portion" can cause substantial contamination of ground-water systems.

The difference in solubility of the BTEX portion of most fuels compared to the C_9 and larger hydrocarbons was emphasized by Dunlap and Beckmann (1988) using a simple experiment. First, they filled containers with 4 liters of deionized water. Next, they placed 400 ml of various fuels on top of the water, sealed the containers, and let them stand for 22 days. At the end of this time, samples of water were withdrawn from the bottom of the containers and analyzed for BTEX, total petroleum hydrocarbons, and several naphthalene isomers.

The results of these analyses illustrate the disproportionate effect that relatively soluble compounds have on the water (Table 12.1). While aliphatic hydrocarbons (alkanes, etc.) were the major component of each fuel, they were just a tiny fraction of what actually dissolved in the water. Conversely, while the BTEX compounds comprised less than 3% of the fuel, they accounted for most of the dissolved fraction in the water.

The presence of fuel oxygenates greatly changes the amount of soluble hydrocarbons present in gasoline. Benzene, the most soluble of the BTEX compounds, has an aqueous solubility of 1,780 mg/L. MTBE, in contrast, has an aqueous solubility of about 50,000 mg/L. In the 1960s, before the widespread use of MTBE, the total soluble fraction of gasoline was about 3%. With MTBE reformulated gasoline, however, this soluble fraction is as high as 20%. The use of fuel oxygenates such as MTBE, therefore, has greatly increased the amount of soluble fuel hydrocarbons present in gasoline. This, in turn, has increased the impact of fuel spills on surface and ground water (Squillace et al., 1997).

The separation of soluble from insoluble hydrocarbons is an important characteristic of all fuel spills that affect ground-water systems. Most of the effect in terms of dissolved components comes from the BTEX and fuel oxygenates fraction. Conversely, the heavier aliphatic components do not dissolve readily and become trapped at or near the water table. These differences in solubility affect both the migration and biodegradation of petroleum hydrocarbons in ground-water systems.

TABLE 12.1 Concentration of Hydrocarbons in Water After Contact with Distillate Products

		Detection Limit	Kerosene	Diesel	Fuel Oil
Benzene	μg/l	0.5	294	344.0	203.0
Ethylbenzene	μg/l	0.5	19	139.0	100.0
Toluene	μg/l	0.5	870	777.0	509.0
Xylenes	μg/l	0.5	1260	875.0	592.0
M-Tert-But. Ether	μg/l	0.5	BDL[a]	BDL	BDL
Ethylene-Dibromide	μg/l	0.005	BDL	BDL	BDL
Tot. Pet. Hydrocarbons	μg/l	0.2	40	16.8	21.1
Naphalene	μg/l	0.5	356	6.6	40.0
1-methyl napthalene	μg/l	0.5	193	66.2	107.0
2-methyl napthalene	μg/l	0.5	225	108.0	152.0
Total BTEX[b]	μg/l		2440	2140.0	1400.0
Total Napthalene[c]	μg/l		774	181.0	299.0

[a]BDL—below detection limit

[b]Summation of benzene, toluene, ethylbenzene, and xylenes

[c]Summation of napthalene, 1-methyl napthalene, and 2-methylnapthalene

Source: Dunlap and Beckman, 1988. Reprinted courtesy of the National Water Well Associaton.

12.4 MICROBIAL DEGRADATION OF ALIPHATIC HYDROCARBONS

Aliphatic hydrocarbons, the straight-chained alkanes, alkenes, and their branched derivatives are important substrates for a variety of microorganisms. The biochemical mechanisms involved in degradation are diverse and depend on the chemical properties of the specific hydrocarbon. Methane oxidation by aerobic bacteria, a special case of alkane oxidation, involves a specialized enzyme system that includes methane monooxygenase. Longer-chained alkanes and alkenes are degraded by converting these compounds to long-chained fatty acids and subsequent degradation via beta-oxidation. In addition to these well-characterized aerobic processes, there are undoubtedly numerous anaerobic pathways for degradation that have yet to be elucidated.

Beginning with the early work of Zobell (1950), it has been possible to enumerate several generalizations concerning microbial oxidation of aliphatic hydrocarbons. These include:

1. Long-chained *n*-alkanes, those greater than C_{10}, are assimilated into cells and oxidized more readily than shorter-chain *n*-alkanes. An important exception to this rule is methane oxidation, which employs a different biochemical pathway for oxidation.

2. Saturated aliphatic hydrocarbons (compounds containing only carbon and hydrogen) are degraded more readily than unsaturated hydrocarbons (compounds containing carbon, hydrogen, and oxygen).

3. Branch-chained hydrocarbons are degraded less readily than are straight-chained ones.

The chief reason for the first observation appears to be related to compound toxicity. Aliphatic hydrocarbons in the C_2–C_{10} range are more soluble in water than the longer paraffinic compounds. This solubility seems to confer greater toxicity, probably because the cell has greater difficulty regulating hydrocarbon transport through the cell membrane. The longer aliphatics are too insoluble for such transport, and cellular uptake can be more readily controlled. The mechanisms of aliphatic uptake are complex and are still a matter of active research. However, it is often observed in nature that microorganisms are found attached to hydrocarbon droplets. The mechanism of cellular attachment to hydrocarbons seems to involve lipopolysaccharides present on the cell wall. In addition, microorganisms acclimated to growth on aliphatics often produce emulsifying compounds that may be involved with regulation of cellular hydrocarbon uptake.

The reasons for these observations are related directly to the biochemistry of aliphatic degradation, which will be considered in some detail in the following sections. In general, however, normal saturated hydrocarbons are more readily converted to long-chained fatty acids for subsequent beta-oxidation than unsaturated or branch-chained hydrocarbons. This, in turn, is the source of considerable recalcitrance to degradation exhibited by some unsaturated and branch-chained aliphatic hydrocarbons.

12.4.1 Methane Oxidation

Methane-oxidizing microorganisms, including bacteria, fungi, and yeasts are common in surface waters, aquatic sediments, soils, and subsurface environments. Among the bacteria, methane-oxidizing bacteria have been isolated and described and classified in various genera, including *Methylobacter, Methylocystis,* and *Methylobacterium*. Because methane-oxidizing autotrophs are so common, considerable effort has been invested in understanding the biochemical mechanisms involved.

Methane oxidation under aerobic conditions proceeds via a sequence of steps that involves methanol, formaldehyde, and formate as intermediate products (Fig. 12.3). The final products of methane oxidation are, of course, carbon dioxide and water. The first step in methane oxidation involves the partial oxidation of methane to methanol, with NADH acting as the electron carrier. This reaction is catalyzed

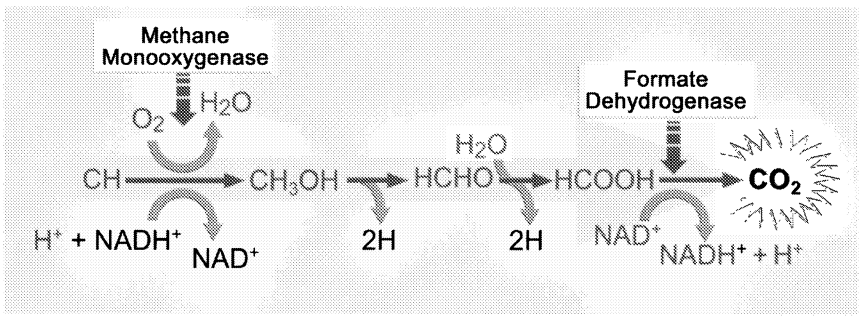


Figure 12.3. Aerobic oxidation of methane in which the initial step is catalyzed by methane monooxygenase.

by the complex enzyme methane monooxygenase. Methane monooxygenase has a very broad substrate specificity and can partially oxidize numerous carbon compounds, including some higher alkanes and alkenes. In addition, methane monooxygenase has been shown to be involved in serendipitous oxidation of chlorinated hydrocarbons, a reaction of considerable environmental importance.

The next steps in this process are the conversion of methanol to formaldehyde by methanol dehydrogenase and the conversion of formaldehyde to formate by means of formaldehyde dehydrogenase. The last step is the oxidation of formate by means of formate dehydrogenase, this time with NAD^+ acting as the electron carrier. From a biochemical point of view, this and other C^1 oxidations are interesting because of the central position of formaldehyde, a relatively uncommon molecule in the environment. From the viewpoint of hydrocarbon degradation, this pathway is very important in the aerobic oxidation of methane and other C_1 and C_2 compounds of environmental interest.

The anaerobic oxidation of methane is a subject that has generated much speculation in the literature but little concrete information. For years, marine sediment geochemists have suggested that the oxidation of methane may be coupled to sulfate reduction. Similarly, it has been suggested that methane can be oxidized by Fe(III)-reducing bacteria. However, no microorganism or consortia of microorganisms have been recovered that will perform these processes under laboratory conditions.

12.4.2 Oxidation of *n*-alkanes

Given the complexity of the biochemical mechanisms involved in oxidizing relatively stable organic compounds, it's not particularly surprising that oxidative mechanisms for many petroleum hydrocarbons make use of mechanisms for oxidizing more "normal" substrates such as organic acids. This is the case for the aerobic oxidation of *n*-alkanes. The strategy for oxidation is to convert the *n*-alkane to a type of organic molecule that can feed into an already established oxidation pathway. In the case of *n*-alkane oxidation, *n*-alkanes are converted to long-chain fatty acids that can then be fed to the beta-oxidation pathway.

Beta-oxidation A large number of respirative bacteria are capable of utilizing long-chained fatty acids as substrates for energy production and growth. These include members of the genera *Pseudomonas, Acinetobacter,* and *Bacillus.* The primary pathway for long-chain fatty-acid degradation is via beta-oxidation.

The biochemistry of beta-oxidation is well-understood and is summarized in Figure 12.4. The first step in this process is the conversion of the organic acid to a coenzyme A (CoA) ester, a reaction catalyzed by acyl-CoA synthetase and requiring energy in the form of ATP. This enzyme is not specific to particular fatty acids and exhibits activity with acids containing as few as six and as many as 20 carbon atoms. Next, the CoA ester is oxidized in the beta position (hence the term "beta-oxidation"), and the two carbon atoms on the end of the molecule are cleaved to yield acetyl-CoA. Acetyl-CoA can then be fed directly to the tricarboxylic acid (TCA) cycle for subsequent complete oxidation. The remainder of the long-chain molecule, which is now shorter by two carbon atoms, is sequentially oxidized until all of the carbon is converted to acetyl-CoA.

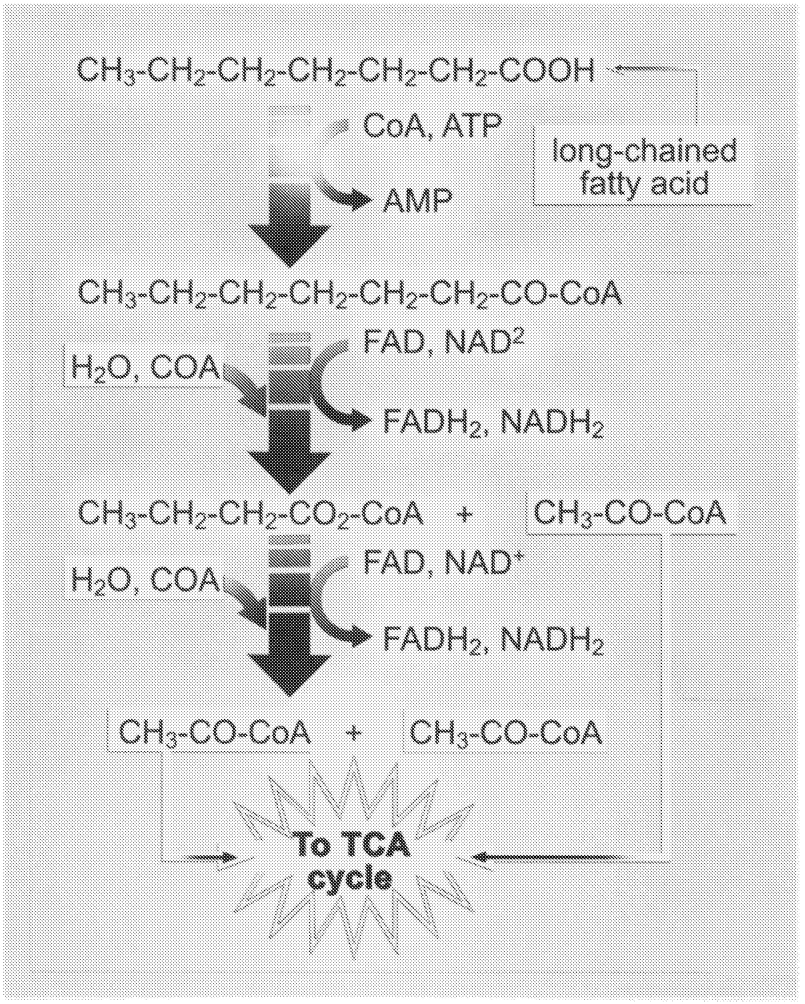


Figure 12.4. Beta oxidation of long-chain fatty acids.

Methyl Group Oxidation Oxidation of a terminal methyl group of a *n*-alkane results in the formation of a long-chained fatty acid. This fatty acid can then be fully degraded via beta-oxidation. Terminal methyl group oxidation is apparently accomplished by means of several mechanisms. One sequence of oxidations and hydroxylations producing either a monocarboxylic or dicarboxylic fatty acid is shown in Figure 12.5a. The sequence begins with the oxidation of the terminal methyl group to an alcohol. Subsequent oxidations then form the carboxylic acid. This carboxylic acid may undergo a hydroxylation reaction with the formation of a dicarboxylic acid. The mono- and dicarboxylic acids are then degraded by beta-oxidation.

n-alkanes may also be fed to beta-oxidation by means of subterminal oxidations (Fig. 12.5b). In this case, a monooxygenase catalyzes the oxidation of a subterminal group in the alkane chain. The chain is then split to form a carboxylic acid and

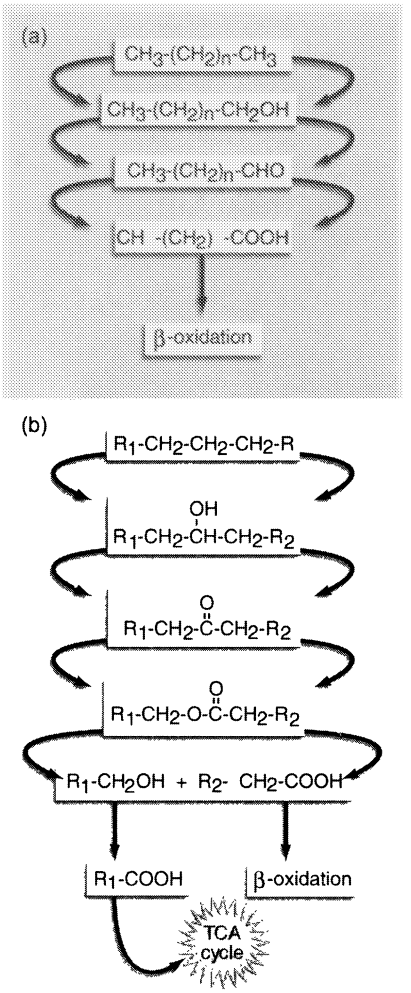


Figure 12.5. Oxidation of *n*-alkanes via (*a*) terminal methyl oxidation or (*b*) subterminal methyl oxidation.

alcohol. The alcohol is further oxidized, and both components are fed to beta-oxidation. There is some evidence that methane monooxygenase may contribute gratuitously to the initial oxidation step. However, it is just as likely that microorganisms acclimated to growth on *n*-alkanes utilize a different monooxygenase.

12.4.3 Alkene Oxidation and Reduction

Alkenes, unsaturated hydrocarbons containing a carbon-to-carbon double bond (C=C), may be degraded via terminal oxidation reactions similar to those found in *n*-alkanes. In addition to terminal oxidation, there appear to be a large number of possible pathways by which alkenes undergo microbially mediated oxidation.

Typically, a large number of intermediate products are detected when even a single culture of cells is grown on alkenes. Although only a few of these pathways have been studied in detail, it seems reasonable to presume that multiple degradation pathways occur in ground-water systems exposed to alkenes. Under anaerobic conditions, alkenes can accept electrons, reducing the double bond to a single bond, and producing alkanes. These alkanes are then subject to oxidation processes under both aerobic and anaerobic conditions.

12.4.4 Branched Aliphatics

Given the central role played by beta-oxidation in the complete oxidation of alkanes and alkenes, it might be expected that the presence of branched chains on hydrocarbon molecules would hinder biodegradation. This is, in fact, observed. Most branch-chained aliphatics exhibit much greater resistance to microbial oxidation than do nonbranched hydrocarbons.

In spite of this recalcitrance, however, microbial mechanisms for the degradation of branched-chain hydrocarbons have been described. One of these, the citroneliol degradation pathway, was described by Seubert and Fass (1964). As one might expect, the basis of this pathway is the removal of one of a branch methyl group, production of a carboxylic acid, and subsequent degradation via beta-oxidation. The biochemistry of such removal mechanisms are complex and specific for specific alkyl groups in specific configurations. Thus, there is not a broad spectrum of compounds that can be degraded by any one pathway. Nevertheless, microorganisms are capable, at least to some degree, of circumventing the biochemical difficulties inherent in branch-chained hydrocarbon degradation.

The relative recalcitrance of branched-chain hydrocarbons to biodegradation has important implications for degrading petroleum hydrocarbons in ground-water systems. As discussed earlier, the burning properties of branched-chain alkanes and alkenes are superior to those of *n*-alkanes and alkenes. Thus, considerable effort is applied during petroleum refining in order to increase the proportion of branch-chained to unbranched compounds. The net effect of this, of course, is the production of petroleum products that are less easily degraded if introduced to the ground-water environment. Considering the vast number of compounds present, it seems inevitable that some portion of petroleum products—probably many branch-chained aliphatics—will resist full oxidation by microorganisms. Thus, complete oxidation of petroleum hydrocarbon products in ground-water systems may not occur.

12.5 MICROBIAL DEGRADATION OF ALICYCLIC HYDROCARBONS

Alicyclic hydrocarbons such as cyclopentane, cyclohexane, and various substituted cycloalkanes are present in petroleum and petroleum-derived fuels. As such, they are regularly delivered to the environment due to natural hydrocarbon seepage as well as fuel spills and leaks. In such environments, alicyclic compounds are not observed to persist. From this it would seem logical that alicyclic hydrocarbon-oxidizing microorganisms are fairly common in the environment. However, it took

several decades of patient work by a number of teams of microbiologists before particular strains of microorganisms were found that could utilize alicyclic compounds as a carbon source in cell growth.

For many years it was assumed that some microorganisms capable of aliphatic hydrocarbon oxidation could also metabolize alicyclic hydrocarbons. To this end, the search for alicyclic-oxidizing hydrocarbons focused on microorganisms that had been previously acclimated to various aliphatic hydrocarbons. This approach met with little success and was eventually abandoned. Finally, Beam and Perry (1974) reported that complete oxidation of cyclohexane could be achieved by consortia of microorganisms in marine sediments. Subsequent to this, de Klerk and van der Linden (1974) showed that, while neither an *n*-alkane nor a cyclohexanol-oxidizing Pseudomonad could oxidize cyclohexane individually, when both species were present cyclohexane was completely degraded to carbon dioxide. Apparently, the observed oxidation of alicyclic compounds in the environment is carried out predominantly by a consortia of microorganisms via commensal (mutually beneficial) relationships.

The study of de Klerk and van der Linden (1974) was important not only because it emphasized the importance of commensal microbial relationships in alicyclic degradation, but also because it gave insight into the mechanisms of degradation. The *Pseudomonas aeruginosa* strain alone did not completely degrade cyclohexane; rather, much of the cyclohexane accumulated as cyclohexanol, a cyclic alcohol compound. Strains of a cyclohexanol-degrading microorganism were known (i.e., *Pseudomonas* "cyclohexanol"), but by itself this strain could not effect complete degradation of cyclohexane. The next logical step was to combine the two kinds of microorganisms. When this was done, the result was the complete oxidation of cyclohexane to CO_2 with the generation of biomass. In addition to elucidating this particular commensal relationship—*P. aeruginosa* partially oxidized cyclohexane to cyclohexanol, providing the substrate for growth of *Pseudomonas* "cyclohexanol"—it also established the importance of partially oxidized intermediate products in cyclohexane degradation.

12.5.1 Pathways for Cyclohexanol Degradation

Once it became clear that formation of cyclohexanols from alicyclic hydrocarbons was an important step in complete biodegradation, it became necessary to elucidate pathways of cyclohexanol transformation. A number of research groups contributed to understanding these transformations, but work by Trudgill and coworkers (Trudgill, 1984) on a strain of cyclohexanol-degrading *Acinetobacter* illustrates the important features of the biochemistry involved.

The first step in the aerobic degradation of cyclohexanol is a dehydrogenation to form an alicyclic ketone. Next, an alicyclic ketone 1,2 monooxygenase catalyzes the insertion of an oxygen into the ring. The ring is then cleaved by means of a hydrolase, and subsequent dehydrogenation reactions result in the formation of a dicarboxylic acid. This acid may then be further degraded via beta-oxidation or other metabolic pathways.

The enzyme systems involved in cyclohexanol degradation are sufficiently nonspecific so that a range of substrates, including *cis*-cyclohexane-1,2 diol, and *trans*-cyclohexane-1,2 diol were degraded in a similar fashion.

Application to Environmental Studies The cyclohexane-cyclohexanol example serves to illustrate some of the processes involved in the degradation of petroleum hydrocarbons by microorganisms. Given the chemical and biological variety of alicyclic molecules, it is certain that degradation proceeds by other pathways as well. Although the anaerobic degradation of alicyclic compounds has apparently received little attention, it is also possible that these compounds are transformed under anaerobic conditions as well.

It is clear, however, that alicyclic compounds are relatively easily degraded by consortia of microorganisms in aquatic sediments. Furthermore, the relationships between microorganisms that effect degradation appear to be both commensal and cooxidative. While degradation of alicyclic compounds in ground-water systems has received little direct attention in the literature, it can reasonably be assumed that similar processes are involved.

12.6 MICROBIAL DEGRADATION OF AROMATIC HYDROCARBONS

Of all the many components of petroleum hydrocarbons, the aromatic compounds such as benzene, toluene, ethylbenzene, and the xylenes (BTEX) are among the most mobile and most potentially toxic when released to the environment. Even though aromatic compounds comprise only a minor portion (2–8% by weight) of gasoline, these compounds are relatively soluble in water. The solubility of benzene in aqueous solution is about 1,700 mg/L, toluene about 500 mg/L, and the various xylene isomers about 135 mg/L. This compares with the solubility of *n*-hexane of about 9 mg/L. So, even though normal and branched alkanes are a much larger component than aromatics of most fuels, the relative solubility of the aromatics leads to a disproportionally large impact on ground-water quality.

In addition to being more readily mobilized into ground water, many aromatic hydrocarbons are now known to be potent carcinogenic (cancer-causing) agents. As early as 1930 it was shown that applying coal tar to mouse skin could induce cancer. Later, this carcinogenic activity was attributed to a particular cyclic hydrocarbon, benzo[a]pyrene. Benzene also has been shown to be carcinogenic and is now considered to be a hazardous substance. The maximum allowable concentration of benzene in drinking water is 0.005 mg/L. In assessing the risk associated with fuel spills, regulatory agencies consider concentrations of benzene to be of primary importance.

Aromatic hydrocarbon compounds are, by definition, characterized by the presence of one or more benzene rings in their structures (Fig. 12.1). The benzene ring itself consists of six carbon atoms linked to form a circle. Unlike cyclohexane, however, the benzene ring contains three C=C double bonds. This forms a very stable chemical structure that poses significant biochemical demands on microorganisms attempting to use it as a carbon and energy source. Because of the abundance of aromatic hydrocarbons in the biosphere, however, many microorganisms have evolved biochemical pathways for utilizing them. In this section, some of what is known about the biochemistry of bacterial aromatic hydrocarbon degradation is summarized.

12.6.1 Benzene Degradation

Benzene is not observed to accumulate in the hydrosphere following spills or leaks of petroleum hydrocarbons. Rather, benzene appears to be readily transformed by microorganisms in a variety of hydrologic settings. Prior to the 1970s, however, little was known about the biochemical mechanisms involved in benzene biodegradation.

Studies by David Gibson and colleagues at the University of Texas resulted in the isolation of a strain of *Pseudomonas putida* that was capable of growth on ethylbenzene and toluene and that also was able to oxidize benzene. The progression of these studies gives an interesting example of the logic involved in understanding biodegradation mechanisms. The first step was to determine some of the intermediate compounds formed during degradation. It was quickly determined that the cells grew on catechol, on *cis*-benzene dihydrodiol, but not on *trans*-benzene dihydrodiol. This suggested that catechol and *cis*-benzene dihydrodiol were intermediate products of benzene oxidation, but that the trans isomer of benzene dihydrodiol was not. Based on this evidence, benzene oxidation appeared to involve sequential oxidations of the benzene ring to catechol (Fig. 12.6).

The presence of a benzene dioxygenase, which performed the initial oxidation of benzene, could be inferred from the observed sequence of reactions. However,

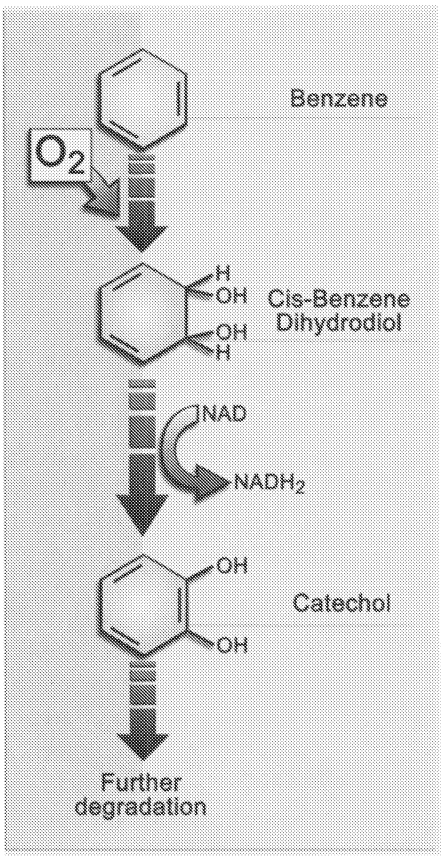


Figure 12.6. Aerobic oxidation of benzene to catechol.

this obscures somewhat the biochemical complexity of the oxidation step. Studies by Axcell and Geary (1975) resulted in the isolation of a *Pseudomonas putida* strain that was capable of growth with benzene as the sole carbon and energy source. This, in turn, led to the characterization of the benzene dioxygenase enzyme. This enzyme turned out to be composed of several subunits: a flavoprotein and two iron-sulfur proteins. The proposed organization of this enzyme complex indicates that oxidation is initiated by electron transfer from NADH to the flavoprotein, through one of the iron-sulfur proteins, to the second iron-sulfur protein where the terminal oxidation of benzene apparently takes place. This sequence of reactions also requires, for reasons that are not clear, the presence of dissolved Fe^{2+} in order to proceed.

The oxidation of benzene by *P. putida* has been studied in some detail and gives an example of how complex the biochemical mechanisms that are involved really are. It's tempting sometimes to think of microbial biodegradation processes as being somewhat magical. In reality, when the biochemical processes are subjected to careful study, it's apparent that the presence of the appropriate enzymatic systems is the real key to biodegradation.

Ortho and Meta Cleavage of Catechol Catechol is a central intermediate product in benzene degradation (Fig. 12.6) as well as in the degradation of many other aromatic hydrocarbons. Two pathways—the ortho and meta cleavage pathways—have been identified that effect the breakdown of catechol. *P. putida* was the microorganism in which these biochemical mechanisms were first identified. However, these pathways have been described in a number of genera, including *Acinetobacter, Bacillus, Alcaligenes,* and *Nocardia.*

In the ortho pathway, also termed the "beta-ketoadipate pathway," catechol is cleaved between the carbon atoms that are carrying the hydroxyl groups (Fig. 12.7a), forming cis,cis-muconate. This compound is then further oxidized, forming beta-ketoadipate. The meta cleavage pathway differs in that the cleavage is initiated between two carbon atoms, only one of which carries a hydroxyl group (Fig.12.7b). The enzyme catechol 2,3-dioxygenase catalyzes the insertion of two oxygen atoms. The ring is then cleaved by means of a hydrolase. The final products are organic acids that can be further degraded to carbon dioxide.

Most strains of *Pseudomonas* utilize either the meta or the ortho pathway for degrading catechol. The meta pathway is thought to be somewhat more flexible and involved in the degradation of substituted isomers of catechol. Some strains, interestingly enough, have been shown to induce enzymes for both pathways. In any case, these biochemical mechanisms are equally important in the complete degradation of aromatic hydrocarbons, as are the initial oxidation steps leading to the formation of catechol.

12.6.2 Degradation of Alkyl Benzenes

Alkyl benzenes, such as toluene and xylene, are also important components of gasolines and other petroleum hydrocarbon fuels. The sequence of reactions involved in the oxidation of these compounds by *Pseudomonas* are analogous to those involved in the oxidation of benzene. In the case of toluene, the initial oxidation leads to formation of a methyl catechol that then undergoes cleavage via the meta pathway. For xylene, the process is similar except that a dimethylcatechol

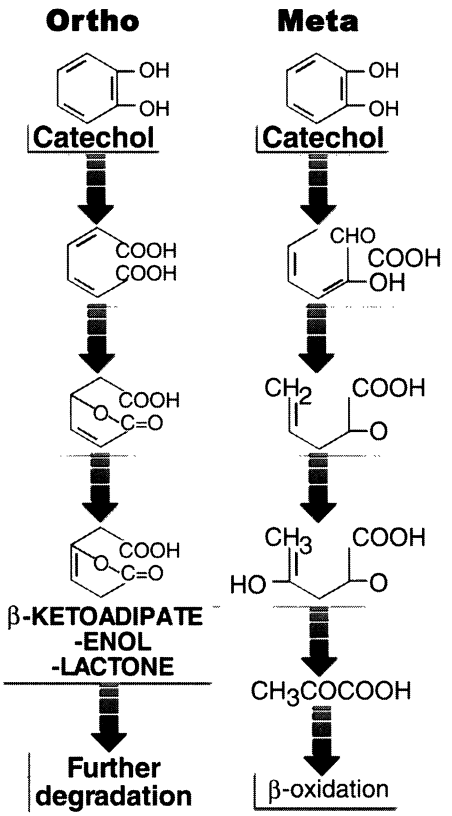


Figure 12.7. The (*a*) ortho and the (*b*) meta pathways for the aerobic oxidation of catechol.

serves as the intermediate product that is fed to the meta pathway. Ethylbenzene appears to follow a similar pathway for oxidation.

From the biochemistry of alkylbenzene degradation, it might be predicted that the ease of degradation is related to the complexity of the catechol intermediate that is formed. This behavior is, in fact, observed. For toluene, the single methyl catechol is easily degraded, and thus toluene is observed to degrade readily. Larger alkyl substituents, such as the ethyl group present in ethylbenzene, are observed to slow down or even to stop degradation. In some cases this slowdown in biodegradation is observed to be accompanied by the accumulation of substituted catachols. Thus, as was observed with the degradation of branched alkanes, more complex molecules are degraded less efficiently. This, in turn, is related to the biochemical limitations of the degradative pathways available to microorganisms and leads to recalcitrance exhibited by some compounds.

Aerobic Degradation of Aromatic Hydrocarbons by Subsurface Bacteria

Although most microorganisms capable of degrading aromatic hydrocarbons have been isolated from soils or aquatic sediments, microorganisms isolated from ground-water systems that carry out these processes have been described. Fredrickson et al. (1991) reported a bacterium, designated strain F199, that was capable

of using alkyl benzenes such as toluene and xylene, as well as other aromatic compounds, as a sole carbon and energy source. This microorganism was recovered from the Middendorf aquifer of South Carolina from a depth of 410 m below land surface.

While the role of various *Pseudomonas* isolates in the catabolism of alkyl benzenes is well-known, strain F199 did not belong to this genera. F199 was gram-positive and exhibited variable cellular morphology that depended upon the particular growth substrate. The ability to degrade aromatic compounds in F199 was inducible with toluene inducing the ability to degrade ^{14}C-toluene and ^{14}C-naphthalene. Curiously, however, ^{14}C-naphthalene did not induce the degradation of ^{14}C-toluene. This in turn, suggested the presence of at least two separate degradative pathways, both of which were capable of degrading naphthalene. Strain F199 was shown to harbor two large plasmids that may code for the observed degradative capability.

There is no particular reason to suppose that aerobic aquifer systems, including such deeply buried aerobic aquifers as the Middendorf aquifer, would not harbor microorganisms with the ability to degrade petroleum hydrocarbons. However, the isolation of F199 is direct evidence that these systems do contain such microorganisms.

12.6.3 Degradation of Polycyclic Aromatic Compounds

In addition to single-ring aromatic hydrocarbons such as the BTEX compounds, petroleum hydrocarbon fuels also contain various polyaromatic hydrocarbon (PAH) compounds such as naphthalene and phenanthrene (Fig. 12.1). As with benzene, the presence of PAHs are of environmental concern because they have been shown to be carcinogenic. Microorganisms that degrade these compounds are widely found in soils. The mechanisms of degradation are analogous to those that are characteristic of benzene degradation.

For the case of naphthalene, an initial oxidation step results in the formation of a dihydroxynaphthalene. Further oxidation results in cleavage of one of the rings with the production of salicylic acid. Degradation of salicylic acid then proceeds through the familiar catechol intermediate. Phenanthrene degradation appears to follow similar mechanisms. The overall strategy is to decouple the rings and to form intermediate products that can feed into other biochemical pathways.

As with other aromatic hydrocarbons, there is evidence that an increase in branched substitutions on one or more of the fused rings leads to increased recalcitrance to bacterial degradation. For example, Heitkamp and Cerniglia (1988) studied PAH degradation by using a strain of bacteria isolated from sediments chronically exposed to aromatic hydrocarbons near an oil field. This bacterium, which had been pre-exposed to pyrene, readily degraded naphthalene, phenanthrene, and pyrene. Curiously, an acclimation period of about one day was required before significant naphthalene degradation was observed. This suggests that the bacterium degrades naphthalene via an enzyme system that is separately induced from the pyrene degradation system. The important point, however, is that the more highly substituted (and more carcinogenic) compounds 1-nitropyrene, 6-nitropyrene, and 3-methylcholanthrene were degraded at a much slower rate.

12.6.4 Anaerobic Degradation of Aromatic Hydrocarbons

Much of what is known about the biochemistry of petroleum hydrocarbon biodegradation has focused on degradation mechanisms. Because many aromatic hydrocarbons, the BTEX compounds for instance, do not contain molecular oxygen in their structures, it is easy to assume that oxidation requires molecular oxygen. For this reason, it was once thought that aromatic hydrocarbons were resistant to microbial attack under anaerobic conditions. If this were true, this would have important impacts upon the fate and transport of these compounds in ground water. Many ground-water systems, even shallow water table aquifers, are anaerobic. Furthermore, the introduction of petroleum hydrocarbons to ground-water systems is invariably followed by the development of anaerobic conditions near the free product. Thus, the absence of anaerobic degradation of petroleum hydrocarbons would greatly limit the biodegradation of these compounds in ground-water systems.

It is interesting from a historical perspective that one of the first observations of anaerobic degradation of aromatic hydrocarbons was made in a contaminated ground-water system (Reinhard et al., 1984). In this study, the selective removal of xylenes from contaminated anaerobic ground water was documented. Furthermore, this removal could not be attributed to hydrodynamic processes such as dispersion or mixing. Apparently, aromatic hydrocarbons could be degraded under anaerobic conditions. Direct experimental evidence of these processes was soon provided. Wilson et al. (1986) showed that ^{14}C-labeled toluene was converted to $^{14}CO_2$ in microcosms of aquifer materials incubated under methanogenic conditions.

As might be expected, however, the microbiology and biochemistry involved with the anaerobic degradation of aromatic hydrocarbons turned out to be much different than that of aerobic degradation. For example, while a large number of aerobic microorganisms that degrade aromatic hydrocarbons have been isolated in pure culture, very few anaerobic microorganisms have been described that perform this function. This is partly due to the greater difficulty involved with isolating anaerobic bacteria. However, it also reflects the importance of mixed cultures and microbial food chains in the complete degradation of organic matter in general, and petroleum hydrocarbons in particular. Furthermore, the biochemical pathways involved in anaerobic degradation are much different from those of aerobic degradation.

Degradation of Toluene and Benzene Under Methanogenic Conditions One of the first studies of toluene and benzene degradation under methanogenic conditions was carried out by Grbić-Galić and Vogel (1987), who used mixed cultures of microorganisms derived from enrichment of sewage sludges. The strategy of this study, which is representative of strategies used to elucidate biochemical pathways, centered on documenting the production of $^{14}CO_2$ from radiolabeled toluene and benzene as well as identifying intermediate compounds produced during degradation.

$^{14}CO_2$ was produced from both ^{14}C-benzene and ^{14}C-labeled toluene, demonstrating that the aromatic ring was being oxidized (Fig. 12.8). This information by itself, however, gives little clue as to the pathways being utilized to effect degradation. By identifying some of the intermediate compounds produced during deg-

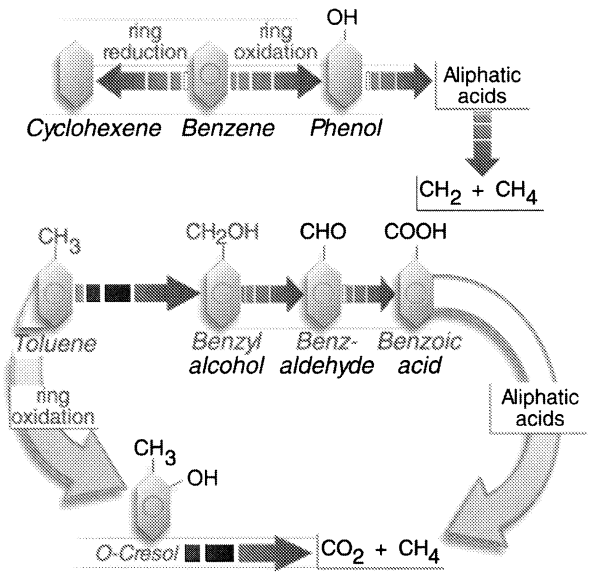


Figure 12.8. Degradation pathways of (*a*) benzene and (*b*) toluene under methanogenic conditions. (Modified from Grbić-Galić and Vogel, 1987, with permission courtesy of the American Society for Microbiology.)

radation, however, it was possible to construct a model of how the degradation of each compound occurs. In this case, numerous intermediate compounds were detected and assigned to particular steps in the degradation process. For example, the appearance of phenol (Fig. 12.8a) suggests that ring oxidation initiates degradation of benzene. Similarly, the appearance of cyclohexene suggests that benzene may also undergo ring reduction. As suggested by studies on benzoate, benzene degradation proceeds through a number of oxidation steps to carboxylic acids. These carboxylic acids are then further oxidized to CO_2 and CH_4.

Toluene, with its single methyl group, apparently may be oxidized in a number of ways. For example, if the molecule initially undergoes ring oxidation (Fig. 12.8b), *p*-cresol or *o*-cresol is produced. Depending on which isomer of cresol is generated, the subsequent oxidation steps are different. Alternatively, the methyl group may be oxidized in the initial step with the formation of benzyl alcohol (Fig. 12.8b). In this sequence, the oxidation proceeds through benzoate and to carboxylic acids. Reduction of the toluene ring is also possible, with the production of methylcyclohexane.

One question that may reasonably be asked when these proposed sequences of oxidations are examined has to do with the source of oxygen inserted into the benzene ring to form phenol or into the toluene ring to form cresol. In the case of aerobic oxidation, insertion of oxygen into ring structures comes exclusively from molecular oxygen. Under anaerobic conditions, however, that seems unlikely. Studies by Vogel and Grbić-Galić (1987) tracing the ^{18}O composition of the intermediate compounds, suggest that the oxygen inserted into the ring structures came from water. Thus, it appears that oxygen-bearing carboxylic acids can be produced from carbon compounds lacking any molecular oxygen under anaerobic conditions.

Degradation of Toluene Under Fe(III)-Reducing Conditions Studies on the biochemistry of aerobic aromatic hydrocarbon oxidation was largely performed with pure cultures of aerobic bacteria. The strategy was to use selective enrichment of water, soil, or sediment samples to isolate and purify cultures, and then to use these purified cultures to understand the biochemical mechanisms of degradation. The literature on the anaerobic degradation of aromatic hydrocarbons, in contrast, focuses largely on the activity of mixed cultures, often termed "consortia," which effect degradation. To a degree, this approach reflects the greater importance of food chains and commensal relationships in anaerobic degradation mechnanisms. However, it also reflects the greater difficulty of working with cultures under strict anaerobic conditions.

In spite of this, pure cultures of microorganisms that are capable of complete oxidation of aromatic hydrocarbons have been described. The first strictly anaerobic microorganism that was shown to oxidize toluene completely to CO_2 grew with Fe(III) as the sole electron acceptor (Lovley and Phillips, 1988). In the course of these studies on Fe(III) reduction, which included consideration of a crude oil spill, it was discovered that phenol and *p*-cresol, in addition to toluene, could be completely oxidized to CO_2 with the reduction of Fe(III) by the dissimilatory Fe(III) reducer *Geobacter metallireducens* (Lovley and Lonergan, 1990; Lonergan et al., 1996).

The inferred biochemistry of aromatic hydrocarbon degradation followed by *G. metallireducens* is shown in Figure 12.9. Toluene is degraded by a series of sequential oxidations in which Fe(III) acts as the sole electron acceptor. As in aromatic oxidation by methanogenic cultures, oxygen is incorporated into the ring structure. This oxygen probably comes from water, as was the case with methanogenic cultures. Alternatively, the oxygen may be derived from Fe(III) oxyhydroxides. The sequence of toluene degradation proposed for *G. metallireducens* (Fig. 12.9) is remarkably similar to that inferred for mixed methanogenic cultures (Fig. 12.8). In both cases, alcohols, aldehydes, and benzoic acid are important intermediate products.

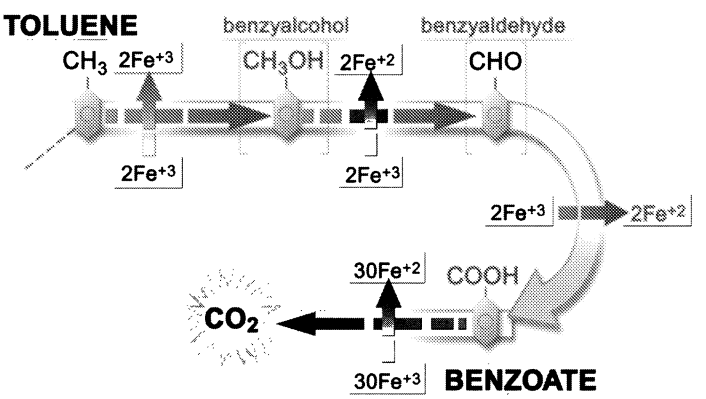


Figure 12.9. Degradation pathway for toluene under Fe(III)-reducing conditions. (Modified from Lovley and Lonergan, 1990, with permission courtesy of the American Society for Microbiology.)

The recognition that Fe(III)-reducing microorganisms can anaerobically oxidize aromatic hydrocarbons is especially important for many ground-water systems. Many shallow sand aquifers—those that are most susceptible to hydrocarbon contamination—contain abundant Fe(III) oxyhydroxides. Thus, once hydrocarbons are delivered to ground-water systems, causing anaerobic conditions to develop, Fe(III) reduction is often the first anaerobic process to actively degrade the hydrocarbon contamination.

Degradation of Alkyl Benzenes Under Denitrifying Conditions Nitrate concentrations in most aquifer systems—either pristine or contaminated—are generally too low for denitrification to be significant relative to other anaerobic microbial processes. Nevertheless, degradation of petroleum hydrocarbons coupled to denitrification is important in bioremediation technology. Because of the limited solubility of oxygen in water, adding oxygen to ground water to increase rates of degradation is difficult. However, because nitrate is relatively soluble, the addition of nitrate is an attractive alternative to the addition of oxygen. Furthermore, the by-product of denitrification, molecular nitrogen gas, is environmentally innocuous. This contrasts with the production of methane, which may create an explosive hazard, or the with production of hydrogen sulfide which creates a noxious odor. Thus, artificially establishing denitrifying conditions can be a useful tool in enhanced biodegradation.

There is evidence that microorganisms present in ground-water systems are capable of degrading benzene, toluene, and xylene (BTX) under denitrifying conditions. Major et al. (1988) showed that, when aquifer solids were incubated under denitrifying conditions, metabolism of BTX compounds were significantly enhanced (Table 12.2). Furthermore, the efficiency of BTX removal under denitrifying conditions approached that of aerobic conditions. Although the results of this experiment can, in the strictest sense, be applied only to the aquifer from which the materials were obtained, they are nevertheless important. If it is assumed that most shallow anaerobic aquifers behave in a similar manner, then nitrate addition could significantly enhance biodegradation rates of petroleum hydrocarbons.

Using a separate experimental approach, Kuhn et al. (1988) placed aquifer material in laboratory columns and acclimated the indigenous bacteria to degrade *m*-xylene with nitrate as the sole electron acceptor. Next, additional alkyl benzenes

TABLE 12.2 Percentage of BTX Remaining After Incubation for 62 Days with Nitrate Under Aerobic or Anaerobic Conditions

	Condition				
Compound	Sterile	Nitrate	Anerobic	Nitrate + Oxygen	Oxygen
Benzene	79	5	66	0	1
Toluene	86	2	65	0	0
o-Xylene	80	15	73	19	15
m-Xylene	80	12	59	8	11

(Initial concentration of compound was 3 mg/l; that is, benzene = 3.84×10^{-5} m; *o*-, and *m*-Xylene = 2.83×10^{-5} M.)

Source: Major, Mayfield, and Barker, 1988. Reprinted courtesy of the National Water Well Association.

were introduced and degradation was monitored over a six-day period. It was found that toluene and *m*-xylene were totally degraded in that period and 3-ethyltoluene was partially degraded. However, little change in the concentrations of benzene, ethylbenzene, and *o*-xylene were found. Because of the short exposure time, it cannot be concluded that the microorganisms present were unable to metabolize these compounds; rather, they may simply not have had enough time to induce enzymatic systems to degrade these new compounds. In any case, these results are consistent with the interpretation that microorganisms indigenous to aquifer systems are capable of degrading some aromatic hydrocarbons under denitrifying conditions.

Another series of laboratory experiments that evaluated BTEX degradation under denitrifying conditions using aquifer material was reported by Hutchins et al. (1991). These experiments are interesting because they were conducted using uncontaminated aquifer material recovered from a shallow ground-water system, as well as material from the same aquifer that had been previously contaminated with JP-4 jet fuel.

In these experiments, degradation of toluene, ethylbenzene, and xylene was observed in both contaminated and uncontaminated aquifer material. Interestingly, however, degradation of benzene was not observed during 160 days of incubation. Perhaps most important of all, rates of alkylbenzene degradation were consistently greatest in uncontaminated aquifer materials. On one hand, this would seem to be counterintuitive as one might expect contaminated sediments to harbor microorganisms acclimated to alkylbenzene degradation. However, at least two other factors may influence alkylbenzene degradation under these conditions. First of all, the contaminated sediments contained aliphatic hydrocarbons in addition to the added alkylbenzenes. This raises the possibility that the microorganisms preferentially use aliphatic hydrocarbons rather than alkylbenzenes. This possibility is plausible, since nitrate was consumed by contaminated sediments even in the absence of alkylbenzenes. Secondly, the extent of contamination may have been sufficient to cause toxic inhibition of metabolism.

The common thread of studies concerning alkylbenzene degradation under denitrifying conditions is that many compounds, particularly toluene and xylenes, are readily degraded. In contrast, most experimental studies indicate that benzene is not significantly degraded under nitrate-reducing conditions. Thus, while the addition of nitrate to petroleum hydrocarbon-contaminated systems as an oxidant can be expected to increase degradation of some petroleum hydrocarbons, it's utility in degrading benzene has not been demonstrated.

12.6.5 Biodegradation of Alkyl Ethers

Alkyl ethers such as methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE), and *tert*-amyl methyl ether (TAME) began to be added to gasolines in the 1970s as octane enhancers. In the 1980s, these compounds began to be added to gasolines as oxygenates to reduce carbon monoxide emissions. As the use of these fuel oxygenates increased, questions concerning their environmental fate arose. Early studies on the biodegradation of MTBE in soils and sludges indicated the MTBE was not significantly degraded (Fujiwara et al., 1984; Moller and Arvin, 1990). This caused a good deal of concern among environmentalists. Given the huge

amounts of MTBE that were being used (24 billion pounds in 1993), it was inevitable that environmental releases would occur. In the absence of biodegradation processes, would MTBE accumulate in ground and surface water? The irony of this, of course, was that the whole point of MTBE was to decrease air pollution. If this actually increased water pollution, then the only thing that that had been accomplished was to transfer pollution from one medium (air) to another medium (water). It was because of this exercise in unintended consequences that the presence or absence of MTBE biodegradation in the environment became a question of considerable importance.

In the early 1990s, the biodegradability of MTBE was not at all clear. Some studies reported little or no biodegradation under either aerobic or anaerobic conditions (Yeh and Novak, 1994). These observations were consistent with the highly stable ether bond present in MTBE (Fig. 12.1). Others reported the isolation of cultures capable of at least partially degrading the MTBE molecule (Salanitro et al., 1994). The MTBE-degrading cultures reported by Salanitro et al. (1994) were originally isolated from sludges taken from a chemical treatment plant. Thus, it appeared that acclimated microorganisms could use MTBE as a source of carbon and energy. It was less clear, however, that microorganisms with MTBE-degrading capability would be present in natural environments such as surface-water sediments or ground-water systems.

One of the first indications that MTBE-degrading microorganisms might indeed be present in natural systems was the observation that propane-oxidizing bacteria were capable of cometabolizing MTBE (Steffan et al., 1997). The mechanism of MTBE-degradation proposed by Steffan et al. (1997) is interesting because it illustrates how complex these degradation processes can be. Beginning with MTBE (Fig. 12.10), the terminal methyl group is oxidized to form TBA. TBA then goes through a series of transformations (including the formation of acetone as an intermediate), leading through pyruvic acid and finally to carbon dioxide. The first step of this possible degradation pathway is indicated by the production of TBA observed by both Steffan et al. (1997) and by Salanitro et al. (1994). The other proposed intermediate compounds shown in Figure 12.10 were observed to be degraded as well, and studies with cell-free extracts indicated that a propane monooxygenase was involved in the observed biodegradation. Because propane-oxidizing bacteria are common in the environment, this raised the possibility that MTBE might be more biodegradable than previously thought.

Most studies of ground-water systems, however, indicate that MTBE is less biodegradable than BTEX compounds. In a direct comparison between BTEX compounds and MTBE, Borden et al. (1997) presented field evidence that MTBE was apparently degrading, but at a slower rate than benzene. Landmeyer et al. (1998) showed laboratory evidence for very slow MTBE biodegradation under aerobic and Fe(III)-reducing conditions. The apparent rates were so slow that MTBE transport was indistinguishable from conservative transport. While MTBE biodegradation appears to be slow in ground-water systems, much more biodegradation apparently occurs where contaminated ground water discharges to surface-water bodies. In a study of several sites where MTBE plumes were discharging into streams, Bradley et al. (1999) showed rapid and complete degradation of MTBE to carbon dioxide under mixed aerobic/anaerobic conditions. This was an important result because it indicates that surface-water sediments have the ability to degrade MTBE. This, in

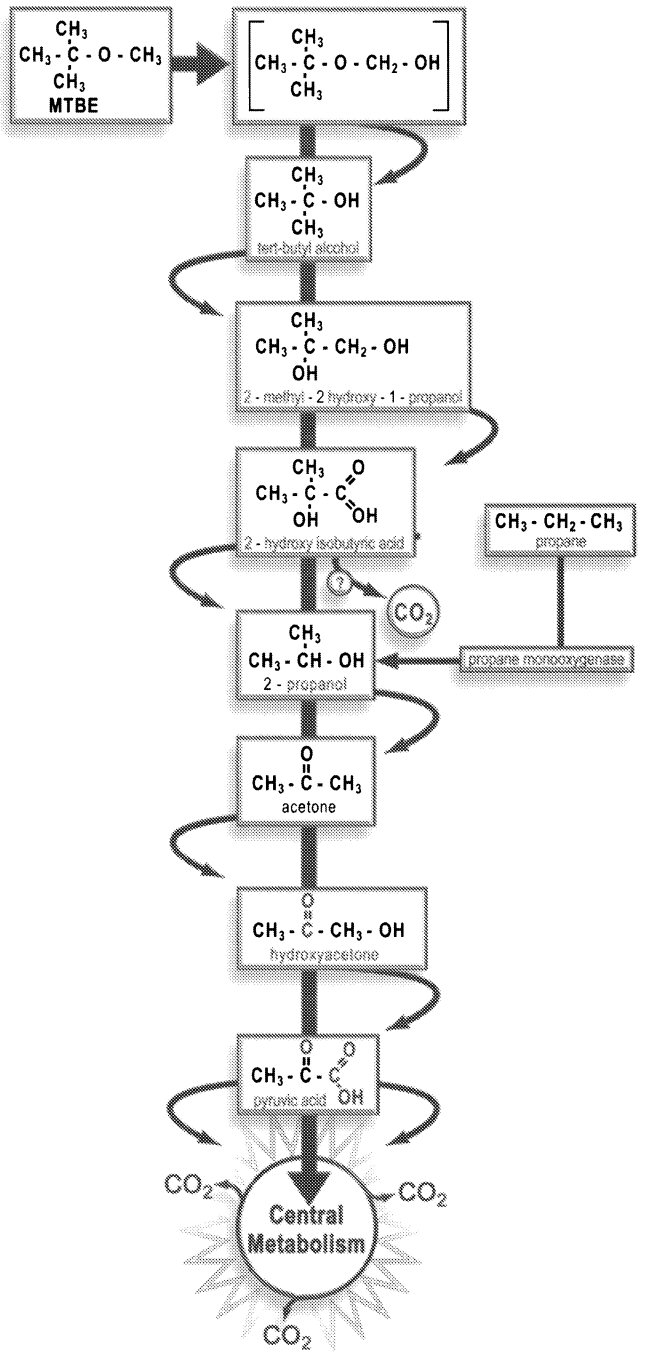


Figure 12.10. Degradation pathway for MTBE by propane monooxygenase as proposed by Steffan et al., 1997.

turn, was the first environmentally significant sink that has been documented for MTBE. Thus, while MTBE biodegradation in ground-water systems appears to occur slowly, biodegradation in surface-water sediments is much more rapid. This, in turn, may help remediate MTBE-contaminated ground water discharging to surface-water bodies, and may prevent MTBE accumulation in surface-water bodies. Nevertheless, because of its recalcitrance to degradation, the U.S. Environmental Protection Agency is presently considering phasing out the use of MTBE and replacing it with alternative fuel oxygenates such as ethanol.

12.7 MICROBIAL DEGRADATION OF PETROLEUM HYDROCARBONS IN GROUND-WATER SYSTEMS

Ground-water contamination by petroleum hydrocarbons was not an issue during much of the twentieth century in the industrialized world. It wasn't so much that people were unaware of petroleum spills, as it wasn't perceived as being much of a problem. With the slow rates of ground-water flow, it typically took years or even decades for the effects of petroleum contamination to become apparent. When they did become apparent, it was usually because of massive contamination of wells used for drinking water. Because many instances of contamination occurred in areas lacking water wells, it's almost certain that the impacts of most spills were simply never detected.

The effects of microbial processes on petroleum hydrocarbons in ground-water systems, particularly the role of these processes in degrading and attenuating contamination, were even slower to be appreciated. As recently as 1981, papers using quantitative modeling techniques for evaluating hydrocarbon mobility made no mention of microbial processes or the effects of such processes on hydrocarbon attenuation (Yazicigil and Sendlein, 1981).

By 1985, microbiologists had gathered clear evidence that biodegradation of petroleum hydrocarbons occurred in both aerobic and anaerobic aquifer systems. However, the most telling evidence that such processes were important came from numerous field studies showing that petroleum hydrocarbons were selectively removed under a variety of conditions. One study in California, for example (Hadley and Armstrong, 1991), surveyed 7,167 water-supply wells for evidence of chemical contamination. Because gasoline spills had occurred from thousands of leaking underground tanks during the previous 50 years, these researchers anticipated that petroleum hydrocarbon contamination, particularly benzene contamination, would be prevalent. To the surprise of the investigators, very little evidence of benzene contamination of ground water was found. After reviewing the possible processes that could remove benzene from ground water, including sorption and evaporation, they concluded that biodegradation processes were the most likely reason for the puzzling "absence" of benzene.

The realization that microbial processes were important in the fate and transport of petroleum hydrocarbons in ground-water systems led to a number of investigations during the late 1980s. These studies form a basis for evaluating biodegradation as a natural attenuation process and as a possible strategy for remediating hydrocarbon contamination in ground-water systems.

12.7.1 Aerobic Degradation of BTX Compounds

The ability of microorganisms to degrade petroleum hydrocarbons has been widely studied and many of the biochemical pathways involved in degradation have been worked out in some detail (see section 12.6). However, it is difficult to predict how complex natural systems, particularly inaccessible ground-water systems, will react to hydrocarbon contamination. The "shrinking" of BTEX plumes in shallow ground-water systems had been widely reported during the 1980s, often with little documenting evidence. However, Barker et al. (1987) reported the results of an experiment in which BTX (benzene, toluene, and xylene) compounds were injected into a sandy water-table aquifer and the resulting plume was monitored for the next year and a half. This study provided reliable evidence for the presence of BTX-degrading microbial activity in water table aquifers.

In the field experiment reported by Barker et al. (1987), about 1,800 liters of ground water were spiked with BTX to give average concentrations of 2.4 mg/L benzene, 1.8 mg/L toluene, 1. I mg/L each of *p*-xylene, *m*-xylene, and *o*-xylene, and 1,280 mg/L chloride. The chloride was added so that it would act as a conservative tracer. The spiked water was injected into a sandy water table aquifer and the resulting plume monitored by means of a dense network of multilevel piezometers. Samples from the monitoring wells were collected each day for three days after injection and each month for 4 months.

The results of this experiment for days 3, 53, and 108 of the experiment are shown in Figure 12.11. By day 53, the combined effects of advection and dispersion had moved the chloride tracer plume between 1 and 5 meters along the natural gradient prevailing in the aquifer, spreading to about three times its initial size as it moved. The benzene component of the plume behaved similarly to chloride in the first 53 days, spreading as it moved downgradient. The toluene component, however, was actually smaller at day 53 than at the beginning of the experiment. By day 108, the toluene component of the plume had virtually disappeared. Furthermore, by day 108, the benzene component of the plume had shrunk noticeably relative to the chloride tracer. Note, however, that the relative position downgradient of the BTX compounds was similar to that of chloride. This observation rules out the possibility that sorption of BTX to aquifer solids was the main attenuating process and implies that biodegradation was the principal process. This conclusion was supported by laboratory scale microcosm experiments.

By vertically integrating concentrations of BTX in the plume, Barker et al. (1987) were able to calculate the mass of each compound present as a function of time (Fig. 12.11b). The mass of chloride, as expected, changed very little. The masses of each BTX compound, however, decreased rapidly. By 108 days, the mass of toluene and the xylene isomers had decreased practically to zero. Although benzene was more resistant to degradation than the other compounds, the mass of remaining benzene was essentially zero by 400 days after injection.

This experiment differs from real petroleum hydrocarbon spills in several ways. First of all, the large mass of aliphatic and alicyclic hydrocarbons present in all fuels were not included in the experiment because of their lack of aqueous solubility. Thus, any effects of these compounds on BTX degradation were absent from the experiment. However, as great quantities of aliphatic compounds are not transported downgradient of petroleum spills, the experiment accurately simulates con-

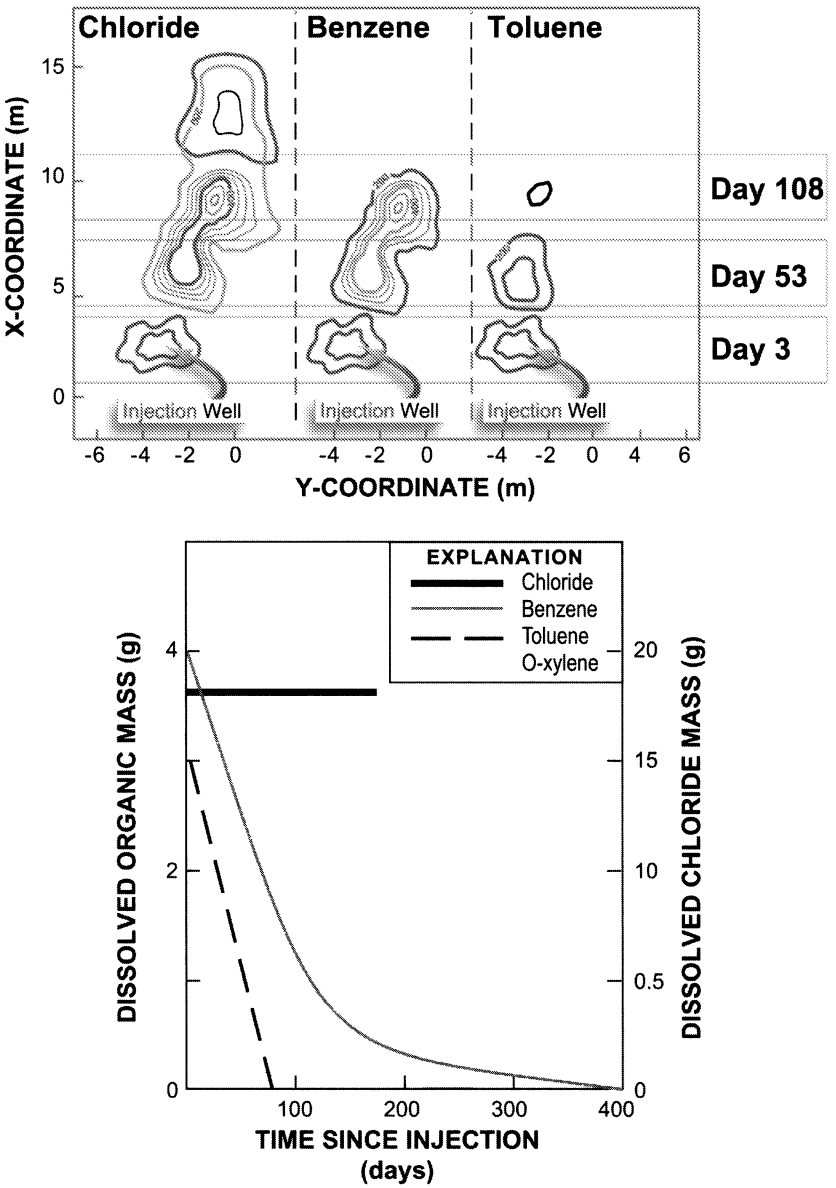


Figure 12.11. (*a*) Disappearance of benzene and toluene relative to chloride in a field scale injection test, and (*b*) decrease in mass of organic compounds relative to chloride in the injection test. (Modified from Barker, Patrick, and Major, 1987.)

ditions downgradient of petroleum spills. In part because of the lack of aliphatic hydrocarbons in the experiment, aerobic conditions prevailed in the aquifer throughout the experiment. As aerobic bacteria may be more efficient hydrocarbon utilizers than anaerobic bacteria, this experiment probably records degradation rates as high as any that would be observed in ground-water systems.

The chief value of the experiment reported by Barker et al. (1987) is that it verified the numerous observations that BTX compounds were actively biodegraded in ground-water systems. However, because of the aerobic conditions and the lack of an immiscible hydrocarbon phase capable of leaching BTX compounds over time, the time required for complete degradation in this experiment was probably less than that required in most petroleum-product spills.

12.7.2 Anaerobic Degradation of BTEX Compounds

One of the first consequences of petroleum hydrocarbon contamination of shallow ground-water systems is the establishment of anaerobic conditions near the plume. One well-documented example of this was a crude oil spill that occurred near Bemidji, Minnesota. Prior to the spill, the aquifer was aerobic (DO $\sim$ 10 mg/L). Soon after the spill, however, increased levels of microbial respiration created anaerobic conditions near the oil lens. This, in turn, provided an opportunity for studying the anaerobic degradation of petroleum hydrocarbons in an anaerobic ground-water system under field conditions (Baedecker et al., 1988; Cozzarelli et al., 1988).

Concentrations of aromatic hydrocarbons in the ground water near at the edge of the lens are shown in Figure 12.l2a and concentrations 10 meters farther downgradient are shown in Figure 12.12b. Concentrations of all of the BTEX compounds were observed to decrease significantly. In fact, all of the alkylbenzene compounds were observed to decrease. Because of the proximity to the oil lens, these concentration decreases could not be attributed to hydrodynamic processes and were interpreted as reflecting anaerobic microbial degradation (Cozzarelli et al., 1988).

Further evidence that microbial processes are involved with degradation are provided by the observed distribution of organic acids in the anaerobic plume. Near the oil lens, fairly high concentrations of organic acids, particularly acetic acid, were observed. These acids have been shown to be intermediate products of anaerobic benzene and toluene degradation in laboratory experiments (Fig. 12.8, Fig. 12.9), and their presence is strong evidence that anaerobic microbial processes are actively degrading the hydrocarbons in this field situation. Downgradient of the oil lens, concentrations of the organic acids decrease because of consumption by a combination of methanogenesis (Baedecker et al., 1988) and Fe(III) reduction (Lovley et al., 1989).

It's interesting to compare the relative degradation rates of each BTEX compound under aerobic conditions (Fig. 12.11) with those observed under anaerobic conditions Fig. (12.12). Under aerobic conditions, it was observed that the relative rates of degradation were xylene > toluene > benzene (Barker et al., 1987). Furthermore, the relative rates of xylene isomer degradation were observed to be *p*-xylene > *m*-xylene > *o*-xylene. Under anaerobic conditions at the Bemidji site, the relative rates of BTEX degradation were similar to those observed under aerobic conditions. However, the order of xylene isomer degradation rates were reversed, with *o*-xylene apparently degrading faster than *m*-xylene or *p*-xylene (Cozzarelli et al., 1988). In any case, this preferential utilization of BTEX compounds probably reflects catabolite repression mechanisms (section 11.2.2). These mechanisms, in turn, can lead to the separation of contaminants within plumes, with the least attractive substrate (benzene or MTBE) present in the leading edge of the plume.

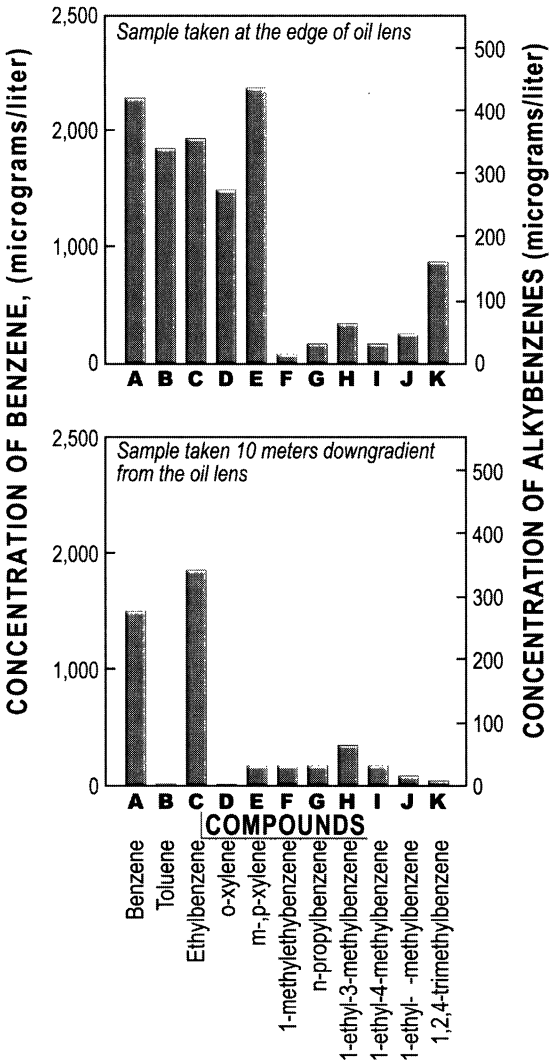


Figure 12.12. Concentrations of aromatic hydrocarbons near a crude oil spill, Bemidjii, Minnesota (*a*) at the edge of the oil lens, and (*b*) 10 meters downgradient of the oil lens. (Modified from Cozzarelli, Eganhouse, and Baedecker, 1988.)

Ethylbenzene was not considered in the study by Barker et al. (1987). However, under the anaerobic conditions of the Bemidji site, ethylbenzene appeared to be relatively resistant to microbial degradation. In the more downgradient part of the plume, where molecular oxygen becomes available, rapid loss of ethylbenzene, as well as benzene, was observed.

12.8 BIOREMEDIATION OF PETROLEUM HYDROCARBON CONTAMINATION IN GROUND-WATER SYSTEMS

In the summer of 1971, a gasoline pipeline in Whitemarsh Township, Pennsylvania, broke and released several hundred thousand gallons of high-octane gasoline. Over

the next year, an estimated one hundred thousand gallons of gasoline seeped to the underlying dolomitic aquifer and dissolved petroleum hydrocarbons migrated to a nearby municipal water-supply well. At that time, it had only recently been proposed that ground-water systems might contain microorganisms capable of degrading petroleum hydrocarbons (Litchfield and Clark, 1973). Furthermore, it was becoming clear that physical methods for removing gasoline from aquifers were not particularly efficient (McKee et al., 1972). A logical approach to this problem, therefore, was to consider using indigenous hydrocarbon-degrading microorganisms to clean up the contaminated aquifer. Accordingly, a group of microbiologists working in the oil industry documented the presence of petroleum-hydrocarbon degrading bacteria in the ground water of the Whitemarsh site, and showed that the growth of these microorganisms could be stimulated in the laboratory by providing oxygen, nitrogen, and phosphate (Jamison et al., 1975). Furthermore, they showed that this same effect could be produced under field conditions by adding nutrients and air-saturated water to contaminated wells. These authors concluded that, if nutrients and oxygen could be efficiently delivered to ground water, then:

> the removal of gasoline might be accomplished in a matter of months rather than the years it would take to physically remove it.

This was one of the first instances where bioremediation was actively considered as a remedial strategy to clean up a petroleum hydrocarbon-contaminated ground-water system.

Since these beginnings in the early 1970s, bioremediation of petroleum hydrocarbons has become an accepted technology and has grown into a $290 million dollar a year industry in the United States alone (Glass et al., 1995). This chapter reviews the history of these technologies specifically as they have been applied to ground-water systems. This history shows that current practice of bioremediation includes many concepts and methods developed at the very beginning of the technology. Conversely, some approaches that were thought to be quite promising in the early 1970s have not come into common use. This history also shows that some of the most useful current practices—monitored natural attenuation, for example—were not anticipated by early investigators in the 1970s.

As bioremediation technology for ground-water systems developed, there has been a gradual realization that hydrologic considerations are as important as microbiologic considerations. Ground-water systems are inherently inaccessible, usually heterogeneous, and difficult to accurately characterize. Experience has shown that favorable hydrologic conditions, and a clear understanding of these conditions, are critical to the success of bioremediation. When this understanding can be properly exploited, either by controlling and directing ground-water movement in engineered bioremediation systems, or by accurately assessing ambient rates and directions of ground-water flow in monitored natural attenuation systems, a reasonable degree of success is possible.

12.8.1 Marine Oil Spills and the Biodegradation of Petroleum Hydrocarbons

The early 1970s was a time of intense scientific interest in the biodegradation of petroleum hydrocarbons (Atlas and Bartha, 1973). This interest stemmed largely

TABLE 12.3 Comparison of Lag Times for the Degradation of Cresol Isomers in Anaerobic Aquifer Slurries

Substrate	Lag Time (Days) for: Sulfate-reducing Incubations	Lag Time (Days) for: Methanogenic Incubations
p-cresol	<10	46
m-cresol	43	46–90
o-cresol	>100	>90

Source: Smolenski and Suflita, 1987. Reprinted courtesy of the American Society for Microbiology.

from environmental concerns surrounding large-scale oil spills in the ocean, such as the sinking of the supertanker *Torrey Canyon* in the English Channel in 1967 (Atlas, 1981). At that time, it was widely feared that contamination from such spills could result in permanent and irreversible damage to the environment. Although it had been known since the 1940s that petroleum hydrocarbons were degraded by microorganisms (Zobell, 1946), the extent to which these processes occurred and the conditions that limited or enhanced biodegradation in aquatic environments were a matter of vigorous inquiry (Atlas and Bartha, 1972; Floodgate, 1972; Bartha and Atlas, 1977).

These studies were instrumental in identifying important metabolic pathways by which petroleum hydrocarbons were degraded. In addition, these studies provided a framework by which the relative biodegradability of different compounds could be understood (McKenna and Kallio, 1964; Gibson, 1971; Pirnik, 1977). It was found, for example, that the *n*-alkanes were the most readily degraded compounds present in petroleum (Treccani, 1964). Degradation of *n*-alkanes proceeded via oxidation of a terminal hydrocarbon forming a long-chain carboxylic acid (Ratledge, 1978). It was also found that methyl branches on aliphatic hydrocarbons tended to increase resistance to microbial attack (Pirnik, 1977). The biodegradation of aromatic compounds was found to involve an oxidative attack upon the ring structure, the formation of a diol intermediate, followed by ring cleavage and the formation of organic acids (Gibson, 1971). For aromatic compounds, simple methyl substitutions on the benzene ring—such as in toluene or various xylene isomers—were found to enhance biodegradability (Gibson and Subramanian, 1984).

These early studies also emphasized the importance of oxygen and trace nutrients for the biodegradation of petroleum hydrocarbons. In particular, it was widely observed that hydrocarbon biodegradation was limited by available nutrients such as nitrogen and phosphorus (Atlas and Bartha, 1972; Floodgate, 1979). Accordingly, it was widely reported that addition of such nutrients could significantly enhance rates of biodegradation (Bridie and Bos, 1971). These studies also suggested that oxygen was the only terminal electron acceptor that supported significant rates of petroleum biodegradation (Bailey et al., 1973). These findings greatly influenced early bioremediation strategies in ground-water systems, where an emphasis was placed on nitrogen, phosphate, and oxygen addition to enhance biodegradation (Jamison et al., 1975).

Studies of oil spills in the marine environment also led to the early development of genetic engineering techniques for enhancing biodegradation. Most notable in this context were the studies of A. M. Chakrabarty and his coworkers (Chakrabarty et al., 1973). These investigators knew that certain strains of *Pseudomonas* had the capability for degrading particular aliphatic hydrocarbons. One strain of *Pseudomonas aeruginosa,* designated strain AC59, was capable of degrading C_6, C_8, and C_{10} compounds, but could not degrade longer aliphatic compounds. Another strain, designated strain AC63, could degrade C_{12} and C_{14} molecules but could not degrade shorter hydrocarbons. Because these degradative capabilities were plasmid-encoded, it was possible to combine them into a single organism using the techniques of genetic engineering. This new microorganism was capable of degrading C_6 through C_{18} hydrocarbons. In the landmark case *Diamond vs. Chakrabarty,* the U.S. Supreme court ruled that such genetically engineered petroleum hydrocarbon-degrading microorganisms constituted a "manufactured" product, and qualified for patent protection. In the early 1970s, and largely because of the work of Chakrabarty and others, it was widely believed that engineered microorganisms would be the basis of many bioremediation strategies. However, fears surrounding the release of genetically engineered microorganisms into the environment have largely prevented such strategies from being applied in the field (Forsyth et al., 1995).

12.8.2 Microbial Degradation Processes in Shallow Water Table Aquifers

By the early 1980s, it was apparent that the contamination of shallow water table aquifers by petroleum hydrocarbons, largely due to leaking underground storage tanks and their transfer lines, was a significant environmental problem (Council on Environmental Quality, 1981). However, methods for remediating such contamination events were problematic. By far the most widely used remediation strategy was to pump contaminated ground water out of the ground and treat it to remove contaminants. Many of these pump-and-treat systems, as they came to be called, did not even consider the possibility that microbial degradation processes occurred (Yazicigil and Sendlein, 1981). The reason for this was that, in the early 1980s, very little was known about the microbiology of shallow water table aquifers (Wilson et al., 1983). There had been a long tradition, beginning in the 1920s, of studying microbial processes in deep petroleum reservoirs (Bastin, 1926). This tradition was carried on by Zobell and his associates throughout the 1930s and 1940s (Davis, 1967). But with few exceptions (Litchfield and Clark, 1973), there were virtually no investigations of the shallow water-table aquifers that were most commonly contaminated by leaking gasoline storage tanks.

Early studies of soil microbiology indicated that numbers of bacteria dropped off sharply with depth (Waksman, 1916), and in the early 1980s it was widely assumed that shallow aquifers were largely sterile. With the increasing concern about petroleum hydrocarbon contamination providing much of the impetus, however, the microbiology of shallow aquifers became an active topic of investigation in the 1980s (White et al., 1983; Hirsch and Rades-Rohkohl, 1983). Several of these studies focused on the ability of subsurface microorganisms to degrade petroleum hydrocarbons (Ehrlich et al., 1985). In particular, Wilson et al. (1983) showed that the potential for microorganisms to degrade toluene, a common component of gasoline, was apparently widespread in shallow aquifers.

Throughout the 1980's, laboratory studies of microbial activity in shallow aquifer systems, and the ability of those microorganisms to degrade petroleum hydrocarbons, was a topic of intense inquiry (B. H. Wilson et al., 1986; Heitkamp and Cerniglia, 1988; Fredrickson et al., 1991). In addition, numerous field studies showed that microbial oxidation of petroleum hydrocarbons was clearly reflected in ground-water chemistry (Cozzarelli et al., 1989; Chiang et al., 1989). These field studies were important because they suggested that petroleum hydrocarbons were being oxidized under anaerobic conditions. Throughout most of the 1970s, it was widely believed that molecular oxygen was necessary to biodegrade petroleum hydrocarbons (Bailey et al., 1973). In fact, the first microorganism capable of completely mineralizing an alkyl benzene under strictly anaerobic conditions was only first isolated in 1988 (Lovley and Phillips, 1988). Laboratory studies have now shown that petroleum hydrocarbons are degraded under nitrate-reducing Grbić-Galić (Hutchins et al., 1991a), Fe(III)-reducing (Lovley et al., 1989), sulfate-reducing (Edwards et al., 1992), and methanogenic (Grbić-Galić and Vogel, 1987) conditions. For many years, the anaerobic oxidation of petroleum hydrocarbons was controversial, largely because aerobic microbial processes had been the focus of the early studies of the marine environment. Nevertheless, the importance of anaerobic oxidation processes in the biodegradation of petroleum hydrocarbons is now firmly established (Lovley, 1997).

In the late 1970s, the use of tetraethyl lead for octane enhancement in gasoline began to be phased out due to environmental concerns about lead in the environment. At that time, some formulations of gasoline began using methyl *tert*-butyl ether (MTBE) as an octane enhancer. The use of MTBE in gasoline was increased significantly by the enactment of the 1990 Clean Air Act, which mandated the seasonal use of fuel oxygenates in certain parts of the United States (Hagin, 1992) to reduce carbon monoxide emissions. Prior to the late 1970s, BTEX compounds were the principal water-soluble component of gasoline. Because of MTBE's relatively high aqueous solubility, and because it can comprise a relatively high percentage of gasoline (between 4 and 15 volume percent), it has become a significant contaminant in many petroleum hydrocarbon spills affecting ground-water systems as well (Squillace et al., 1997).

These laboratory and field studies provided a basis for the design and operation of engineered bioremediation systems for petroleum hydrocarbon-contaminated ground water (Hutchins et al., 1991b). In this regard, these studies were important and very successful. However, because the laboratory studies were carried out under controlled (and therefore artificial) conditions, they could not anticipate the difficulties that would be encountered when attempting to deliver nutrients and electron acceptors to ground-water systems. These difficulties would only become fully evident during field-scale testing of different bioremediation systems.

12.8.3 Engineered Bioremediation of Petroleum Hydrocarbons in Ground-Water Systems

The engineered bioremediation systems for treating petroleum hydrocarbon-contaminated ground water first conceived in the early 1970s were based largely on observations made from marine petroleum spills. The growth and metabolism of microorganisms in natural environments is always limited by the availability of

electron donors, electron acceptors, or other essential nutrients (Atlas, 1981). In petroleum spills, there is no lack of electron donors (petroleum hydrocarbons), so microbial metabolism is generally limited by the availability of electron acceptors (such as oxygen, nitrate, sulfate, or Fe(III)), or by the availability of essential nutrients (such as nitrogen, potassium, and phosphate). The basic principle of engineered bioremediation systems is to relieve the lack of electron acceptors and nutrients and thereby increase rates of petroleum hydrocarbon degradation.

In 1974, a patent was issued to Richard Raymond of Suntech Oil (Raymond, 1974) describing a method for the addition of nutrients and an electron acceptor (oxygen) to aquifers in order to stimulate petroleum hydrocarbon biodegradation. This patent, entitled "Reclamation of Hydrocarbon Contaminated Ground Waters," describes how nutrients and oxygen can be introduced to aquifers by injection wells and the water circulated through contaminated aquifer materials by pumping wells. A schematic diagram of this bioremediation system is shown in Figure 12.13. Many engineered field-scale systems that have been built and tested in the United States are variations on the concept originally patented by Suntech Oil.

Many of the early case studies that used the Suntech Oil process were not described in the peer-reviewed scientific literature, although it was reported that the process enjoyed "reasonable" success in several instances (J. T. Wilson et al., 1986). One of the first case studies using this basic methodology reported in the

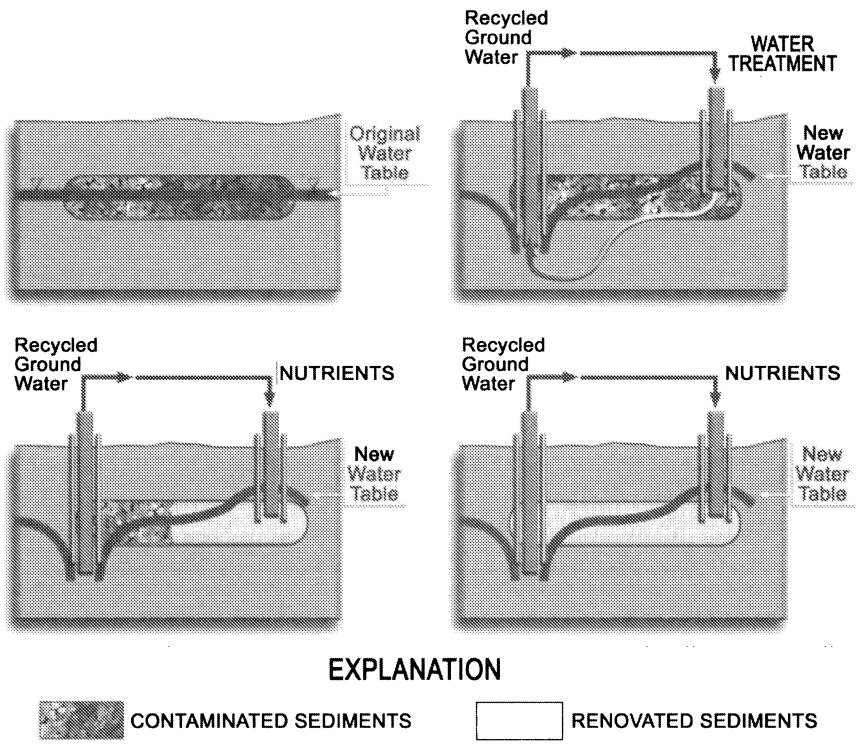


Figure 12.13. Steps present in many in-situ bioremediation systems for petroleum hydrocarbon-contaminated ground-water systems.

peer-reviewed literature was described by Wilson and Brown (1989). In this case, approximately 900 gallons of leaded gasoline had leaked into a fractured dolomite overlain by silty sands and gravels and was impacting a nearby municipal well. The first part of the remediation effort was designed to collect free product using a dual-pump extraction system that recovered about 100 gallons of free-phase gasoline. After a laboratory pilot study showed that addition of ammonium, phosphate, and hydrogen peroxide (to serve as a source of oxygen) could stimulate microbial degradation of hydrocarbons, a full-scale field system was constructed. The nutrients were added through injection wells and an infiltration gallery so that soils above the water table would also be treated, and a pumping well was used to separate free-phase fuel and to draw nutrients toward the zone of contamination. After 18 months of system operation, the zone of contamination was reported to have decreased in size by a factor of 10, with concentrations of total petroleum hydrocarbons reduced below 1 part per billion.

The use of hydrogen peroxide as a source of oxygen was widely applied in many early bioremediation systems. Molecular oxygen is only slightly soluble in water (~8.0 mg/L for water in contact with air at 20°C) and so oxygen delivery from air-saturated water from injection wells or infiltration galleries is inherently limited. Hydrogen peroxide (H_2O_2), in contrast, is completely miscible in water and will spontaneously decompose, giving a source of oxygen:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

By adding hydrogen peroxide to infiltrating water, it was hoped to greatly increase the delivery of oxygen to contaminated aquifer sediments.

One hydrogen peroxide–based bioremediation system, designed to remediate an aviation gasoline–contaminated aquifer in Traverse City, Michigan, was described by Wilson et al. (1994a). This study was designed, in part, to compare the results of oxygen addition by ground-water aeration with oxygen enhancement from hydrogen peroxide. For both oxygen-delivery methods ground water was also amended with ammonium chloride, disodium phosphate, and potassium phosphate to provide essential nutrients. For the first 90 days of operation, oxygen was delivered solely by aeration. In this time, concentrations of oxygen 31 feet downgradient of the infiltration wells did not increase and concentrations of benzene, toluene, ethylbenzene, and xylene (BTEX) were not observed to decrease. After the addition of hydrogen peroxide, dissolved oxygen concentrations in the downgradient well increased substantially and BTEX compounds were completely removed. However, as treatment continued, dissolved oxygen concentrations declined over time even though hydrogen peroxide continued to be added to the system. Oxygen was again detected in this observation well, but only after the amount of hydrogen peroxide added to the treatment stream was increased. This observed behavior suggested that hydrogen peroxide consumption in the treatment system increased over time, and that this increased consumption led to decreased oxygen delivery to contaminated aquifer sediments.

The microbial processes affecting hydrogen peroxide decomposition in jet fuel–contaminated aquifer sediments were studied in detail by Spain et al. (1989). The delivery system in this study consisted of an infiltration gallery overlying the zone of contamination. Hydrogen peroxide was added to treated ground water, dispersed

to the infiltration gallery, and allowed to circulate through fuel-contaminated sediments. Spain et al. (1989) measured rates of hydrogen peroxide decomposition in sediments underlying the infiltration gallery and in recirculated ground water. It was found that sediments rapidly decomposed hydrogen peroxide, with a first-order decomposition rate constant of 7.0 hr^{-1}. This decomposition rate indicated that the half-life of hydrogen peroxide in sediments underlying the infiltration gallery was only about six minutes. This high rate of hydrogen peroxide decomposition, much of which was shown to be catalyzed by microbial activity, greatly decreased the effectiveness of hydrogen peroxide delivery to sediments deep in the zone of jet fuel contamination. This study (Spain et al.,1989) showed that using hydrogen peroxide as an oxidant to facilitate petroleum hydrocarbon bioremediation could be problematic. This, in turn, led some investigators to consider alternative oxidants for use in engineered bioremediation systems.

Nitrate can serve as an energetically favorable electron acceptor under anoxic conditions (Tiedje, 1988). Furthermore, by the late 1980s, a number of investigators had shown that petroleum hydrocarbons were biodegraded under nitrate-reducing conditions (Kuhn et al., 1988; Hutchins et al., 1991a). It was logical, therefore, to test the feasibility of using nitrate as an electron acceptor in engineered bioremediation systems. Several early field studies indicated at least partial success in removing BTEX compounds (Batterman, 1986; Sheehan et al., 1988; Lee et al., 1988) with nitrate as the primary electron acceptor. These early studies also showed, however, that monitoring the effectiveness of nitrate-based bioremediation was extremely difficult. In particular, it was difficult to separate the effects of hydrodynamic processes (such as dilution) from biological degradation processes on concentrations of contaminants and nitrate. As such, the effectiveness of nitrate-based engineered bioremediation systems remained uncertain.

Faced with these uncertainties, the U.S. Environmental Protection Agency initiated a detailed study of nitrate-based bioremediation of a jet fuel–contaminated aquifer in Traverse City, Michigan (Hutchins et al., 1991b). This study was designed to separate the hydraulic effects of circulating ground water from biological effects of nitrate addition. The bioremediation system was built to circulate relatively large volumes of ground water (~200 gallons per minute) through an infiltration gallery. This water percolated to the water table, was transported laterally through fuel-contaminated sediments, and was recovered by pumping wells. This recovered water was then continuously circulated to the zone of contamination through the infiltration galleries. The bioremediation system was also designed to continuously add nitrate to the circulating water. The results of this study are shown in Figure 12.14.

When the system was started up, ground water was circulated for 42 days before nitrate addition was initiated. During this time, a significant amount of aerobic biodegradation occurred due to oxygen added during ground-water recirculation. In addition, significant leaching of soluble BTEX compounds was documented. In particular, benzene was almost completely leached from ground water at the site and initially accumulated in the circulating water. There was a net mass loss of 2.9 kg of benzene during the prenitrate treatment phase. Much of this mass loss could be attributed to the aerobic decomposition of benzene. After 42 days of water circulation, nitrate addition to the system was initiated. Concentrations of nitrate initially increased to about 8.0 mg/L and fluctuated thereafter (Fig. 12.14). Con-

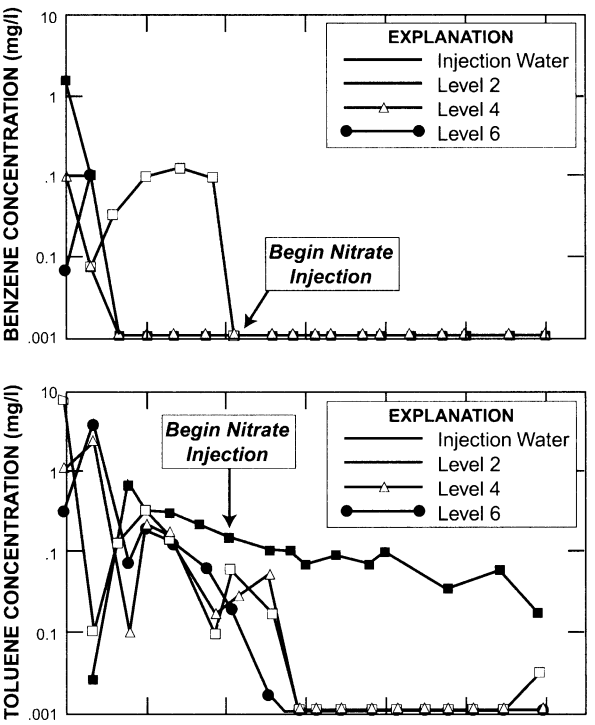


Figure 12.14. Concentration changes of (*a*) benzene, and (*b*) toluene at different sampling levels during operation of an in-situ bioremediation system, Traverse City, Michigan. (Modified from Hutchins et al., 1991; with permission courtesy of the National Ground Water Association.)

centrations of toluene decreased rapidly in ground water after nitrate addition. Concentrations of *m*- and *p*-xylene also decreased, but at a slower rate. Concomitant with the biodegradation of BTEX compounds, it was shown that numbers of denitrifying bacteria in aquifer sediments increased from about 10^6 to 10^7 cells per gram of dry sediment. In addition, the amount of jet fuel was shown to decrease in aquifer sediments. In shallower sediments (which had been most impacted by recirculating water), concentrations of jet fuel decreased from about 5,000 to about 1,000 mg/kg. In deeper sediments, however, decreases in jet fuel concentrations were not as substantial. In spite of the residual petroleum hydrocarbons that remained, aqueous concentrations of BTEX compounds were reduced below maximum concentration levels specified by the State of Michigan (Hutchins et al., 1991b).

Engineered bioremediation systems using nitrate as an oxidant have been widely used in other hydrologic environments (Barbaro et al., 1992; Hutchins et al., 1995; Sweed et al., 1996; Vroblesky et al., 1997). Because nitrate itself in ground water is of regulatory concern, the amount of nitrate added to a contaminant aquifer in some instances was restricted to the 15 mg/L maximum concentration limit for drinking water (Vroblesky et al., 1997). The collective experience of these studies indicates that, in many cases, hydrologic constraints were the principal limitations

on the use of the technology. For example, Hutchins et al. (1995) found that nitrate-amended recharge water tended to remediate high-permeability sediments where ground-water flow was concentrated. In contrast, nitrate-amended recharge water was not delivered as efficiently to lower-permeability sediments and, as a consequence, bioremediation was not as efficient. Vroblesky et al. (1997) reported that hydrogeologic factors such as seasonal fluctuations in ground-water levels, and changes in ground-water flow directions, affected the efficiency of nitrate delivery to zones of contamination. In addition, Vroblesky et al. (1995) showed that variations in hydraulic conductivity could lead to "stratigraphic trapping" of fuel in the subsurface. Such trapping made it much more difficult to introduce nitrate-amended water to the most contaminated sediments, and was a significant constraint on engineered bioremediation in this system. In other systems, it was found that colloidal material mobilized by recirculating ground water and nutrients could plug aquifer materials, cause significant decreases in sediment hydraulic conductivity, and interfere with bioremediation (Wiesner et al., 1996).

As it became generally recognized that hydrologic constraints had a large effect on the efficiency of bioremediation systems (Baker and Herson, 1990; Mercer and Cohen, 1990), techniques for maximizing the efficiency of treatment water delivery to the subsurface were investigated. For example, incomplete mixing of injected treatment water with contaminated water in the formation was often found to be a problem (Hutchins et al., 1995). To deal with this, Devlin and Barker (1994) proposed injecting nutrients into a constructed high-permeability wall, and allowing the natural hydrologic gradient to carry the nutrients to the zone of treatment. By pulsing nutrient injection, Devlin and Barker (1994) reported that it was possible to maximize mixing efficiency, and thus to maximize delivery of nutrients to the contaminated aquifer.

Microbiologic constraints associated with nitrate-based bioremediation systems were also reported. For example, Barbaro et al. (1992) found that toluene, ethylbenzene, and toluene were efficiently remediated by nitrate addition. Benzene, however, was recalcitrant. Laboratory studies have consistently indicated that benzene does not degrade efficiently under nitrate-reducing conditions (Hutchins et al., 1991a). Because benzene often drives regulatory concern about BTEX-contaminated ground water, this may limit the effectiveness of nitrate-based bioremediation.

Much of the cost associated with engineered bioremediation system designs that are derived from the original Suntech method are associated with drilling and maintaining nutrient injection wells, or constructing infiltration galleries. For this reason, several investigators have considered land-surface application systems for remediating contaminated ground water (O'Leary et al., 1995; Sweed et al., 1996). This concept involves pumping BTEX-contaminated ground water from an aquifer, applying this water to surface soils, and allowing it to percolate back to the underlying ground-water system. During transport through the soils and unsaturated zone, BTEX compounds are degraded by aerobic microbial processes. In addition, BTEX compounds are volatilized and lost to the atmosphere. O'Leary et al. (1995) reported BTEX mass losses of between 65% and 100% by the time the applied water reached the water table. Sweed et al. (1996) showed by means of tracer tests that this method might also be used to deliver nitrate to an underlying ground-water system. However, a study of nitrate addition to this system indicated that consid-

erable amounts of nitrate were taken up by vegetation at land surface (Hutchins et al., 1995), and that such uptake might reduce the effectiveness of nitrate delivery.

The original Suntech Oil design injected air into the water column of a well to oxygenate ground water. In addition to oxygenating the water, this had the desirable effect of purging volatile and semivolatile hydrocarbons from the water. The combination of adding oxygen to stimulate microbial hydrocarbon degradation while purging contaminants physically from the ground water has developed into a widely used technology called "air sparging" (Brown, 1994). Modern practice of air sparging, however, differs substantially from the original Suntech Oil design. Rather than injecting air into the water column of a well, air is injected directly into a water-saturated aquifer. This technology has been used extensively in Europe (Hiller and Gudemann, 1988) and has found widespread acceptance in the United States (Brown et al., 1995).

Soil vapor extraction has been widely used to remediate soils and unsaturated zone sediments contaminated by petroleum hydrocarbons (U.S. Environmental Protection Agency, 1991). A variation of this technology, called "bioslurping," has been developed for use with contaminated ground-water systems as well (Keet, 1995). To apply this technology, a well is screened across the water table in a contaminated zone, and a vacuum intake pipe positioned at the water table surface. When vacuum is applied to the pipe, a mixture of oil, water, and soil gas is removed and brought to the surface for treatment. This physically removes free-product hydrocarbons floating on the water table surface, but also draws in air to oxygenate ground water, which facilitates aerobic biodegradation (Air Force Center for Environmental Excellence, 1994). Because bioslurping combines free product recovery with enhancement of biodegradation processes near the water table, it has proven to be widely useful (Baker, 1995). One of the most useful aspects of the bioslurper design is that it minimizes the volume of contaminated ground water withdrawn from the aquifer, thus minimizing the volume of water requiring treatment at land surface. Also, this technology can be applied to fractured-rock systems where other oxidant-delivery systems are difficult to engineer.

Bioaugmentation, the process of adding specially adapted microorganisms to a system to enhance rates of biodegradation, has been widely applied to remediating contaminated surface soils and in various ex situ remediation methods (Forsyth et al., 1995; Baud-Grasset and Vogel, 1995). However, it has not proven widely useful for contaminated ground-water systems. This largely reflects the hydrologic difficulties inherent in delivering microorganisms efficiently to contaminated aquifer sediments (Thomas and Ward, 1994). In one study of a relatively homogeneous sand aquifer, Burlage et al. (1995) reported that the transport and recovery of specific strains of injected bacteria—as identified using PCR amplification of DNA—was much more erratic than that of a dissolved chemical tracer. These researchers suggested that this observed behavior was due to complex patterns of bacterial attachment and disattachment from sediment grain surfaces. It is also possible that the injected microorganisms could not compete successfully with microorganisms indigenous to the ground-water system for available carbon and nutrient resources. Because of these difficulties, and because hydrocarbon-degrading bacteria are ubiquitous in near-surface environments (Atlas, 1977), the costs associated with delivering laboratory-grown hydrocarbon-degrading bacteria to contaminated zones are often not justified.

In summary, engineered bioremediation systems for petroleum hydrocarbon–contaminated aquifers are most commonly designed to increase the availability of electron acceptors, usually oxygen or nitrate. In some cases nutrients such as nitrogen, phosphate, and potassium are added as well. The success of such systems for removing petroleum hydrocarbons is directly related to the efficiency that the electron acceptor and nutrients are delivered to contaminated water and aquifer solids.

12.8.4 Monitored Natural Attenuation of Petroleum Hydrocarbons

During the 1980s, there was an enormous effort to assess and monitor petroleum hydrocarbon contamination of ground water in the United States and Europe. From this mass of information came several unanticipated and surprising results. It was widely observed, for example, that plumes of petroleum hydrocarbon–contaminated ground water stopped expanding over time and assumed a dynamic steady-state configuration. Perhaps the best-documented example of this behavior was a crude oil spill in northern Minnesota near the town of Bemidji (Baedecker et al., 1988; Cozzarelli et al., 1989; Baedecker et al., 1993). In 1979 an oil pipeline ruptured and spilled 1,670 m^3 of crude oil onto land surface. Over the next year, oil migrated downward and formed a lens floating on the water table. The site was instrumented with observation wells and monitored throughout the 1980s and 1990s. A plume of dissolved hydrocarbons, principally BTEX compounds, was observed to develop downgradient of the oil lens. However, by 1985, the BTEX plume had stopped spreading, extending only about 150 meters downgradient of the oil lens. Subsequent studies showed that a dynamic steady state had developed between the rate that soluble hydrocarbons were leaching into the ground water and the rate that anaerobic and aerobic biodegradation processes consumed the hydrocarbons (Lovley et al., 1989; Baedecker et al., 1993). Thus, after about 1985, the position and size of the BTEX plume did not change significantly.

Similar observations were being made in other hydrologic systems as well. In a controlled experimental release to a shallow sandy aquifer (Fig.12.11), Barker et al. (1987) showed that naturally occurring biodegradation processes effectively removed benzene, toluene, and all three xylene isomers from solution in about one year. Barker et al. (1987) used the term "natural attenuation" to describe the combined dilution, dispersion, sorption, and biodegradation processes that caused contaminant concentrations to decrease. Similarly, Chiang et al. (1989) showed significant losses of benzene mass over a three-year period in a contaminated aquifer underlying a gas plant, and attributed this to natural attenuation processes.

On a much larger scale, a study of 7,167 municipal supply wells in California showed that, while leaking gasoline was by far the most common contaminant being released into ground-water systems, benzene was found in only 10 wells (Hadley and Armstrong, 1991). This study concluded that biodegradation processes were actively consuming BTEX compounds and thereby protecting California ground-water supplies from widespread petroleum hydrocarbon contamination.

These and other studies clearly showed that, in many instances, natural biodegradation processes were far more important in preventing the migration of soluble hydrocarbons than had been foreseen in the early 1980s. Because of this realization, a committee of the National Research Council (NRC) first made the distinction

between engineered bioremediation—where human intervention was used to enhance microbial processes in ground-water systems, and intrinsic bioremediation—where natural microbial processes alone could renovate contaminated ground water (National Research Council, 1993).

In their definition of intrinsic bioremediation, the NRC committee also made a distinction between natural attenuation, which occurs ubiquitously, and intrinsic bioremediation, where these natural processes are protective of human health and the environment. More recently, the U.S. Environmental Protection Agency (1999) replaced the term "intrinsic bioremediation" with monitored natural attenuation. This was done to emphasize the fact that environmental monitoring was always required to show that natural processes were meeting remediation goals, and that this was not a no-action cleanup alternative. In addition, monitored natural attenuation requires a rigorous site evaluation to demonstrate that natural processes will prevent contaminant migration to sensitive receptors such as wells or nearby wetlands. As such, monitored natural attenuation can be considered just like any other technology for treating BTEX-contaminated ground water (Weidemeier et al., 1999).

Before monitored natural attenuation could be applied routinely to contaminated sites, however, it was first necessary to develop methods for assessing the efficiency of natural biodegradation processes. Salanitro (1993) emphasized the importance of hydrologic and microbiologic site characterization in assessing natural attenuation processes in aquifers. McAllister and Chiang (1994) pointed out the importance of assessing the availability of potential electron acceptors, especially dissolved oxygen, in contaminated aquifers.

To date, numerous procedures and protocols have been developed for assessing monitored natural attenuation of petroleum hydrocarbons. One of the first efforts was undertaken by J. T. Wilson of the U.S. EPA and the U.S. Air Force Center for Environmental Excellence (AFCEE). This approach relied on three independent lines of evidence: (1) documenting mass loss of contaminants at the field scale, (2) geochemical evidence of biodegradation processes, and (3) laboratory microcosm studies using aquifer materials collected from the site in order to establish that natural attenuation was occurring (Wiedemeier et al., 1994). This approach was codified into a protocol for use by Air Force personnel at field sites (Wiedemeier et al., 1995a) that has been widely used in the United States. A subsequent effort by the Naval Facilities Engineering Command moved beyond demonstrating the occurrence of natural attenuation processes, and focused on documenting the efficiency of these processes and gave a step-by-step approach to assessing monitored natural attenuation (Wiedemeier and Chapelle, 1998).

Many early approaches to assessing monitored natural attenuation relied on qualitative indicators of biodegradation processes. In order to make such assessments more quantitative, Wiedemeier et al. (1995b) proposed making mass balance calculations based on the stoichiometry of both aerobic and anaerobic biodegradation processes. In this approach, the available supply of oxidants (oxygen, nitrate, Fe(III), and sulfate) was used to calculate the degradation "capacity" of a given aquifer system. Chapelle (1994) showed that assessments of monitored natural attenuation could be made quantitative by comparing rates of contaminant transport to rates of biodegradation. To make such comparisons quantitative required consideration of hydrologic (rates of ground-water flow and aquifer dispersion char-

acteristics), geochemical (extent of contaminant sorption), and microbiologic (rates of contaminant biodegradation) factors. If rates of contaminant transport were large relative to biodegradation rates, then contaminant plumes could migrate considerable distances and possibly impact receptors. On the other hand, if rates of contaminant transport were small relative to biodegradation rates, the plume would be more contained. This framework for assessing the efficiency of monitored natural attenuation is shown in Figure 12.15. These hydrologic, geochemical, and microbiological factors can be integrated using digital solute-transport models (Chapelle et al., 1995).

As quantitative studies showed that monitored natural attenuation could be an effective treatment strategy for petroleum hydrocarbon–contaminated ground water, this method became increasingly acceptable to the regulatory community. Prior to 1994, very few states considered monitored natural attenuation to be an acceptable strategy for renovating petroleum hydrocarbon–contaminated ground water. By 1996, however, most states had developed either formal or informal policies for considering monitored natural attenuation (Ritz, 1996). In California, a study by Lawrence Livermore National Laboratory (1995) recommended that "passive" (i.e., intrinsic) bioremediation be utilized whenever possible in dealing with leaking underground fuel tanks.

Because of increasing regulatory acceptance, monitored natural attenuation of petroleum hydrocarbon–contaminanted ground-water systems has become routine. In some cases, monitored natural attenuation has been used as a stand-alone method (Rifai et al., 1995; Wiedemeier et al., 1995; Wilson et al., 1995; Ginn et al., 1995; Chapelle et al., 1995; Landmeyer et al., 1996). In other cases, monitored natural attenuation has been used in conjunction with other remediation technologies (Brown et al., 1995). The conjunctive use of monitored natural attenuation with other more intrusive remediation technologies is probably the most efficient way to completely remediate petroleum hydrocarbon spills in a relatively short time frame.

12.8.5 Estimating Biodegradation Rates in Ground-Water Systems

Analytical or digital solute-transport modeling methods for assessing monitored natural attenuation rely heavily on having estimates of petroleum hydrocarbon degradation rates in aquifer systems. There are two general methods for estimating biodegradation rates in ground-water systems. One approach is to collect aquifer sediments, construct either static or flow-through microcosms in the laboratory, and measure the disappearance of particular compounds (Wilson et al., 1994) or the production of $^{14}CO_2$ from radiolabled compounds (Klecka et al., 1990). However, this approach introduces uncertainties due to sediment disturbance and difficulties in reproducing in situ conditions (Madsen, 1991). It has been found, for example, that laboratory methods may greatly overestimate rates of microbial metabolism in some ground-water systems (Chapelle and Lovley, 1990).

Another approach for measuring biodegradation rates is to track the removal of electron donors (soluble petroleum hydrocarbons such as benzene or toluene) or electron acceptors (such as oxygen, nitrate, or sulfate) along ground-water flowpaths, and apply a dispersion model approach to separate out the effects of biodegradation from those of dispersion and advection (Buscheck and Alcantar, 1995).

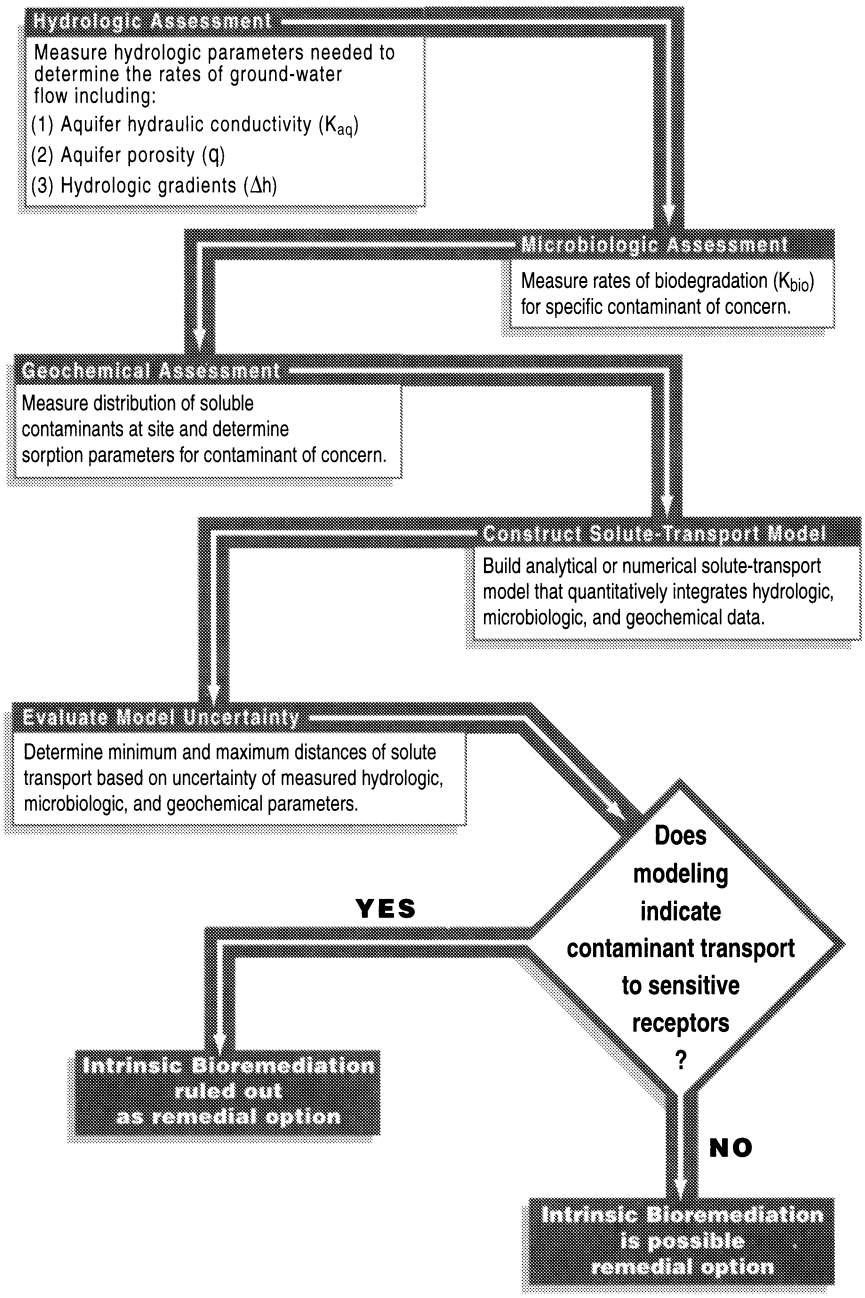


Figure 12.15. Framework for assessing the efficiency of natural attenuation in ground-water systems.

Chapelle et al. (1996) used a combination of the laboratory approach and dispersion model approach to estimate biodegradation rates in a petroleum hydrocarbon aquifer. This example illustrates the advantages and disadvantages of both methods, as well as illustrating the uncertainties inherent in biodegradation rate estimates. A map of the site is shown in Figure 12.16. A leak from tank 1 led to the contamination of aquifer sediments, and a plume of BTEX-contaminated ground water extended downgradient. The plume is uniformly anoxic and lacks measurable concentrations of nitrate. Near the source area, however (well EW-7), concentrations of sulfate are relatively high and decrease downgradient. This suggests that sulfate reduction is the predominant terminal electron-accepting process in this system. Concentrations of dissolved hydrogen are in the 1–5-nM range characteristic of sulfate reduction, also indicating the predominance of sulfate reduction at this site. Toluene concentrations are relatively high near the source area and decrease downgradient. Benzene concentrations, however, are much more variable and no trend of decreasing concentrations downgradient is apparent.

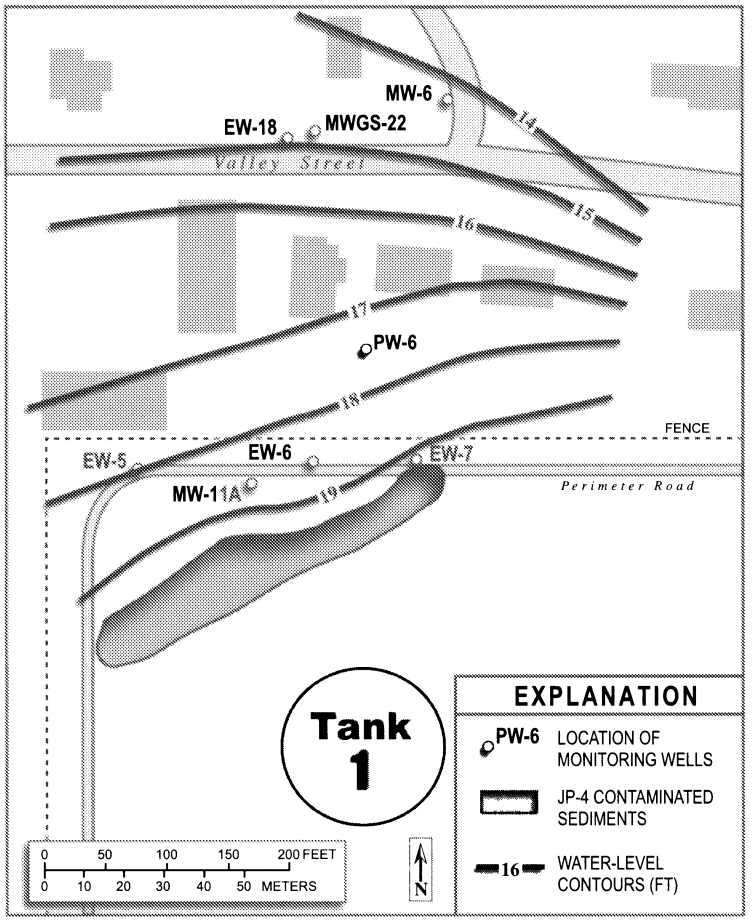


Figure 12.16. Map showing the configuration of the water table and locations of monitoring wells at the Hanahan, South Carolina site.

If it is assumed that the decrease in toluene concentrations downgradient is due primarily to the combined affects of dispersion and biodegradation, then it may be described by the solute-transport equation:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} - v\frac{\partial C}{\partial x} - \frac{\rho_b K_d}{n}\frac{\partial C}{\partial x} - kC \tag{1}$$

where D is the coefficient of hydrodynamic dispersion (m^2/d), v is the velocity of ground-water flow (M/d), ρ_b is bulk density, K_d is a linear sorption distribution coefficient, n is porosity, and k is a first-order biodegradation rate constant (d^{-1}) (Freeze and Cherry, 1979, p. 402 and p. 552). The coefficient of hydrodynamic dispersion, in turn, is proportional to ground-water velocity and scale-dependent aquifer dispersivity D^* (m):

$$D = vD^*$$

Appropriate procedures for solving equation 1 depend on the ground-water system in question, and the specific problems being addressed. When a contaminant plume has reached approximate steady-state conditions (that is, the plume is not expanding or contracting with time and $\partial C/\partial t = 0$), the sorption term becomes small relative to the other three terms, and the solute-transport equation simplifies to the ordinary differential equation

$$D\frac{d^2 C}{dx^2} - v\frac{dC}{dx} - kC = 0 \tag{2}$$

For boundary conditions of $C = C_0$ at $x = 0$, and $C = 0$ as $x \rightarrow \infty$, equation 3 has the particular solution

$$C(x) = C_0 \exp^{-(-v+\sqrt{v^2+4Dk}/2D)x} \tag{3}$$

It is convenient to evaluate equation 3 using a spreadsheet program, as given in Table 12.5. By entering the average velocity of ground water, the dispersivity characteristic of the site, and an estimated biodegradtation rate, concentrations of solutes as a function of distance along the flowpath (x in equation 12.3) is calculated. Assuming D and v are known, values of k can be fit to observed data (Table 12.4). By bracketing observed concentrations of solutes as a function of distance along the flowpath, values of k can be estimated.

When applied to this site, where ground-water velocity (v) was approximately 2 feet per day, and where the dispersivity based on the scale of the plume (Gelhar, 1992), this procedure yields estimates of a first-order biodegradation rate constant (k_{tol}) of between 0.01 and 0.02 d^{-1} (Fig. 12.17a) for toluene. Note, however, that the data for benzene (Fig. 12.17b), which are much more variable than the toluene data, do not yield an unambiguous biodegradation rate constant.

The dispersion model approach can be applied to the consumption of electron acceptors as well as to the consumption of electron donors in ground-water systems. For example, the consumption of sulfate in this system can be fitted to the disper-

TABLE 12.4 Spreadsheet Transform for Evaluating Equation 12.3

v = Input value for ground-water velocity
d = Input value for aquifier dispersivity
k = Input value for biodegradation rate; this number is positive
D = d*v
C = Input contaminant concentration at the source area
fd = Input flowpath distance from the source area to a specified downgradient point
dis = fd/10
col(2,1) = 0
for i = 2 to 10 do
col(2,i) = col(2,i−1)+dis
N = (v*v) + (4*D*k)
NA = sqrt(N)
NC = (−v+NA)
NAC = NC/(2*D)
col(1,1) = NAC
A = −NAC
col(3) = (exp(A*col(2)))*C

sion model, yielding a biodegradation rate estimate of 0.04 d^{-1} (Fig. 12.18). Because the stoichiometry of toluene oxidation coupled to sulfate reduction indicates that 4.5 moles of sulfate are consumed for each mole of toluene,

$$C_7H_8 + 4.5\ SO_4^{=} + 3H_2O \rightarrow 7HCO_3^{-} + 4.5\ HS^{-} + 2.5\ H^{+} \qquad (4)$$

this suggests a toluene biodegradation rate of 0.04/4.5 or 0.009 d^{-1}, which is fairly close to estimated toluene biodegradation rates. However, because many other petroleum hydrocarbons are coupled to sulfate metabolism as well, this correspondence may simply be chance. It's interesting to note that the decrease in pH predicted by equation 12.4 is in fact observed at this site (Table 12.4).

These dispersion-model estimates for the biodegradation rate constant were compared to estimates generated by laboratory radiotracer experiments. Figure 12.19 shows the mineralization of toluene and benzene under different electron-accepting conditions. For toluene, there was no significant mineralization for nitrate-amended, Fe(III)-amended, and unamended (methanogenic) microcosms. However, for the sulfate-amended microcosm, the slope of the $^{14}CO_2$ recovery curve yields a toluene biodegradation rate of 0.01 d^{-1}. Similarly, the degradation of benzene under sulfate-amended conditions was approximately 0.003 d^{-1}.

A comparison of the field-derived and the laboratory-derived biodegradation rate estimates illustrates several points. First, toluene biodegradation rate estimates derived from the dispersion model approach and from the radiotracer experiments conducted under sulfate-reducing conditions agree reasonably well. Note, however, that there is no agreement between the field estimates and the laboratory experiments conducted under nitrate-reducing, Fe(III)-reducing, or methanogenic conditions. Water chemistry data at this site indicate that sulfate-reduction is the predominant electron-accepting condition at this site (Table 12.4). Thus, microorganisms present in the sediments collected for laboratory incubations were accli-

TABLE 12.5 Ground-water Chemistry of the Hanahan Site

Well Number	Distance Along Flowpath (feet)	DIC[1] (mg/l)	pH (units)	[2]DO (mg/l)	Nitrate (mg/l)	[3]Sulfate (mg/l)	Hydrogen [4](nM)	[3]Benzene (μg/l)	[3]Toluene (μg/l)
EW-7	0	85 ± 1.4	6.6	<0.1	<0.1	14.3 ± 1.4	—	19 ± 5.0	345 ± 74
MW-11A	20	109 ± 7.0	6.25	<0.1	<0.1	9.0 ± 1.9	1.8	5.2 ± 10	39 ± 20
EW-6	40	110 ± 7.3	6.49	<0.1	<0.1	6.0 ± 0.9	—	74.7 ± 27	223 ± 47
EW-5	70	119 ± 12	6.49	<0.1	<0.1	4.2 ± 0.68	—	353 ± 64	219 ± 64
PW-2	100	155 ± 8.5	5.61	<0.1	<0.1	1.6 ± 0.27	3.5	27 ± 3.5	190 ± 26
EW-18	270	182 ± 6.3	6.42	<0.1	<0.1	0.47 ± 0.2	—	46 ± 10	38 ± 19
MWGS-22	280	175 ± 21	5.92	<0.1	<0.1	0.19 ± .04	4.2	248 ± 128	90 ± 43
MW-6	330	112 ± 2.0	5.71	<0.1	<0.1	0.09 ± .09	1.6	23 ± 7.0	19 ± 5

[1]Dissolved inorganic carbon.
[2]Dissolved oxygen.
[3]Mean of three samples ± SD.
[4]Nanomoles per liter.

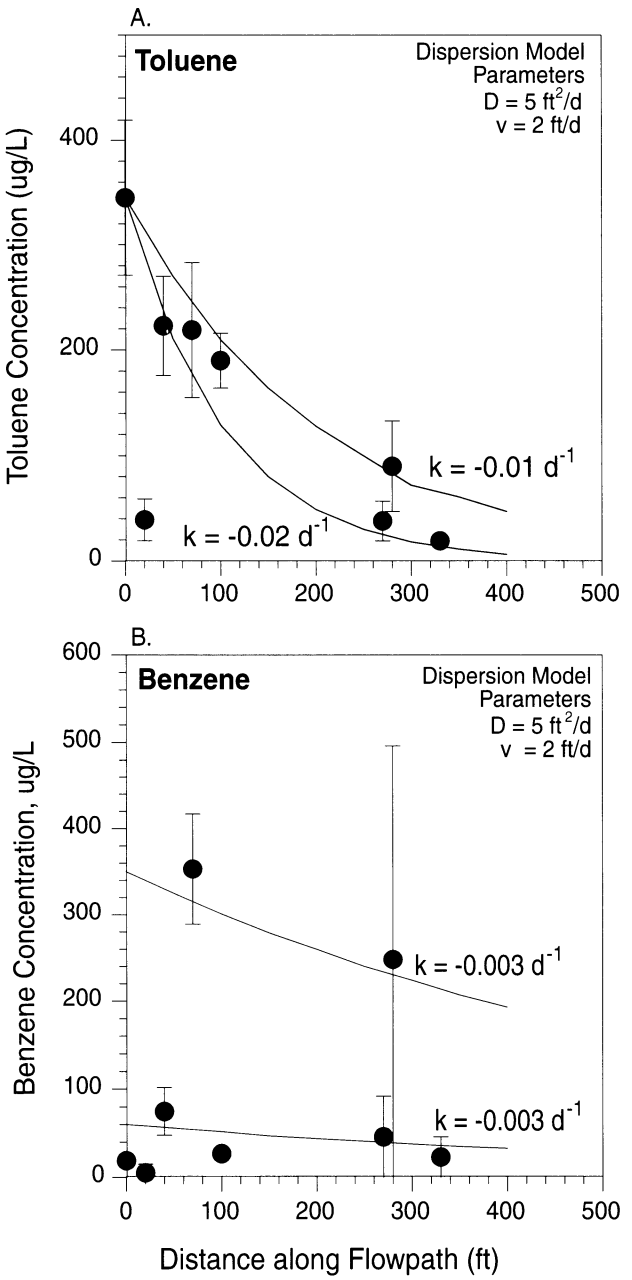


Figure 12.17. Dispersion model estimates of biodegradation rates at the Hanahan, South Carolina, site.

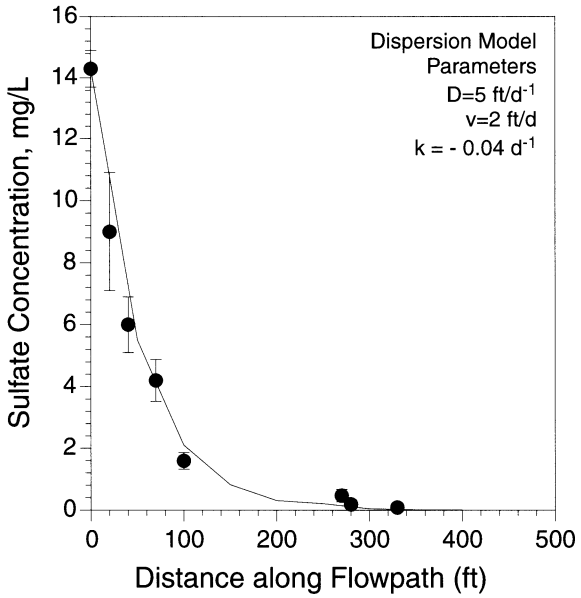


Figure 12.18. Concentration changes of sulfate along the flowpath as an estimate of sulfate respiration rates at the Hanahan, South Carolina, site.

mated primarily to sulfate-based metabolism. This explains the apparent lack of toluene and benzene mineralization under different electron-accepting conditions—the microorganisms were simply not acclimated to those electron acceptors. This indicates, however, that if the electron-accepting conditions of laboratory experiments are not carefully matched to ambient conditions in the field, such experiments can yield unreliable results. In these experiments, for example, the experiments conducted without added sulfate inaccurately indicated a lack of contaminant mineralization in this system. Thus, it is clear that laboratory-derived biodegradation rates must be based on experiments that carefully match field conditions.

This comparison also shows that field methods for estimating biodegradation rates are subject to numerous uncertainties, including:

1. Variability in contaminant source concentrations (C_0), which must be assumed to be constant.
2. Variations in ground-water flow velocity (v).
3. Variations in contaminant concentrations within the plume.
4. Adequate placement of monitoring wells.

Because of these uncertainties, biodegradation rates derived from field must always be considered rough estimates. Laboratory experiments are also subject to numerous sources of uncertainty, including:

1. Sediment disturbance during sample collection and transport.
2. Differences between electron-accepting conditions in microcosms and ambient conditions in the field.

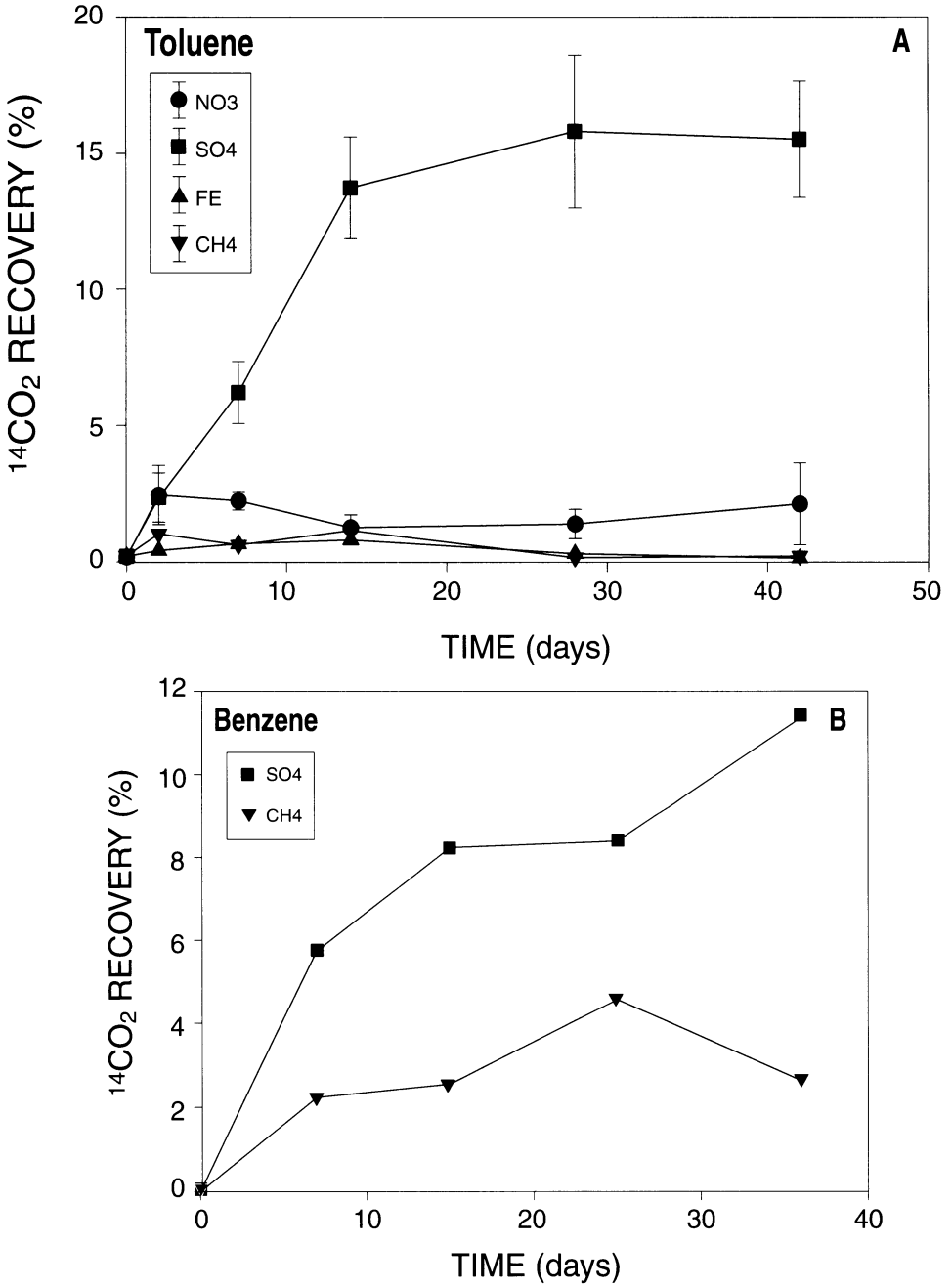


Figure 12.19. Mineralization of (*a*) ^{14}C-toluene, and (*b*) ^{14}C-benzene to $^{14}CO_2$ over time in laboratory incubations as estimates of biodegradation rates at the Hanahan, South Carolina, site.

These uncertainties must be carefully considered when using rate constants for particular applications.

12.9 SUMMARY

Crude oils are composed of a wide variety of organic components and are classified based on the relative amounts of paraffins (alkanes), aromatics, and asphaltic (sulfur-bearing) compounds present. Pennsylvania crude oil consists mainly of paraffins and has less than 0.5% sulfur. In contrast, some of the California crude oils contain up to 5% sulfur. Refined fuels are derived from crude oil by a variety of processes. Fractional distillation of crude oil yields a variety of products, including gasoline, the components of which boil between 30°C and 200°C, and lubrication oils, which boil at temperatures greater than 400°C. In addition to distillation, numerous other processes, such as high-temperature "cracking" and catalytic processes, are employed to increase the yield of gasoline from crude oil. The burning efficiency of gasoline, as measured by the octane rating scale, may be enhanced by catalytically increasing the number of branch-chained hydrocarbons present.

Refined gasolines consist mainly of aliphatic (16–25%), cycloalkane (35–55%), aromatic (10–20%) hydrocarbons, and fuel oxygenates (up to 15%). The aromatic fraction normally consists of 1–3% benzene, toluene, ethylbenzene, and xylene (BTEX). Although they are a minor component of most fuels, BTEX compounds have a disproportionately large environmental impact on ground-water systems because they are the most water soluble of the petroleum hydrocarbons. In addition, because many fuel oxygenates such as MTBE are also highly soluble, they also have a large impact on ground-water quality.

The aerobic degradation of aliphatic hydrocarbons often proceeds by oxidizing a terminal methyl group, forming a long-chain fatty acid, followed by beta-oxidation of the organic acid. The presence of branched chains on an aliphatic molecule interferes with beta-oxidation so that these compounds are normally more refractory than straight-chained aliphatic compounds. Degradation of cycloalkanes under aerobic conditions appears to be often mediated by communities of microorganisms, each of which derives some benefit from catalyzing a portion of the process. The first step in cycloalkane oxidation is the oxidation of one of the carbons to form cyclic alcohols such as cyclohexanol. The cyclic ring is then cleaved with the formation of fatty acids that are further degraded via beta-oxidation.

Aromatic hydrocarbons are degraded by a number of aerobic and anaerobic mechanisms. Under aerobic conditions, benzene and its derivatives are partially oxidized to catechols, with subsequent ring cleavage and beta-oxidation. Under anaerobic conditions, phenols and cresols are partially oxidized to intermediate products. Most anaerobic biodegradation of aromatic hydrocarbons is effected by communities of microorganisms. However, one strictly anaerobic bacterium, the Fe(Ill) reducer *Geobacter metallireducens,* has been shown to oxidize toluene completely in pure culture under anaerobic conditions.

Much of what is known about the biochemistry of petroleum hydrocarbon oxidation has been learned from aquatic sediment or soil microorganisms. However, subsurface microorganisms appear to follow many of the same degradative path-

ways. Under aerobic conditions, BTEX compounds have been shown to quickly (1–2 years) degrade. Similar behavior has been observed in anaerobic ground-water systems.

In many cases, rates of microbial degradation are limited mainly by the lack of suitable electron acceptors. Artificially adding electron acceptors and other essential nutrients to ground-water systems is termed "engineered bioremediation" and is a technology that can be effective in restoring hydrocarbon-contaminated shallow aquifer systems. The keys to effective bioremediation are often more hydrologic than biologic and include (1) accurate assessment of the contamination, (2) reliable information on the hydrologic properties of the aquifer, and (3) adequate control of recharging water so that the entire contaminated area can be remediated. In the few documented instances in which these factors have been adequately considered, bioremediation of petroleum hydrocarbon contamination has been highly successful. Experience has shown that natural biodegradation processes can, in some cases, remediate petroleum hydrocarbon contamination in ground-water systems. This technology, most commonly referred to as "monitored natural attenuation," requires a quantitative assessment of rates of contaminant biodegradation relative to rates of contaminant transport by ground-water flow. Where rates of contaminant biodegradation are large relative to rates of contaminant transport, and where contaminant transport to environmentally sensitive receptors is prevented, monitored natural attenuation can be an effective remediation strategy.

QUESTIONS TO CONSIDER

1. What kinds of microbial habitats are formed when a gasoline spill moves through the unsaturated zone and accumulates on the water table? Where are biodegradation rates the highest? Why?
2. Why is it that long-chain aliphatic hydrocarbons are observed to be more biodegradable than short-chain hydrocarbons?
3. Aerobic oxidation of benzene results in the formation of what cyclic alcohol? How does this facilitate further biodegradation?
4. Anaerobic oxidation of benzene under both methanogenic and Fe(III)-reducing conditions results in the formation of what unstable intermediate products?
5. What observations during the 1980s indicated that microbial processes were involved in the degradation of BTEX compounds? How was this demonstrated experimentally?
6. Is it necessary to seed ground-water systems with hydrocarbon-degrading bacteria in order to successfully engineer a bioremediation system? Why or why not?
7. Is methyl *tert*-butyl ether more or less biodegradable than BTEX compounds? Why do you think this is?
8. The term "natural attenuation" was first used in what year by what authors?
9. What methods are available to estimate biodegradation rates for BTEX compounds in ground-water systems? How precise are measurements made with these methods?
10. What is the principal obstacle to implementing successful engineered bioremediation of a ground-water system?

CHAPTER 13

BIODEGRADATION AND BIOREMEDIATION OF HALOGENATED ORGANIC COMPOUNDS IN GROUND-WATER SYSTEMS

Halogenated organic compounds include some of the most useful and economically important chemicals available to industry and agriculture. Alkyl halides—aliphatic organic compounds that are substituted with chlorine, bromine, iodine, or fluorine—are used in many applications. Chlorinated alkanes and alkenes, such as trichloroethane and trichloroethene, are used as dry-cleaning fluids, refrigerants, degreasing agents, solvents, and in the production of caffeine-free coffee. Brominated alkyl compounds, such as ethylene dibromide (EDB), are used as pesticides. Aryl halides—aromatic compounds such as chlorobenzenes, chlorophenols, and chlorinated biphenyls—are extensively used as herbicides, insecticides, fungicides, heat transfer media, insulators, and lubricants.

Some of the uses of these compounds—pesticides, for example—involve intentional release to the environment. Pesticides used in agriculture may be mobilized from the soil zone and carried to shallow ground-water aquifers by percolating recharge. Many uses for these compounds—solvents or refrigerants, for example—do not involve intentional release to the environment. Nevertheless, improper disposal techniques (seepage pits) and inadvertent chemical spills have led to extensive release of these compounds to ground-water systems. In either case, the potential for subsurface microorganisms to degrade these compounds influences their mobility in ground-water systems. This chapter considers the various microbially mediated processes that affect the fate and transport of halogenated organic compounds in ground-water systems.

13.1 CHEMISTRY AND USES OF HALOGENATED ORGANIC COMPOUNDS

There are several classes of halogenated organic compounds that are commonly used in industry and agriculture. It is useful to distinguish between these broad

classes of compounds because each one has characteristic properties and uses. In addition, each class exhibits characteristic patterns of microbial degradation. In this discussion, it is useful to distinguish between these compounds on the basis of chemical structure, chemical properties, and uses. Thus, the halogenated aliphatic, monoaromatic, and biphenyl compounds are considered separately. In addition, chlorinated insecticides and herbicides are considered separately.

13.1.1 Aliphatic Compounds

The chemical structures of some representative halogenated aliphatic compounds are shown in Figure 13.1. Halogenated aliphatic compounds, which are often re-

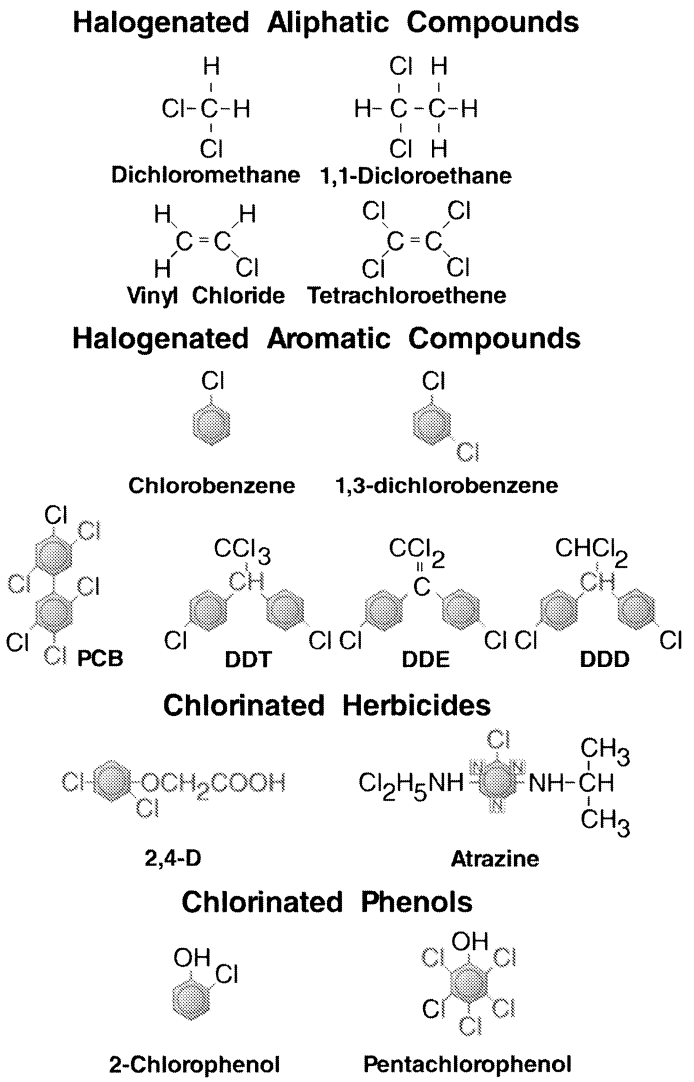
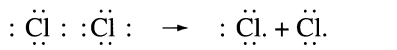


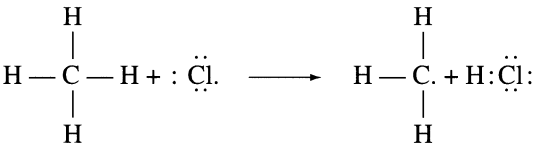
Figure 13.1 Chemical structures of some representative halogenated organic compounds.

ferred to as alkyl halides, are produced from alkanes and alkenes by means of halogenation reactions. For example, if methane and chlorine gas are heated together, a small proportion of the chlorine molecules will split into two highly reactive chlorine atoms.



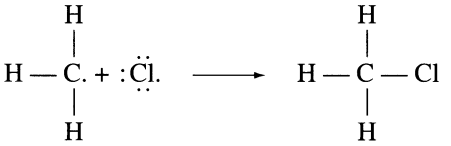
Chlorine molecule Chlorine atoms

These chlorine atoms, which lack the full complement of eight orbital electrons, tend to remove hydrogens from the methane molecules, forming a methyl radical and hydrogen chloride.

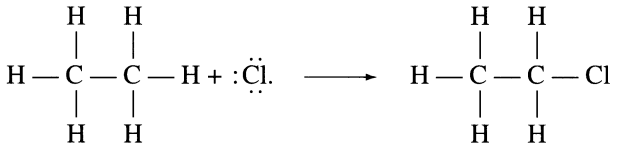


Methyl radical + hydrogen chloride

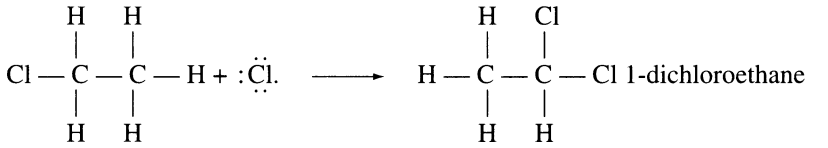
This methyl radical then reacts with chlorine to form methyl chloride.



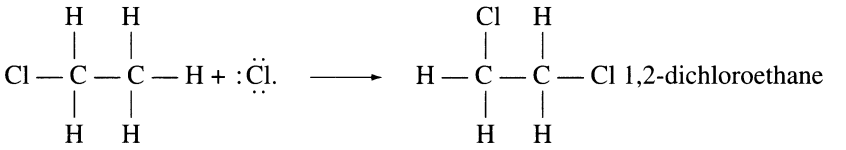
Halogenation reactions are exothermic and, once the reaction sequence begins, tend to continue as a chain reaction. A common source of halogenated methanes in drinking water supplies is the chlorination of methane-containing ground water. Trihalomethanes, which are potentially carcinogenic and which are regulated in water supplies, are produced by sequential halogenation when methane-containing ground water is chlorinated as described above.



Halogenation reactions can be initiated with alkanes or alkenes of any length, and they do not necessarily terminate after one halogenation reaction has occurred. For example, if ethane reacts with chlorine gas, the first product is chloroethane. With a subsequent chlorination, two alternatives are possible



or



Thus, halogenation reactions typically yield a mixture of products that must be subsequently separated. Alkyl halides have chemical properties that are useful for a wide range of industrial applications: having a high vapor pressure, having a high aqueous solubility, and serving as excellent solvents for nonpolar organic compounds. The solubility of selected alkyl halides, which contributes to their mobility in ground-water systems, is given in Table 13.1.

Chloromethane, dichloromethane, and trichloromethane (chloroform) are used in manufacturing silicones, synthetic rubbers, and refrigerants. Carbon tetrachloride is used in the manufacture of fire extinguishers and in dry-cleaning operations. 1,2-dichloroethane is used as an insecticidal fumigant and in the manufacture of paints.

TABLE 13.1 Octanol-water Partition Coefficients (K_{ow}) and Solubilities of Selected Halogenated Hydrocarbons

Compound	Log K_{ow}	Solubility (mg/L)
Dichloromethane	—	20,000
Chloroform	1.97	7,950
Carbon tetrachloride	2.64	800
1,2-Dichloroethane	—	8,450
1,1,1-Trichloroethane	—	1,360
1,1,2,2-Tetrachloroethane	—	3,230
1,2-Dibromoethane	—	3,520
Vinyl chloride	—	2,700
1,1,2,2-Tetrachloroethylene	2.60	400
Bromoform	—	3,190
1,2-Dichloropropane	—	3,570
Chlorobenzene	2.84	448
1,2-Dichlorobenzene	3.38	148
1,4-Dichlorobenzene	3.38	79
1,2,4-Trichlorobenzene	4.02	19
Fluorobenzene	2.27	1,540
Bromobenzene	2.99	446
Iodobenzene	3.25	340

Source: From Smith, Witkowski, and Fusillo, 1987.

Vinyl chloride (1-chloroethene) is widely used in the manufacture of polyvinyl polymers. Ethylene dibromide (EDB) is used as a solvent, in the manufacture of waterproofing agents, and as a soil fumigant for controlling nematodes in agriculture.

Because of the many uses to which these compounds are put, large amounts are manufactured yearly in the industrialized nations. The yearly production of some alkyl halides in the United States is given in Table 13.2. The amounts of these chemicals that are produced are staggering. The production of just dichloroethane, for example, exceeded 4.5 million metric tons in 1981.

With such large quantities of alkyl halides being produced, it is inevitable that some will be released into the environment. When released to soils or to surface water, the primary fate of most alkyl halides is volatilization. This is due to the relatively high vapor pressure of these compounds. In addition, the relatively low octanol-water partition coefficients (Table 13.1) of these compounds results in their having relatively little affinity for sorption onto organic matter and minerals in soils. Experimental studies have shown that evaporative losses of alkyl halides from soils and surface water generally exceeds 80% in less than ten days.

Once these compounds have been delivered to ground-water systems, however, rates of volatilization are greatly diminished. In subsurface environments, the principal attenuating mechanisms for alkyl halides are sorption, biodegradation, and volatilization to the unsaturated zone.

13.1.2 Monocyclic Aromatic Compounds

The chemical structures of some common halogenated monocyclic aromatic compounds are shown in Figure 13.1. Halogenated monocyclic aromatic compounds, which are often termed "aryl halides," may be produced from benzene by means of halogenation reactions. Although these halogenation reactions are analogous to those of the aliphatic compounds, the details of reaction are different. Aryl halides, such as chlorobenzene, may be produced from benzene and chlorine gas in the presence of a ferric chloride catalyst. The ferric chloride serves to produce a chloronium ion that is highly electrophilic and attacks the electrons of the carbon-carbon double bond present in benzene. The net result is the exchange of the chloronium ion for a hydrogen ion in the benzene structure, with the consequent formation of chlorobenzene. Similar reactions are involved in producing a variety of substituted benzene structures.

Halogenated benzenes are used for a variety of industrial and agricultural applications. Chlorobenzene is used as an insecticide, as well as in the manufacture of dyes and more complex aryl halides. Dichlorobenzene (DCB) is used as a fumigant and insecticide (DCB is the active ingredient in mothballs), as well as in industrial odor control and in metal polishing. DCB is also used as an active ingredient in toilet bowl cleaners—a use that delivers large amounts of DCB to sewage treatment plants and septic systems. Brominated benzene structures, such as bromobenzene, are used as solvents for waxes and fats and as additives to motor oil and fuels.

Aryl halides are manufactured in large quantities in the United States (Table 13.2) for these and other uses. Although the quantities of aryl halides produced are

TABLE 13.2 U.S. Production (metric tons × 10^4) of Selected Halogenated Aliphatic and Monocyclic Aromatic Hydrocarbons

Compound	1961–65	1966–70	1971–75	1976	1977	1978	1979	1980	1981	1982
Chloromethane	5.5	14.9	20.8	17.1	21.6	20.6	21.0	16.4	18.4	16.0
Dichloromethane	6.9	14.5	19.0	24.4	21.7	25.9	28.7	25.5	26.8	23.8
Chloroform	3.6	9.1	11.6	13.2	13.7	15.8	16.1	16.0	18.3	13.5
Carbon tetrachloride	21.8	36.5	45.9	38.9	36.7	33.4	32.4	32.1	32.9	nd
Chloroethane	26.6	23.1	28.1	30.4	27.8	24.5	26.4	17.9	14.7	13.1
1,2-dichloroethane	82.1	234.8	379.2	264.6	498.7	498.9	534.9	503.7	452.3	nd
1,1,1-trichloroethane	nd	14.0	21.9	28.6	28.8	29.2	32.5	31.4	27.8	25.0
Chlorodifluoromethane	nd	nd	nd	7.7	8.1	9.3	9.6	10.3	11.4	nd
Trichlorofluoromethane	5.6	10.4	13.6	11.6	9.7	8.8	7.6	10.3	7.4	nd
Dichlorodifluoromethane	9.6	15.1	19.9	17.8	16.2	14.8	13.3	13.4	14.7	nd
Vinyl chloride	63.8	142.1	223.1	257.5	271.5	315.8	289.7	293.2	311.7	nd
Trichloroethylene	13.9	24.5	18.8	14.3	13.5	13.6	14.5	12.1	11.7	8.1
Tetrachloroethylene	14.2	27.0	32.3	16.3	27.8	32.9	35.1	34.7	31.3	nd
Bromoform	0.6	0.9	1.2	nd	1.6	1.6	nd	nd	nd	nd
Ethylene dibromide	nd	13.8	14.0	9.1	11.1	10.4	13.0	8.8	7.6	nd
Monochlorobenzene	25.0	25.0	17.2	14.9	14.8	13.4	14.7	12.8	2.9	nd
1,2-dichlorobenzene	1.9	2.7	2.7	2.2	2.1	1.9	2.6	2.2	2.3	nd
1,4-dichlorobenzene	3.2	2.9	2.9	1.7	3.0	1.9	3.8	3.4	3.3	nd
Aklyl benzens	28.3	29.4	23.6	nd	23.6	23.8	28.4	40.6	24.2	nd
2,4 + 2,6-dinitrotoluene	nd	12.6	18.6	18.0	nd	29.8	31.2	nd	22.8	nd

Source: From Smith, Witkowski, and Fusillo, 1987.

large, they are substantially less than the amounts of alkyl halides produced. Nevertheless, large quantities of these compounds are released to the environment by means of accidental spills or pesticide applications.

The aryl halides are less soluble than the alkyl halides and are less volatile. However, the environmental fate of these two classes of compounds is similar. The aryl halides, when spilled at land surface or in surface-water bodies, tend to volatilize to the atmosphere. Because they are moderately soluble, they tend to be mobile once they have been delivered to ground-water systems. The relatively low octanol-water partition coefficients of these compounds indicate that they are not strongly sorbed by mineral particles or organic material. Once they have reached ground-water systems, biodegradation processes are the principal attenuation mechanisms for these compounds.

13.1.3 Polychlorinated Biphenols

The chemical structure of one common polychlorinated biphenyl (PCB), 2,2′,4,4′,5,5′ PCB, is shown in Figure 13.1. PCBs are produced by chlorination, a chemical reaction that requires catalysis, of the biphenyl structure. PCBs are chemically and thermally quite stable. In addition, they exhibit excellent heat transfer and dielectric properties. Because of these properties, PCBs have been extensively utilized as dielectric fluids in electrical capacitors and transformers, in heat transfer systems, and as lubricants in turbine engines and vacuum pumps.

PCBs were first used in industrial applications during the 1930s. Production of PCBs peaked during the 1960s and early 1970s, with about 35,000 metric tons being produced per year in the United States alone. Because of their environmental persistence and toxicity, PCBs were first regulated in 1976, and in 1979 the manufacture and sale of PCBs was discontinued in the United States. In spite of this action, many operating transformers still contain PCBs. Furthermore, because of their persistence in the environment, PCBs remain a serious environmental problem in some areas.

PCBs exhibit significantly different characteristics of aqueous solubility and mobility than do halogenated benzenes. Table 13.3 shows the solubility and octanol-water partition coefficients of selected PCBS. The observed octanol-water partition coefficients are much higher and the solubilities are much lower than chlorinated benzenes. Because of these properties, PCBs are strongly adsorbed by mineral and organic materials in soils and are seldom found in significant aqueous concentrations in ground-water systems. Nevertheless, their presence in sediments associated with shallow water-table aquifers is a matter of environmental concern in some hydrologic systems. As such, the microbial degradation of PCBs in these environments is an important issue.

13.1.4 Organochlorine Insecticides

The structure of dichlorodiphenyltrichloroethane (DDT), the first organochlorine insecticide ever developed, is shown in Figure 13.1. Since the introduction of DDT in 1939, numerous other chlorinated insecticides have been developed and used to varying degrees. Chlordane was developed in 1945, followed by heptachlor, aldrin, dieldrin, and toxaphene in 1948. Many of these chlorinated compounds do not

TABLE 13.3 Octanol-water Partition Coefficients (K_{ow}) and solubilites of selected PCB's and Organochlorine Insecticides

Compound	Log K_{ow}	Solubility (mg/L)
Biphenyl	4.09	7.5
2,4′-PCB	5.10	—
4,4′-PCB	5.58	0.062
2,5,2′,5′-PCB	5.81	0.016
2,4,5,2′5′-PCB	6.11	0.010
2,4,5,2′4′,5′-PCB	6.72	0.00095
p,p′-DDT	6.36	0.0031
o,p′-DDT	—	0.026
p,p′-DDD	5.99	0.020
o,p′-DDD	6.08	—
p,p′-DDE	5.69	0.040
o,p′-DDE	5.78	—
Lindane	3.70	7.87
Aldrin	—	0.017
Dieldrin	—	0.195
Chlordane	5.48	0.056
Toxaphene	—	0.74
Endrin	—	0.26
Heptachlor	—	0.056
α-Endosilfan	—	0.53

Source: From Smith, Witkowski, and Fusillo, 1987.

contain the alicyclic ring, but are treated together in this discussion because of their common usage.

Organochlorine insecticides have been widely distributed in the environment by agricultural spraying. However, because of their persistence in the environment, toxic effects, and tendency to bioaccumulate in higher organisms, their use has been greatly decreased over the last several decades. During the 1960s, the annual production of these compounds in the United States exceeded 70,000 tons. However, by the mid-1990s, U.S. production was virtually nonexistent.

The solubility and octanol-water partition coefficients of organochlorine insecticides are similar to those of PCBs (Table 13.3). Lindane, with an aqueous solubility of 7.87 mg/L, is the most soluble of these compounds, but most exhibit solubilities of under 0.01 mg/L. This low solubility, as well as the high affinity of these compounds for minerals and organic matter in soils, greatly limits their delivery to ground-water systems. However, because these compounds were extensively applied to agricultural areas, some transport to ground-water systems has occurred. Because of this, the biodegradation characteristics of these compounds in ground-water systems are important in some systems.

13.1.5 Chlorinated Herbicides

Herbicides are extensively used in the United States to control weed growth in agriculture and to control aquatic vegetation in navigable surface-water bodies. The

structures of atrazine and 2,4-dichlorophenoxyacetic acid (2,4-D) are shown in Figure 13.1.

Chlorinated herbicides are characterized by relatively high aqueous solubilities (Table 13.4) and do not tend to sorb strongly to mineral surfaces or soil organic matter. Because these chemicals are applied extensively to agricultural areas, they are often found in underlying water-table aquifers. As such, the ability of subsurface microorganisms to degrade these substances is a topic of considerable interest.

Most herbicides are rapidly degraded in surface-water systems. Investigations have shown that the half-lives of 2,4-D and atrazine in surface-water systems are on the order of 3 to 12 days. 2,4-D has been reported to degrade rapidly under the aerobic conditions characteristic of most surface-water environments. However, the degradation of 2,4-D appears to be slowed under anaerobic conditions. In contrast, atrazine appears to degrade, at least partially, under either aerobic or anaerobic conditions.

13.1.6 Chlorinated Phenols

The phenols are a group of organic compounds characterized by a benzene ring with one or more hydroxyl groups attached. Chlorinated phenols have one or more chlorines attached (Fig. 13.1). Monochlorophenols are seldom used directly in industrial applications but are important intermediates in the production of di-, tri-, and pentachlorophenols. 2,4-dichlorophenol is used as an antimildew agent in the textile industry and in the manufacture of wood preservatives. In addition, 2,4-dichlorophenol is used to manufacture the herbicide 2,4-D and 2,4,5 trichlorophenol is used to manufacture the herbicide 2,4,5-T. The total production of di-, tri-, tetra-, and pentachlorophenol is about 25,000 metric tons.

TABLE 13.4 Aqueous solubilities of some common herbicides

Compound	Solubility (mg/L)
2,4-D	890
2,4,5-T	278
Atrazine	70
Simazine	5
Monuron	230
Diuron	42
Linuron	75
Monolinuron	735
Propanil (Stam F-34)	50–225
Picloram	430
Benefin	0.1
Profluralin	0.1
Trifluralin	4
Silvex	140
Diquat	700,000
Paraquat	miscible

Source: Smith, Witkowski, and Fusillo, 1987.

Phenolic compounds are important components of creosote, which is used extensively as a wood preservative and particularly as a preservative of telephone poles and railroad ties. Creosote is a volatile, oily substance that is obtained by distillation of wood tar. Many coniferous (evergreen) trees produce phenolic compounds naturally to protect themselves against molds, fungi, and insects. Thus, by concentrating these compounds by distillation to creosote, humans have simply harnessed a natural pesticide (phenolic compounds) and made them more potent. Prior to about 1950, creosote was the principal material used as a preservative for telephone poles and related products. After 1950, chemical engineering technologies for producing pentachlorophenols (PCPs) became more efficient, and PCPs began to replace raw creosote as a wood-preserving material.

Chlorophenols have very high solubilities and moderately low octanol-water partition coefficients (Table 13.5). As such, sorption to mineral grain and organic matter in aquatic sediments and ground-water systems is generally small. On the other hand, chlorophenols are susceptible to microbial degradation once their concentrations fall below toxic thresholds. Because phenols are present naturally in the environment, microbial biodegradation processes for these compounds are well-developed. This built-in phenol-degrading capability, which is present in all aquatic sediments and ground-water systems, is an important process affecting chlorophenol mobility in subsurface environments.

13.2 MICROBIAL DEGRADATION OF HALOGENATED ORGANIC COMPOUNDS

Because environmental contamination by halogenated organic compounds is so common, considerable efforts have been made to understand the microbial processes that affect their fate and transport in aquatic systems. In general, the kinds of operative microbial degradation processes depend upon the chemical characteristics of the individual compounds and upon the aerobic or anaerobic nature of the

TABLE 13.5 Solubilities and Octanol-Water Partition Coefficients (K_{ow}) of Selected Phenolic Compounds

Compound	Solubility (mg/L)	Log K_{ow}
Phenol	93,000	1.46
2-Chlorophenol	28,500	2.17
2,4-Dichlorophenol	4,500	2.75
2,4,6-Trichlorophenol	800	3.38
Pentachlorophenol	14	5.01
2-Nitrophenol	2,100	1.76
4-Nitrophenol	16,000	1.91
2,4-Dinitrophenol	5,600	1.53
2,4-Dimethylphenol	17,000	2.50
p-Chloro-m-cresol	3,860	2.95
4,6-Dinitro-o-cresol	—	2.85

environment. It is useful, therefore, to consider the microbial degradation of each class of compound separately.

13.2.1 Chlorinated Ethenes

Chlorinated ethenes are subject to a variety of microbial degradation processes that include reductive dechlorination (Vogel and McCarty, 1985; Barrio-Lage et al., 1987; Odum et al., 1995), aerobic oxidation (Hartmans et al., 1985, Davis and Carpenter, 1990), anaerobic oxidation (Bradley and Chapelle, 1996), and aerobic cometabolism (Wilson and Wilson, 1985; McCarty and Semprini, 1994; Semprini, 1995). Because of this variety, assessing ongoing degradation processes in groundwater systems is often complex. However, because the conditions under which these processes occur have now been well-documented, it is often possible to identify the presence or absence of particular degradation processes at a given site.

Reductive Dechlorination Chloroethenes contain chlorine atoms in their molecular structure, and this makes them relatively oxidized compounds. Because of this, chloroethenes can act as oxidants (that is, electron acceptors) in microbial metabolism (Vogel et al., 1987). In reductive dechlorination, molecular hydrogen reacts to replace a chlorine on a chlorinated ethene molecule. Because this causes a net reduction of the molecule by removing a chlorine, it is called reductive dechlorination. In addition, because it is now known that these reduction processes can be used by some microorganisms to support respiration, they are also referred to as chlororespiration.

In the early 1980s a number of reports described the accumulation of daughter products such as *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC) in anaerobic ground-water systems and attributed this phenomenon to microbial reductive dechlorination. The consensus today is that microbial reductive dechlorination is ubiquitous in anaerobic, chloroethene-contaminated aquifers, but that the extent of dechlorination is highly variable from site to site (Chapelle, 1996; Bouwer, 1994; Gossett and Zinder, 1996; McCarty, 1996; McCarty and Semprini, 1994; Vogel, 1994).

For chlorinated ethenes, the tendency to undergo reductive dechlorination decreases with decreasing number of chlorine atoms present in the molecule (Bouwer, 1994; McCarty and Semprini, 1994). Perchloroethene (PCE), with its four chlorine atoms (Fig. 13.1), is a stronger oxidant than most naturally occurring in electron-accepting species normally present in ground water, with the notable exception of O_2 (Vogel et al., 1987). Thus PCE readily undergoes reductive dechlorination to TCE except in oxygenated ground water. Reductive dechlorination of TCE to *cis*-dichloroethene occurs under Fe(III)-reducing, sulfate-reducing, and methanogenic conditions. Reductive dechlorination of *cis*-DCE to yield VC apparently requires at least SO_4-reducing conditions (Chapelle, 1996; Vogel et al., 1987), but proceeds more readily in methanogenic environments. VC, on the other hand, is the least oxidized of the chloroethenes, and the reductive dechlorination of VC to nonchlorinated ethene is often slow and is significant only under highly reducing, methanogenic conditions (DiStefano et al., 1991; Freedman and Gossett, 1989). As a result of this decreasing reductive potential with decreasing number of chlorine

atoms, reductive dechlorination of chloroethene contaminants is often incomplete and often leads to the accumulation of *cis*-DCE and VC in ground water.

The first pure cultures shown to catalyze reductive dechlorination were methanogens that did not actually grow using chloroethenes as electron acceptors and apparently did not gain energy from the reaction. These organisms mediated slow and partial reductive dechlorination of PCE and TCE to yield DCE. Based on these observations, reductive dechlorination was thought to be a strictly cometabolic process brought about by the accidental interaction of chloroethenes with enzymes and cofactors produced by the microorganisms for other metabolic purposes (McCarty and Semprini, 1994). Thus, microorganisms were not thought to obtain any direct benefits from these processes.

This thinking began to change in the early 1990s, when a number of investigations demonstrated that reductive dechlorination can be carried out by microorganisms that use chloroethenes as terminal electron acceptors for their metabolism. Specifically, it was shown that molecular hydrogen acted as the electron donor in these processes, with chloroethenes acting as electron acceptors as shown in Figure 13.2 (DiStefano et al., 1991). These microorganisms, collectively termed halorespirers, are able to grow using chloroethenes as sole terminal electron acceptors. Although halorespirers capable of reducing PCE or TCE to DCE are relatively common (Holliger et al., 1993; Maymo-Gatell et al., 1997; Sharma et al., 1996) to date only one, *Dehalococcus ethenogenes*, has been shown to completely degrade PCE to ethene (Maymo-Gatell et al., 1997). When microorganisms like *D. ethenogenes* are present in ground-water systems, then complete reductive dechlorination to ethene may be a significant biodegradation process in anaerobic aquifers.

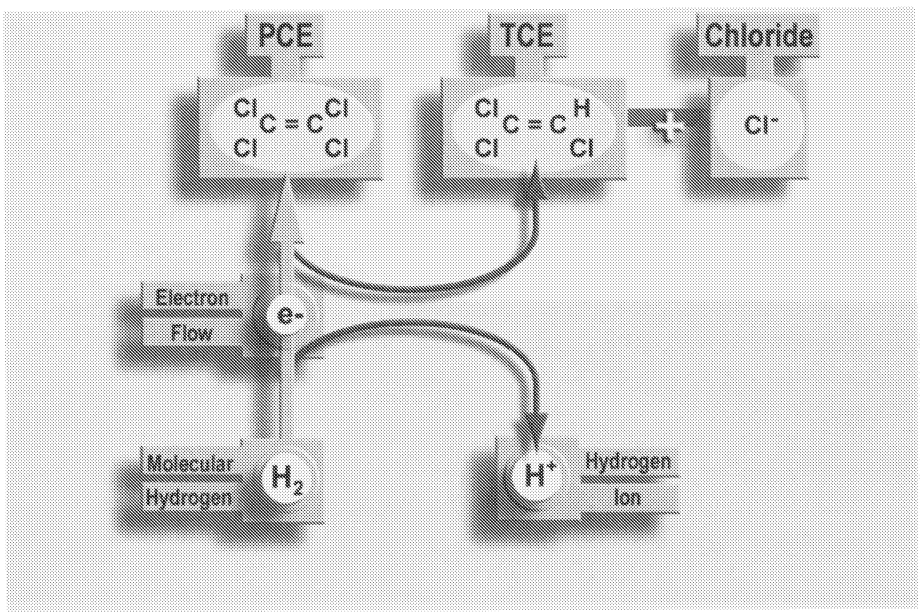


Figure 13.2 Molecular hydrogen (H_2) driving the reductive dechlorination of perchloroethene (PCE) to trichloroethene (TCE).

At present, the general consensus is that reductive dechlorination includes both cometabolic and chlororespiration processes. Furthermore, although complete microbial reductive dechlorination is possible in anaerobic aquifers, complete reductive dechlorination to ethene is rare due to low electron donor availability and thus to unfavorable redox conditions. Thus reductive dechlorination alone does not usually completely remediate chloroethene contamination in ground-water systems. However, in combination with aerobic and anaerobic mechanisms for microbial oxidation of DCE and VC, the process of reductive dechlorination can be an important component of the natural attenuation of chlorinated ethenes in ground-water systems.

Aerobic Oxidation While the tendency of chloroethenes to undergo reductive dechlorination decreases as the number of chlorines present in the molecule decreases, the tendency to undergo oxidation increases (Vogel et al., 1987). As the least chlorinated of the chloroethenes, VC has the greatest tendency to undergo oxidation. Rapid microbial degradation of vinyl chloride, including mineralization, has been observed in laboratory cultures and aquifer samples under aerobic conditions (Hartmans et al., 1985; Davis and Carpenter, 1990; Phelps et al., 1991; Bradley and Chapelle, 1996). Moreover, under aerobic conditions, VC can be used as a sole carbon source for growth and metabolism (Hartmans et al., 1985; Hartmans and de Bont, 1992). DCE, on the other hand, has been shown to oxidize under aerobic conditions in liquid culture, but this oxidation apparently does not support microbial growth (Bradley and Chapelle, 1999).

Because the production of DCE and VC generally occurs by reductive dechlorination under anaerobic conditions, the aerobic oxidation of these compounds is often limited in ground-water systems. However, where anaerobic conditions that produce DCE and VC grade to more oxic conditions, which often happens on the fringes of contaminant plumes, aerobic oxidation of these compounds can be significant. Furthermore, the aerobic degradation of VC and DCE can be stimulated in ground-water systems by providing a source of oxygen.

Anaerobic Oxidation The anaerobic oxidation of DCE and VC is a process that has only been recognized recently (Bradley and Chapelle, 1996). Given the early emphasis on reductive dechlorination under anaerobic conditions as a principal degradation pathway, the fact that these compounds also oxidize anaerobically was unexpected. Nevertheless, in a series of papers, it was shown that VC, and to a lessor extent DCE, could oxidize to carbon dioxide under Fe(III)-reducing conditions (Bradley and Chapelle, 1996; Bradley and Chapelle, 1997; Bradley et al., 1998).

At first, the fact that Fe(III) reduction could support the anaerobic oxidation of VC was attributed to the fact that Fe(III) is a fairly strong oxidant. However, further investigation showed that a portion of both ^{14}C-VC and ^{14}C-DCE was mineralized to $^{14}CO_2$ not just under Fe(III)-reducing conditions but under sulfate-reducing conditions and even methanogenic conditions (Fig. 13.3). In addition, it was shown that humic acids could serve as an electron acceptor for VC and DCE oxidation (Bradley et al., 1998). Clearly, the simple explanation that a strong oxidant such as Fe(III) was solely responsible for anaerobic oxidation could not account for observed oxidation under methanogenic conditions (Bradley and Chapelle, 1999).

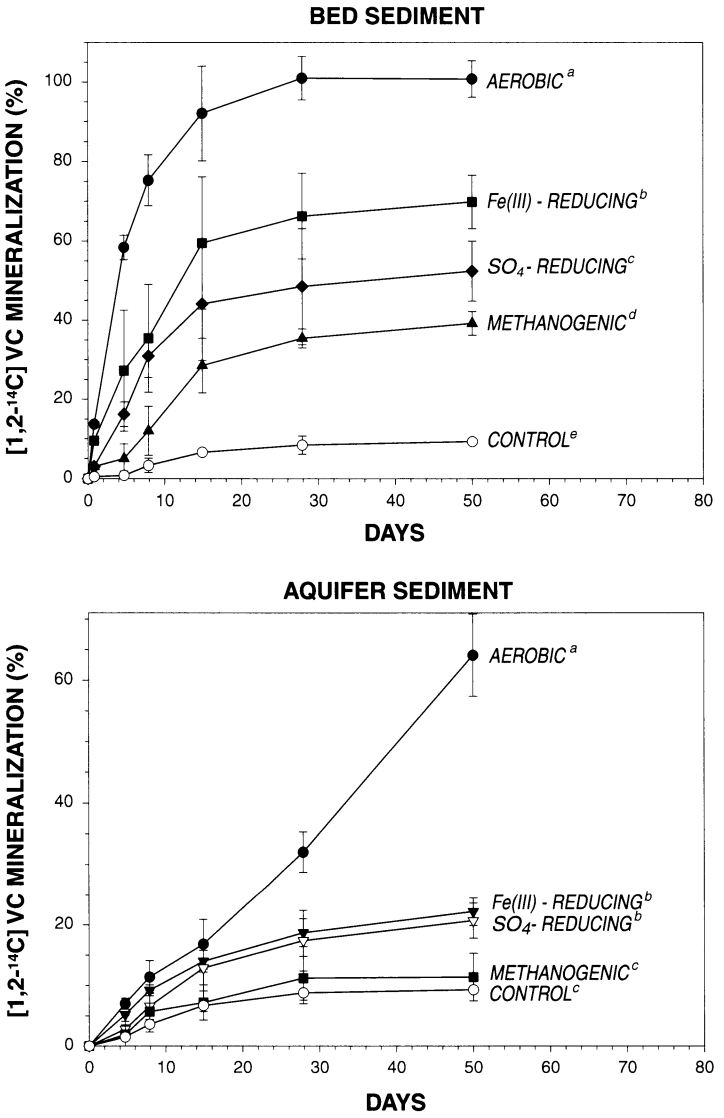


Figure 13.3 Relative rates of ^{14}C-labeled vinyl chloride ([1,2-^{14}C]VC) mineralization under different electron-accepting conditions for (a) stream-bed sediments, and (b) aquifer sediments. (Modified from Bradley and Chapelle, 1998).

An explanation for these observations became evident with the discovery that ^{14}C-acetate was an intermediate of ^{14}C-VC mineralization under methanogenic conditions (Bradley and Chapelle, 1999; Bradley and Chapelle, 2000). This suggested that the actual degradation of the VC was not being carried out directly by any particular terminal electron-accepting process, but rather by fermentative acetogenic microorganisms. This suggestion was later confirmed by the observed accumulation of ^{14}C-acetate from ^{14}C-VC when methanogenesis and reductive dechlorination were blocked by addition of BES to the microcosms (Bradley and Chapelle, 2000).

Acetogens are known to metabolize a wide variety of organic compounds while producing acetate as a waste product (Drake, 1994). Once acetogenic bacteria transform VC to acetate, the acetate can then be readily oxidized to CO_2 by a wide variety of terminal electron-accepting processes (Fig.13.4).

This model (Fig. 13.4) is consistent with the observation that the rate of anoxic VC mineralization decreases with the oxidizing potential of the electron acceptor. Because Fe(III)-reducing bacteria have a higher affinity for acetate than methanogens, they maintain lower concentrations of acetate. This, in turn, allows acetogenic microorganisms to produce acetate (their waste product) at a faster rate, leading to faster $^{14}CO_2$ production (Fig. 13.3). However, the observed presence of acetate as an intermediate in VC mineralization does not rule out the possibility that VC can also be directly oxidized to CO_2. Such direct oxidation would also be consistent with the pattern shown in Figure 13.3. It is now clear that VC can be degraded by both reductive dechlorination and by acetogenic fermentation under anaerobic conditions (Fig. 13.4). The acetogenic fermentation leads to a net oxidation of VC to CO_2. However, the possibility remains that VC can also be directly oxidized by respirative NO_3-reducing, Mn(IV)-reducing, Fe(III)-reducing, or sulfate-reducing bacteria (Fig. 13.4).

While it has been shown that DCE is also subject to a net oxidation under anaerobic conditions (Bradley and Chapelle, 1998), it is presently unclear whether DCE can be transformed directly, or whether DCE transformation is initiated by reductive dechlorination to VC. The fact that the kinetics of DCE mineralization are different from those for VC, however, suggests that the processes differ, and this may indicate that reductive dechlorination to VC is a necessary step in the complete biodegradation of DCE.

Aerobic Cometabolism Another process that leads to microbial degradation of chloroethenes in ground-water systems is cometabolic oxidation. In 1985, J. T. and B. B. Wilson reported that methanotrophic (methane-oxidizing) bacteria were capable of oxidizing TCE to CO_2 under aerobic conditions. Since then, a wide variety

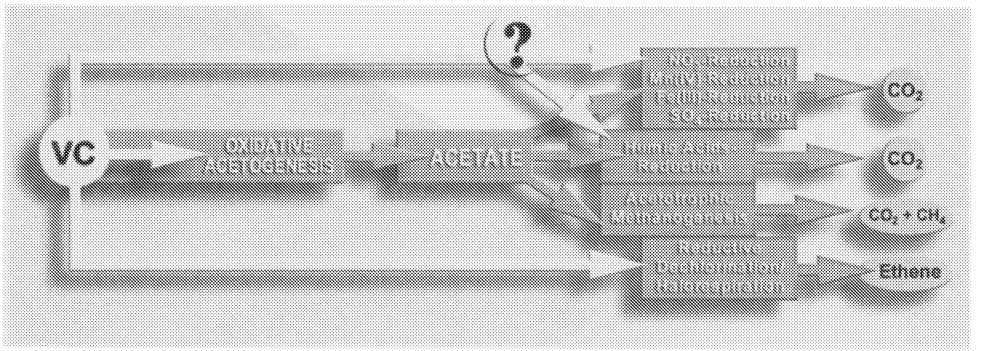


Figure 13.4 Model showing possible degradation pathways of vinyl chloride (VC) including acetogenic transformation to acetate with with subsequent complete oxidation of acetate by different electron-accepting processes, and reductive dechlorination. Direct oxidation of VC is possible, particularly when coupled to Mn(IV) or humic acids reduction, but has not yet been demonstrated.

of aerobic microorganisms, which are able to oxidize TCE, DCE, and VC to CO_2 without accumulation of toxic intermediates, have been identified. These include methane oxidizers, propane oxidizers, ethene oxidizers, aromatic compound oxidizers, ammonium oxidizers, isoprene oxidizers, and vinyl chloride oxidizers (see McCarty and Semprini, 1994). Cometabolic oxidation of chloroethenes does not supply energy for microbial growth or metabolism. Rather, for each of these processes, the responsible microorganisms contain nonspecific oxygenases that fortuitously oxidize chloroethenes to CO_2. Consequently, aerobic cometabolism of chloroethenes requires the presence of oxygen and a primary substrate to initiate the production of a suitable oxygenase.

Because it is rare for significant concentrations of methane (or other cosubstrates) to coexist with dissolved oxygen, cometabolic oxidation is probably rare in ground-water systems. However, cometabolic oxidation of chloroethenes by subsurface microorganisms has been successfully exploited for engineered remediation of chloroethene-contaminants in ground water (McCarty and Semprini, 1994; Semprini, 1995; Semprini et al., 1990; 1991). TCE contamination in aerobic aquifers can be biodegraded by methanotrophic microorganisms when methane is supplied to the subsurface in sufficient quantity to stimulate and support methanotrophic activity. Because methane and oxygen do not typically co-occur in ground-water systems, however, methanotrophic oxidation of chloroethenes is unlikely under nonengineered circumstances. A number of ex situ investigations have demonstrated that toluene oxidizers can cometabolize chloroethenes under aerobic conditions (Fan and Scow, 1993; Fuller et al., 1995; Hopkins and McCarty, 1995; Mars et al., 1996). Moreover, many ground-water contaminant plumes are the result of mixed waste releases and, as a consequence, contain chloroethenes and aromatic compounds like toluene. Unfortunately, contaminant plumes that contain hydrocarbon compounds in sufficient concentration to support cometabolic oxidation of chloroethenes are characteristically anaerobic because of oxygen depletion by aerobic respiration. Consequently, significant aerobic cometabolic oxidation of chloroethenes in such systems would be, at most, a transient phenomenon. However, it is possible to artificially create conditions favorable to cometabolic oxidation by injecting methane (or some other cometabolic substrate) together with oxygen into ground-water systems (Semprini et al., 1990).

Redox Conditions and the Biodegradation of Chlorinated Ethenes in Ground-Water Systems Although microbial degradation processes of chlorinated ethenes are complex, they are predictable. Under highly reducing conditions, all chlorinated ethenes can serve as electron acceptors in microbial metabolism. In this case, microorganisms reduce chlorinated ethenes by transferring electrons to them. Under other redox conditions, microorganisms can use lightly chlorinated ethenes such as VC as electron donors. In this case there is a net oxidation of chlorinated ethenes. Under aerobic conditions, this appears to be a direct oxidation process. However, under reducing conditions this oxidation appears to proceed with acetogenic microorganisms (and possibly other fermentative microorganisms) playing an intermediate role (Fig. 13.4).

Under anoxic conditions, highly chlorinated ethenes such as PCE and TCE are subject to reductive dechlorination according to the sequence

$$PCE \rightarrow TCE + Cl \rightarrow DCE + 2Cl \rightarrow VC + 3Cl \rightarrow ethene + 4Cl$$

However, the *efficiency* of dechlorination differs for different redox conditions. Dechlorination of PCE and TCE to DCE occurs under mild nitrate- or Fe(III)-reducing conditions, whereas the transformation of DCE to VC, or the transformation from VC to ethene, requires the more strongly reducing conditions of methanogenesis or sulfate reduction (Vogel et al., 1987). It has been shown (DiStefano et al., 1991; Concordia et al., 1996) that reductive dechlorination is driven by molecular hydrogen (Figure 13.2). It follows, therefore, that the efficiency of reductive dechlorination is directly related to the availability of H_2.

Concentrations of H_2 in ground-water systems (see Chapter 10) are controlled by ambient microbial terminal electron-accepting processes (TEAPs). Under anaerobic conditions, H_2 is continuously produced by fermentative microorganisms metabolizing natural or anthropogenic organic matter. This H_2 is then utilized by respirative microorganisms that most commonly use Fe(III), sulfate, or CO_2 as terminal electron acceptors. Significantly, each of these TEAPs has a different affinity for H_2 uptake (Lovley and Goodwin, 1988). CO_2 reduction (methanogenesis) has the lowest H_2 affinity, and steady-state H_2 concentrations in methanogenic aquifers are therefore relatively high (~10 nanomoles per liter (nM)). Sulfate reduction has a slightly greater affinity for H_2 than methanogenesis, and is characterized by slightly lower H_2 concentrations (1–4 nM). Fe(III) reduction (0.2–0.8 nM) and nitrate reduction (<0.10 nM) have even greater affinitites for H_2 and are characterized by progressively lower steady-state H_2 concentrations (Table 10.1).

Thus, the observation that reductive dechlorination is more efficient under methanogenic or sulfate-reducing conditions is due to the greater availability of H_2 to chlororespiring bacteria than under Fe(III)- or nitrate-reducing conditions, not to the direct activity of methanogenic or sulfate-reducing microorganisms.

Once reductive dechlorination has initiated biodegradation, the lightly chlorinated ethenes VC and DCE produced by reductive dechlorination can be oxidized to CO_2 under oxic conditions:

VC

$$ClC_2H_3 + 5/2\ O_2 \rightarrow 2CO_2 + H_2O + H^+ + Cl^-$$

DCE

$$Cl_2C_2H_2 + 2O_2 \rightarrow 2CO_2 + 2H^+ + 2Cl^-$$

Thus, complete degradation of chlorinated ethenes is favored by sequential anoxic-oxic conditions. First, under reducing conditions, PCE and TCE is transformed to DCE and VC. Then, as ground water migrates to more oxic condtions, DCE and VC can be oxidized to carbon dioxide:

anoxic (methanogenic, sulfate-reducing) → oxic, Fe(III)-reducing conditions

PCE, TCE → DCE and VC DCE, VC → $2CO_2$ and Cl^-

The efficiency with which chlorinated ethenes are completely degraded, there-

fore, depends on the redox conditions present in an aquifer, and to the succession of redox processes that chlorinated ethenes are exposed to along particular ground-water flowpaths. Thus, assessing redox conditions is an important component to assessing the biodegradation of chlorinated ethenes in ground-water systems (Chapter 10).

13.2.2 Chlorinated Benzenes

Chlorinated benzenes, like halogenated aliphatic compounds, are subject to biodegradation under both aerobic and anaerobic conditions. Outwardly, there is a good deal of similarity between biodegradation of chlorinated benzenes and ethenes. Under aerobic conditions, lightly chlorinated benzenes and ethenes can both serve as electron donors to support microbial growth. Similarly, under anaerobic conditions more heavily chlorinated ethenes and benzenes are both subject to reductive dechlorination. However, the biochemistry of these processes is different, and this leads to differences in the efficiency of chlorinated ethene and chlorinated benzene biodegradation.

Aerobic Degradation A number of microorganisms have been described that are capable of utilizing chlorinated benzenes as primary substrates. For example, Reineke and Knackmuss (1984) described a bacterium capable of utilizing chlorobenzene as a sole carbon and energy source. Studies with this chlorobenzene-utilizing bacterium led to the elucidation of the operative degradation pathway.

The general pathway of chlorobenzene degradation as indicated by the studies of Reineke and his colleagues is shown in Figure 13.5. The general strategy is the formation of chlorocatechol followed by ortho cleavage of the ring. The substituted muconic acid is then dechlorinated and the nonchlorinated intermediate products metabolized to 3-oxoadipate, which then enters the cell's tricarboxylic acid cycle. Note that this is virtually identical to the pathway of aerobic benzene degradation. The only substantial difference is the necessary elimination of the chloride from the organic compound at some point in the pathway.

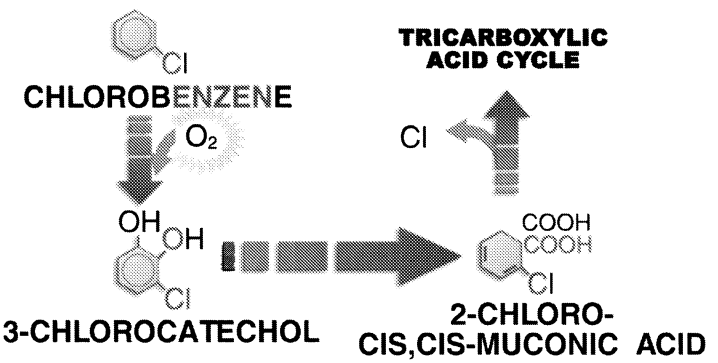


Figure 13.5 Aerobic biodegradation of chlorobenzene.

More recently (Sander et al., 1991) has demonstrated that similar mechanisms are involved in the degradation of trichloro- (TCB) and tetrachlorobenzene (TeCB). In the case of 1,2,4-TCB, degradation is initiated by dioxygenation of the aromatic nuclei forming 3,4,6-trichlorocatechol. Subsequent ortho cleavage results in formation of the corresponding 2,3,5-trichloromuconate, which channels into the tricarboxylic cycle, as was the case with chlorobenzene (Fig. 13.5).

The similarity of chlorobenzene degradation to that of benzene has not been lost on microbial ecologists. Because chlorinated benzenes have been produced only in the last few decades, it seems unlikely that degradation pathways existed prior to widespread availability of aryl halides to microbial populations via environmental contamination. To some investigators, this suggests that the enzyme systems involved in aryl halide degradation evolved from preexisting systems designed to degrade the more commonly occurring benzene molecule. This, in turn, implies an extremely rapid rate of microbial evolution to xenobiotic compounds.

To other investigators, however, such rapid evolution seems unlikely. They point to the fact that there are numerous naturally occurring chlorinated aromatic compounds—2,4-dichlorophenol, for instance—that could form the basis for the observed degradation pathways. The important issue in this unresolved controversy deals with understanding the rate at which microorganisms adapt genetically to new potential substrates. If, as some would argue, microorganisms have evolved the ability to grow on chlorinated benzenes during the last 20 years, this implies that new degradative pathways may evolve fairly quickly. On the other hand, if microorganisms already have the enzymatic capability to deal with chlorinated benzenes, then the implication is that degradative pathways may not evolve as quickly as some would like to think.

Anaerobic Degradation In contrast to aerobic biodegradation, processes affecting chlorinated benzenes in ground-water systems is less well understood. Qualitatively, it seems reasonable that highly chlorinated benzenes such as di- and trichlorobenzene would be subject to reductive dechlorination under anaerobic conditions. This expectation comes mainly from the fact that di- and trichlorinated benzenes are fairly oxidized compounds and it seems logical they would have a relatively high affinity for molecular hydrogen. However, in addition to such transformations being thermodynamically feasible, biochemical mechanisms for catalyzing them must also be in place. Experience has shown that assuming such mechanisms in the absence of hard evidence is not a sound practice. Studies of ground-water systems (section 13.3.3) indicate that, while reductive dechlorination may occur, it is not usually observed to be a significant attenuation mechanism.

13.2.3 Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBS) were used extensively as transformer fluids between about 1935 and 1978 in the United States. The Monsanto Company, the sole U.S. manufacturer, marketed these products under the trade name Aroclor. Several varieties of Aroclor were produced that differed primarily in the weight percent of chlorine per molecule. In Monsanto's nomenclature, Aroclor was denoted by a four-digit number in which "12" referred to a biphenyl and the second two digits

referred to the weight percent of chlorine in the molecule. Thus, Aroclor 1254 was a product consisting largely of biphenyls that were 54% chlorine. This translates to about five chlorine atoms per molecule. In practice, however, Aroclors were mixtures of many different PCBs containing anywhere from one to 10 chlorines per molecule.

The resistance of PCBs to microbial degradation is one property that made these compounds so useful in industrial applications. Nevertheless, they are subject to degradation under certain conditions. PCBs with relatively few chlorines per molecule are subject to oxidation reactions under aerobic conditions. For example, Clark et al. (1979) described degradation of PCBs by mixed microbial cultures taken from soils and aquatic sediments. Furukawa et al. (1978) described an *Acinetobacter* species capable of degrading 2,4,4′-trichlorobiphenyl. The pathway of degradation implied by these studies involves the familiar pattern of hydroxylation followed by *meta* cleavage of the alicyclic ring. These studies have documented the relative recalcitrance of heavily chlorinated PCBs to aerobic microbial oxidation. This may reflect interference of the hydroxylation and ring cleavage steps by chlorine molecules. Alternatively, this may simply reflect the fact that heavily chlorinated PCBs are more oxidized compounds than lightly chlorinated PCBs, and that this makes oxidation less energetically favorable.

Under anaerobic conditions, the predominant microbially mediated degradation process is reductive dechlorination. In anaerobic sediments of the Hudson River, which were extensively contaminated by Aroclor 1242, a marked increase in the proportion of biphenyls exhibiting 1, 2, and 3 chlorines relative to the parent product was noted (Brown et al., 1984). These authors suggest that the observed reductive dehalogenation is analogous to the reductive processes documented for halogenated benzoic acids.

The picture that emerges from consideration of microbial attack on PCBs under aerobic and anaerobic conditions is that highly chlorinated molecules are resistant to aerobic oxidation but are subject to reductive dehalogenation. Conversely, lightly chlorinated PCBs are resistant to reductive dehalogenation but are subject to aerobic oxidation. This pattern has not been lost on researchers investigating possible bioremediation strategies. Specifically, it has been suggested that a sequence of anaerobic degradation followed by aerobic degradation can result in complete mineralization of PCBs. Unfortunately, the sequence normally followed in natural aquatic sediments—initial shallow burial and aerobic degradation, followed by deeper anaerobic degradation—is just the reverse of the pattern that would be most effective. However, it is evident that microbial processes have the potential to completely degrade these compounds if the proper sequence of redox conditions can be achieved.

13.2.4 Organochlorine Insecticides

Organochlorine insecticides such as DDT, lindane, and chlordane were extensively used in agriculture during the 1940s, 1950s, and 1960s. However, because of their environmental persistence and because of their tendency to accumulate in the lipids of aquatic organisms, their use has been largely discontinued in the United States. They have been replaced by such insecticides as the pyrethroids, permethrin, and

fenvalerate, which require much lower application rates. Nevertheless, because of DDT's environmental persistence it remains an issue in some areas.

Although DDT is very persistent in the environment, degradation does occur. The first step that must occur in order for degradation to proceed is the reductive dechlorination of DDT to DDD (dichlorodiphenyldichloroethane). This process occurs only under highly reducing conditions and does not appear to require microbial mediation. However, this reaction may be facilitated by reduced iron porphyrins present in mixed populations of anaerobic bacteria. In addition, DDT may be reduced to DDE (dichlorodiphenyldichlorethene). This appears to be a dead-end pathway, as further degradation of DDE is not generally observed.

Like DDT, other organochlorine insecticides are rather recalcitrant to degradation. The cyclodiene insecticides aldrin, chlordane, endrine, and heptachlor are generally considered to be persistent in most environments. Heptachlor may be oxidized to heptachlor epoxide by soil microorganisms, and endrin is metabolized to form ketones and aldehydes that retain the attached chlorines. Lindane, the most soluble of the chlorinated insecticides, is also one of the most degradable, forming chlorinated benzenes with the mediation of fermentative soil bacteria such as *Bacillus* and *Clostridium.*

13.2.5 Chlorinated Herbicides

While PCBs and organochlorine insecticides are relatively toxic and resistant to microbial degradation, their mobility in ground-water systems is limited by their tendency to partition onto sediments and organic matter. Chlorinated herbicides such as atrazine and 2,4-D are much more soluble and tend to be transported more easily in the unsaturated and saturated zones of ground-water systems. Because of this relative mobility, relative rates of microbial degradation are important to their fate and transport.

Atrazine belongs to the general class of herbicides known as the s-triazines, a name that refers to the central ring structure that contains three nitrogen groups. Attached to the ring structure of atrazine is a chlorine atom, an isopropylamino group, and an ethylamino group. Atrazine is degraded by a number of biotic and abiotic mechanisms that involve removal of one or more of the functional groups as well as ring cleavage.

The most important mechanisms in atrazine degradation are hydroxylation (H) and dealkylation (D) reactions (Fig. 13.6). The dechlorination and hydroxylation of atrazine to produce hydroxyatrazine occurs both biotically and abiotically. When occurring abiotically, this process has been shown to be pH-dependent, with maximal rates occurring at a pH of about 8.0. The formation of hydroxyatrazine results in detoxification of atrazine. Dealkylation of both the isopropyl and ethyl groups has been reported. Behki and Khan (1986) showed that particular strains of *Pseudomonas* were capable of utilizing atrazine as a sole carbon source and that atrazine was metabolized via dealkylation reactions. This study also suggested that formation of deisopropylatrazine was favored over deethylatrazine and that particular species were capable of dechlorinating either of these metabolites when another carbon source was provided. It was also observed that dechlorination of atrazine tended to proceed after dealkylation.

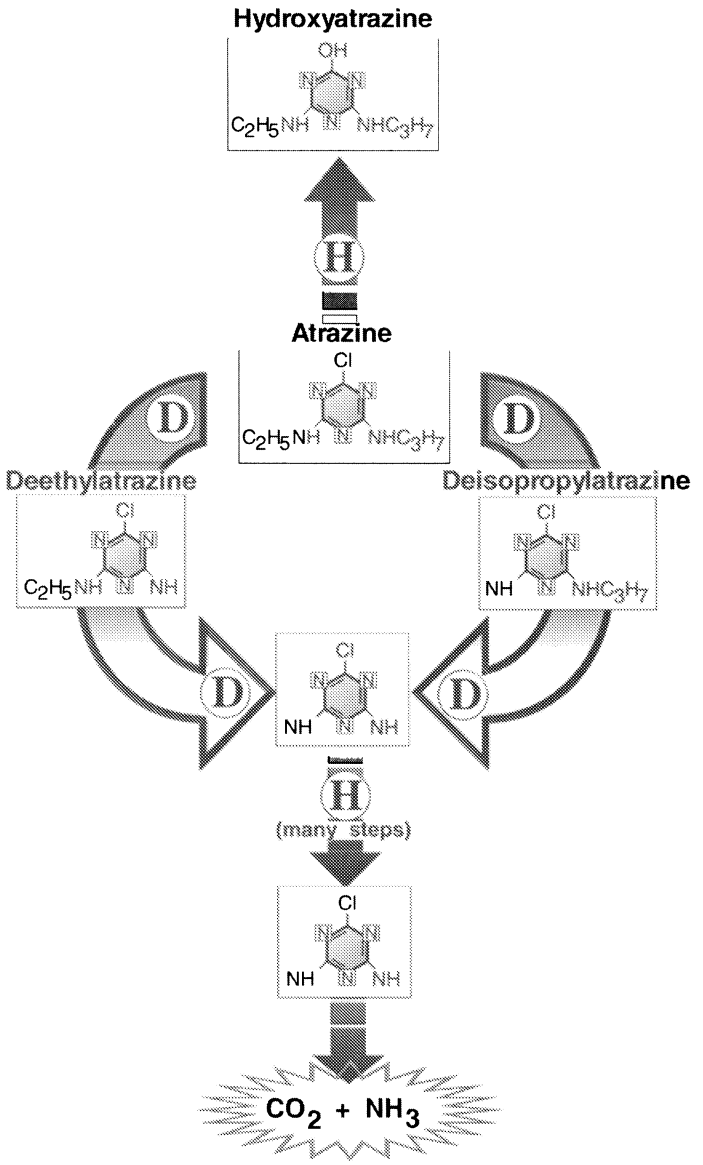


Figure 13.6 Hydroxylation (H) and dealkylation (D) reactions in the degradation of atrazine.

13.2.6 Chlorinated Phenols

Chlorinated phenols released into surface and subsurface sediments from wood-treatment facilities or as degradation products of chlorinated herbicides are subject to both aerobic and anaerobic biodegradation. As with other chlorinated organic compounds, however, the mechanisms and pathways involved differ substantially under aerobic and anaerobic conditions.

Aerobic Degradation Under aerobic conditions, two distinct mechanisms for the metabolism of chlorophenols have been identified. The first of these mechanisms

is the methylation of one or more hydroxyl groups associated with the phenolic compound. Neilson, Allard, and Hynning (1983) showed that a species of *Arthrobacter* carries out this process. The chloroanisoles that are produced by this process are volatile and tend to escape to the atmosphere at land surface. As chlorophenols are typically present in the wood shavings used for litter in poultry-raising operations, the production of volatile chloroanisoles may result in contamination of chicken eggs or meat.

The second mechanism for the metabolism of chlorophenols that has been extensively studied involves the formation of chlorocatechols from chlorophenols. A number of *Pseudomonas, Atcaligenes,* and *Nocardia* species have been described that carry out this and similar biochemical transformations. For example, Knackmuss and Hellwig (1978) showed that a *Pseudomonas* species could grow using 4-chlorophenol as a sole carbon and energy source with the complete degradation of the 4-chlorophenol. The initial step in this metabolic pathway was the conversion of 4-chlorophenol to 4-chlorocatechol with subsequent cleavage of the alicylic ring via either the ortho or the meta pathways.

Whether these or other biochemical mechanisms are involved in the aerobic degradation of chlorophenols in natural systems is difficult to say. However, the aerobic degradation of chlorinated phenols has been observed in soils, and this process has been suggested as a means for the bioreclamation of chlorophenol-contaminated soils.

Anaerobic Degradation The anaerobic degradation of chlorinated phenols has been extensively studied because of the delivery of these compounds to sewage treatment plants and their subsequent degradation by activated-sludge microorganisms. Mikesell and Boyd (1986) showed that chlorinated phenols were completely degraded by a combination of reductive dehalogenation and anaerobic metabolism.

The reductive dechlorination of chlorophenols was studied by Bryant, Hale, and Rogers (1991), who showed that the order of chlorine removal from pentachlorophenol was predictable. In the case of one adapted sediment community, the chlorine in the para position tended to be removed preferentially to that in the ortho position and that the chlorine in the meta position tended to be removed last. These or similar reductive processes are probably the principal mechanisms involved in chlorophenol degradation in anaerobic aquifer systems.

13.3 BIODEGRADATION OF HALOGENATED ORGANIC COMPOUNDS IN GROUND-WATER SYSTEMS

Microbiologic studies such as those outlined in the previous sections have demonstrated the potential for microorganisms to transform a number of halogenated organic compounds. More important, however, these studies form the basis for understanding the biochemical mechanisms involved in these transformations. The importance of such understanding is difficult to overestimate. On one hand, it provides a framework for evaluating how microbial processes may affect contaminant fate and transport in a variety of environments, including ground-water systems. On the other hand, such understanding forms the basis for developing practical in situ and ex situ bioremediation strategies.

Although laboratory studies are a necessary first step in understanding the microbial degradation of chlorinated hydrocarbons under particular conditions, field studies are also necessary. Laboratory studies can never perfectly reproduce the variety of conditions present in even the simplest ground-water system. Thus, determining the extent and rates of biodegradation processes for a particular system is best accomplished using ground-water and/or sediment chemistry data. Ideally, laboratory studies and field studies can be used in conjunction to form a better understanding of microbial processes in contaminated ground-water systems.

13.3.1 Reductive and Oxidative Biodegradation Patterns for Chlorinated Ethenes

It has been shown (Section 13.2.1) that chlorinated ethenes are subject to a variety of microbial biodegradation processes, including reductive dechlorination, oxidation, and cometabolism. The way that these different processes combine in ground-water systems determines the efficiency of biodegradation. Because ground-water systems exhibit such a variety of redox conditions, the behavior of chlorinated ethenes and ethanes is similarly varied. The following examples serve to illustrate this variety.

Biodegradation Patterns in an Oxic Aquifer, Albany, Georgia The Marine Corps Logistics Base (MCLB) at Albany, Georgia, is used to rebuild and refurbish armored vehicles. An important part of these kinds of operations is to use degreasing agents to clean machine parts. Thus, from the 1950s through the present time, large amounts of solvents have been used at this military installation. Over the years, and particularly in the 1960s, solvents were spilled or disposed of in the ground, and have migrated to the underlying ground-water system.

A schematic diagram of MCLB Albany is shown in Fig. 13.7. The base is underlain by the Floridan aquifer system, which is an important source of drinking water in Georgia. The Floridan aquifer consists of marine limestones and dolomites that were deposited in an offshore depositional environment. Because of this, these carbonate sediments contain very little organic carbon (Fig. 13.7A). Weathering and dissolution of carbonate material has formed a layer of residum that extends from land surface to a depth of approximately 50 feet at this site. This residum is underlain by a relatively fine-grained, white carbonate mud. This low-permeability carbonate mud is underlain by the highly permeable Ocala Limestone, which serves as an important source of ground water (Fig. 13.7A).

Because of the lack of natural organic matter in this aquifer, the ground water is fully aerobic and contains between 7 and 8 mg/L dissolved oxygen. In addition, concentrations of dissolved hydrogen are very low, and there is no measurable methane present in the ground water (Fig. 13.7A). These highly oxic, low-methane conditions preclude reductive dechlorination or cometabolic transformations of TCE. As a result, TCE does not biodegrade significantly at this site. This lack of biodegradation is indicated by the presence of TCE, but the lack of any observable tranformation products such as VC or *cis*-DCE (Fig. 13.7A) even though the TCE contamination has been present in the aquifer for at least 30 years. This behavior is characteristic of fully oxic ground-water systems, and has been observed at many sites around the world.

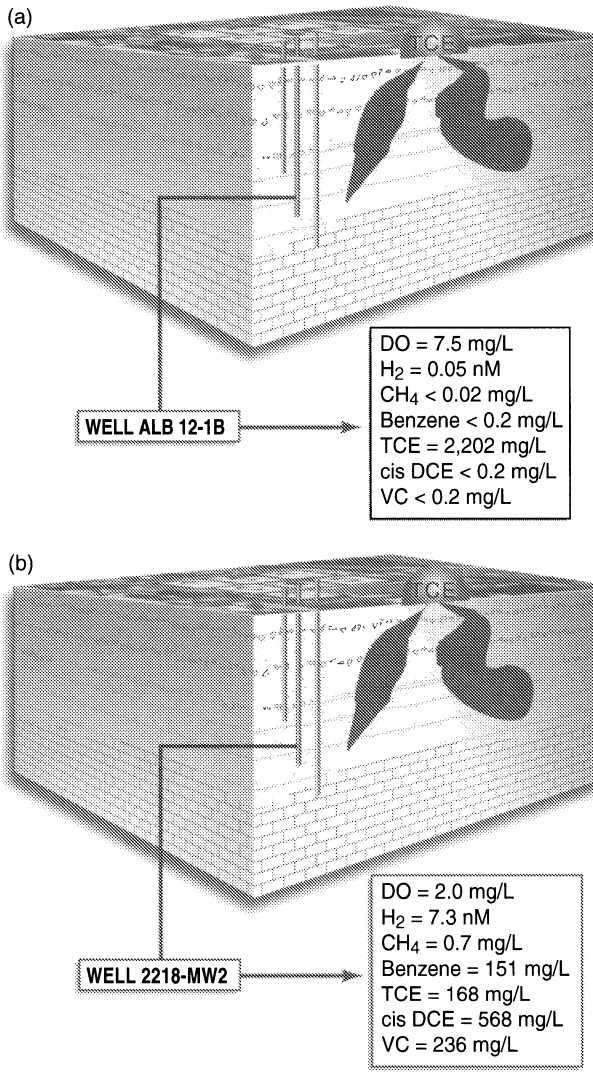


Figure 13.7 Biodegradation patterns of trichloroethene (TCE) in the Floridan aquifer (*a*) when TCE is the sole contaminant and biodegradation is negligible, and (*b*) when TCE is co-disposed with petroleum hydrocarbon contaminants and biodegradation produces dichloroethene (cis-DCE) and vinyl chloride (VC).

This observed behavior can be explained in at least two ways. The first is that the oxygen present in this system serves as a more efficient electron acceptor than TCE, and thus there is no advantage for microorganisms to transform TCE. Thus, the observed behavior of TCE can be explained by the ambient redox conditions of the site. Alternatively, it might be argued that the presence of fully oxic conditions excludes the anaerobic bacteria necessary to transform TCE. Thus, it may be that the kinds of microorganisms necessary to carry out TCE transformation are simply not present in this aquifer.

The presence of petroleum hydrocarbon contamination in some places at MCLB Albany may shed light on this question. In parts of the aquifer where BTEX compounds are found together with TCE contamination, a much different water-chemistry pattern emerges (Fig. 13.7B). Concentrations of dissolved oxygen are lower, while concentrations of dissolved hydrogen are higher. More important, in addition to TCE, the daughter products *cis*-DCE and vinyl chloride are also present. This indicates that, if a suitable organic substrate is present in an otherwise organic matter–free aquifer, biodegradation of TCE is observed to proceed. This suggests that the kind of microorganisms carrying out these transformations are present in this system in spite of the highly oxic conditions. Given a suitable organic substrate (BTEX), these microorganisms are then able to biodegrade TCE.

Biodegradation Patterns in an Oxic Aquifer Contaminated with Fuels and Solvents, Plattsburgh, New York We have seen in the previous example that biodegradation of chlorinated ethenes does not occur under fully oxic conditions, but that it can be stimulated by the addition of a suitable organic substrate such as petroleum hydrocarbons. A good example of how this general behavior can lead to highly efficient biodegradation of TCE was shown at a former Air Force Base located near Plattsburgh, New York (Bradley et al., 1998).

The former Plattsburgh Air Force Base was used to operate B-52 bombers during the Cold War. An important part of flight procedures at any airport is to train personnel in firefighting techniques. During the 1950s and 1960s, fire-training pits were operated at Plattsburgh for this purpose. In these pits, models of aircraft were doused with flammable liquids and set on fire so that firefighters could practice extinguishing them. Any kind of flammable liquid could be used for this, and over the years a mixture of waste solvents such as TCE and jet fuel were used.

Plattsburgh Air Force Base is located in a glacial outwash terrain. The glacial sediments contain very little organic matter, and the shallow ground-water system is fully aerobic under pristine conditions. However, the firefighting procedures at the fire training pits led to the release of thousands of gallons of petroleum hydrocarbons and solvents. A portion of these contaminants migrated downward, forming a lens of free product floating on the water table. The dissolution of this free product has caused an extensive plume of mixed BTEX and chlorinated solvents to form (Fig. 13.8).

Outside of the contaminant plume, concentrations of dissolved oxygen are relatively high (Fig. 13.8A) and concentrations of dissolved iron are relatively low (Fig. 13.8B). Inside the core of the plume, however, ground water is anoxic and concentrations of dissolved iron and methane (Fig. 13.8C) are relatively high. Concentrations of dissolved hydrogen in the 10 nM range indicate that methanogenic conditions predominate immediately downgradient of the free-product lens, whereas farther away from the contaminant source area hydrogen concentrations drop into the 0.5 nM range characteristic of Fe(III) reduction (Fig. 13.8D). Thus, this system is characterized by initially methanogenic conditions followed by more oxidizing Fe(III)-reducing conditions. Based solely on this observed succession of redox conditions, it would be expected that TCE would be subject to rapid reductive dechlorination near the source area, causing the formation of cis-DCE and VC. Farther downgradient, it would be expected that the Fe(III)-reducing conditions would facilitate oxidation of VC to carbon dioxide.

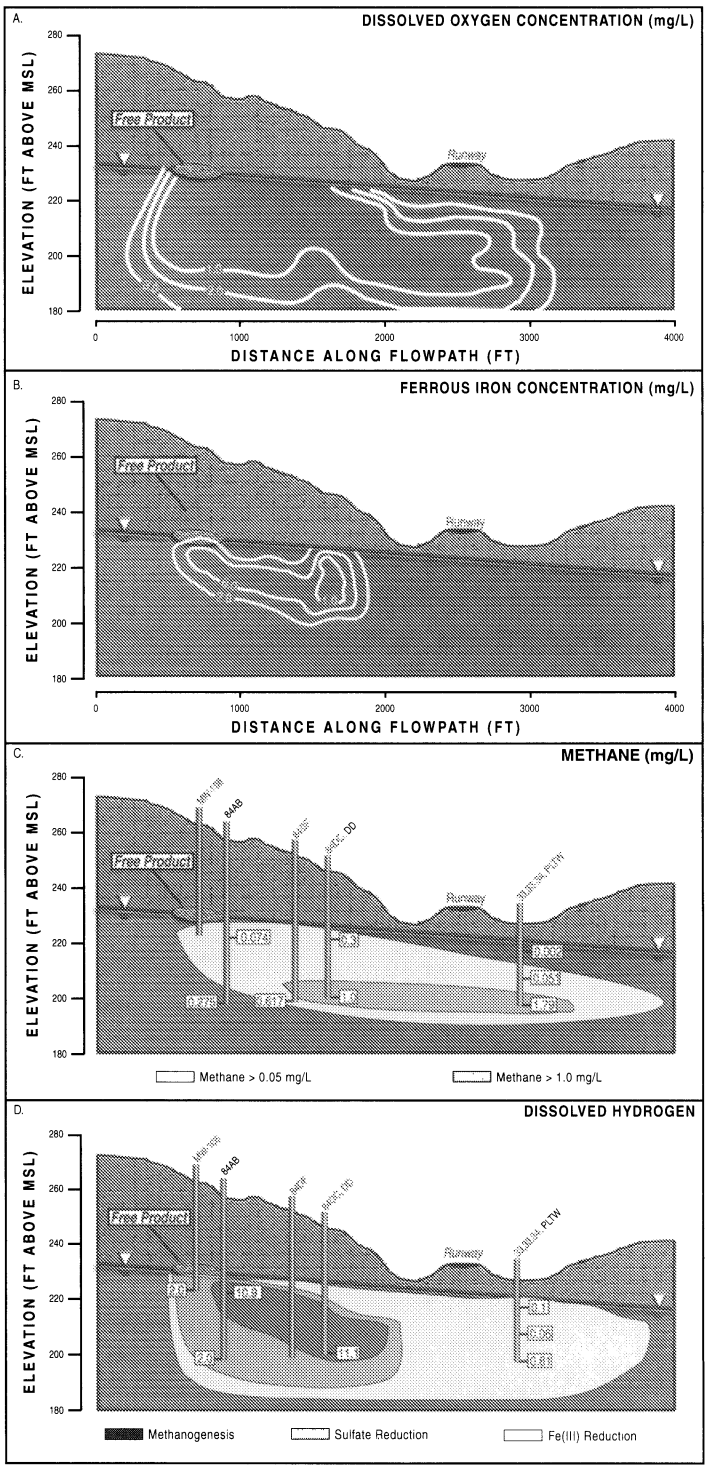


Figure 13.8 Concentrations of (*a*) dissolved oxygen, (*b*)dissolved ferrous iron, (*c*) dissolved methane, and (*d*) dissolved hydrogen in the ground-water system underlying the Plattsburgh site showing methanogenic conditions near the free product grading to sulfate-reducing and Fe(III)-reducing conditions downgradient. This progression of reducing to more oxidizing conditions is favorable to the complete biodegradation of TCE.

This predicted behavior is born out by observed concentrations of contaminants in this system. Near the source area, concentrations of TCE decrease rapidly along the flowpath (Fig. 13.9A), whereas concentrations of VC increase (Fig. 13.9B). However, once the aquifer grades into Fe(III)-reducing conditions, VC concentrations decrease rapidly (Fig. 13.9B).

These ground-water chemistry patterns clearly suggest the initial production, and then biodegradation, of VC as water moves along the hydrologic gradient. However, as is common with field data, they do not shed light on the mechanism of biodegradation. For this reason, a series of controlled laboratory studies, using sediments cored from the site, were undertaken at this site to investigate the mechanism of VC biodegradation (Bradley et al., 1998). The results of this investigation clearly showed oxidation of ^{14}C-VC to $^{14}CO_2$ (as in Fig. 13.3). These studies at Plattsburgh, New York, were the first to show that lightly chlorinated ethenes such as VC are subject to net oxidation under Fe(III)-reducing conditions in ground-water systems.

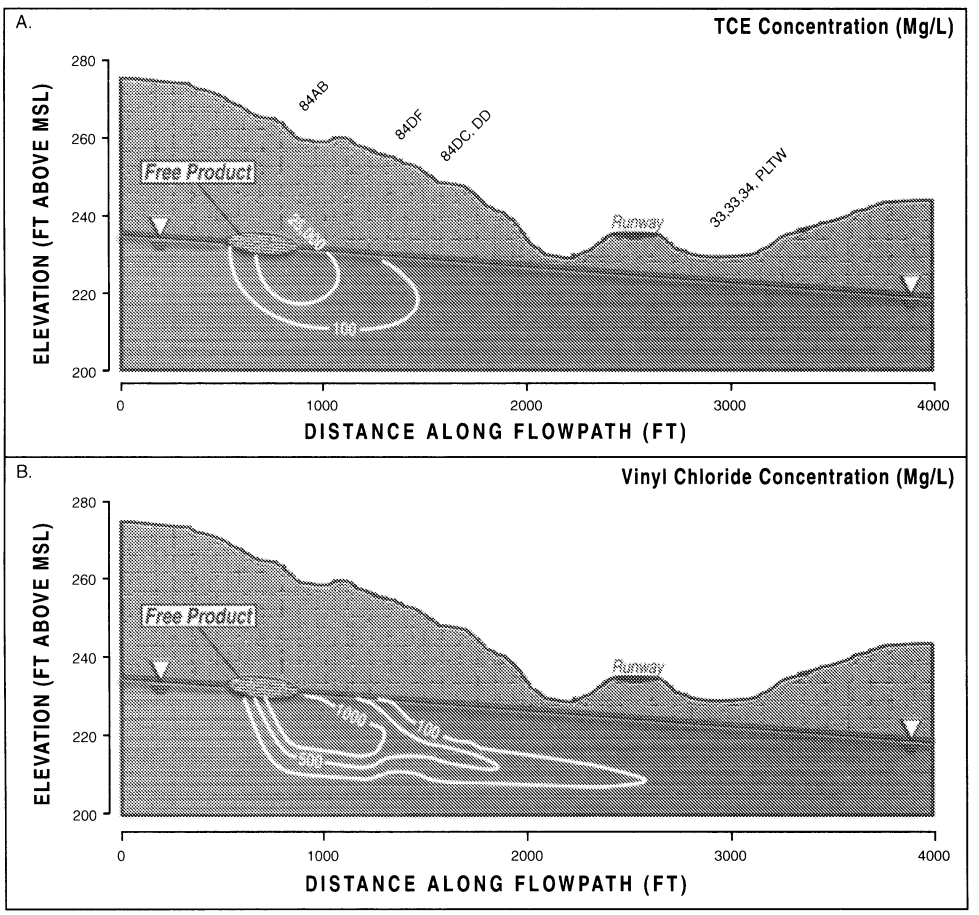


Figure 13.9 Concentrations of trichloroethene (TCE) and vinyl chloride (VC) at the Plattsburgh site. Note that TCE concentrations decrease rapidly in the highly reduced methanogenic zone and that VC concentrations decrease rapidly in the Fe(III)-reducing zone.

Biodegradation Patterns of Chlorinated Ethenes in a Naturally Anoxic Aquifer, Kings Bay, Georgia The two preceding examples show that reductive dechlorination can be driven by the presence of petroleum hydrocarbons. Experience has shown, however, that natural organic carbon present in an aquifer can also drive the initial reductive dechlorination of chlorinated ethenes and lead to relatively efficient biodegradation. One example of this behavior was observed at a site in Kings Bay, Georgia (Chapelle and Bradley, 1998).

The aquifer underlying Kings Bay, like many aquifers located near the shoreline of the Atlantic Ocean, was deposited by prograding barrier islands. On the ocean side of these barrier islands, sands are deposited under relatively high-energy beachfront conditions. This leads to the deposition of relatively clean, well-sorted sands. The lagoon sides of these barrier islands, however, are characterized by low-energy marshes and swamps that deposit finer-grained sediments containing a large amount of organic matter. As the shoreline progrades seaward, a characteristic succession of high-permeability, low-organic-matter sands overlain by low-permeability, high-organic-matter silts and clays develops organic-rich sediments, which overlie the more permeable beach sands, remove dissolved oxygen from vertically percolating recharge water so that the high-permeability sands tend to be anoxic (Fig. 13.10). These anoxic conditions, in turn, are capable of driving the reductive dechlorination of chlorinated ethenes.

This kind of behavior was illustrated by the disposal of perchloroethene (PCE) in a landfill near Kings Bay. The PCE was apparently disposed of directly into the landfill, and migrated as a dense nonaqueous liquid to the underlying anoxic aquifer. Concentrations of hydrogen, methane, iron, and sulfide (Fig. 13.11) indicate

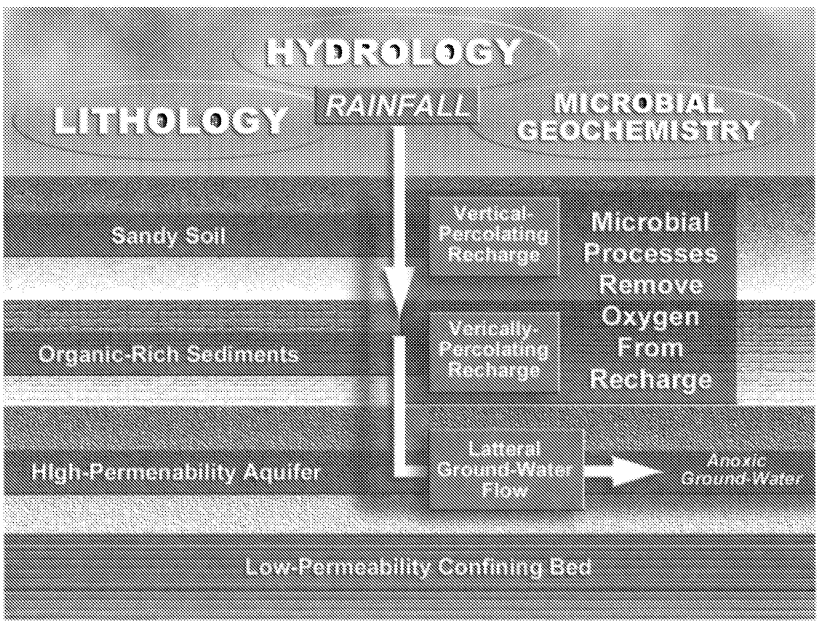


Figure 13.10 Schematic diagram showing how organic-rich sediments strip dissolved oxygen out of vertically percolating recharge at the Kings Bay, Georgia, site.

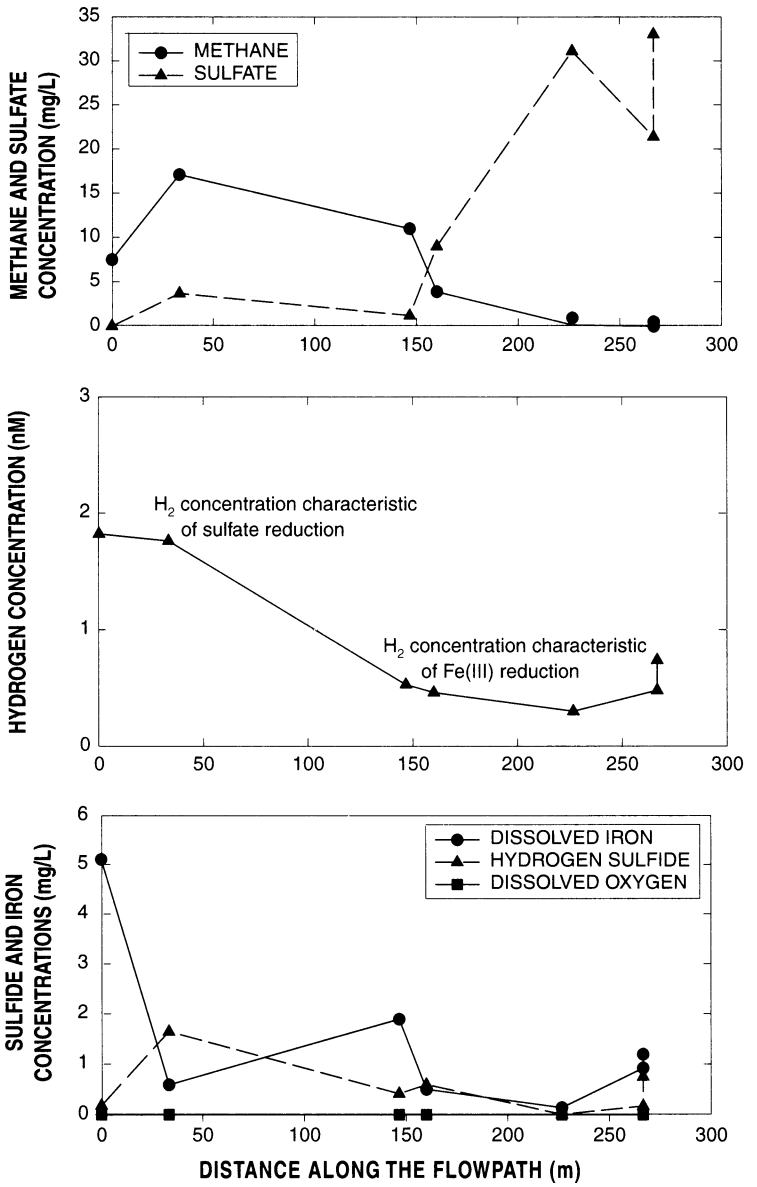


Figure 13.11 Concentrations of (*a*) methane and sulfate, (*b*) dissolved hydrogen (H_2), and (*c*) dissolved iron, hydrogen sulfide, and dissolved oxygen at the Kings Bay, Georgia site showing the progression of redox conditions from predominately sulfate-reducing near the landfill to predominantly Fe(III)-reducing downgradient (Modified from Chapelle and Bradley, 1998.)

that sulfate-reducing conditions predominate underneath the landfill, and grade to Fe(III)-reducing conditions downgradient. This pattern of reducing conditions grading to more oxidizing conditions would be expected to facilitate initial reductive dechlorination of PCE near the landfill, followed by oxidative degradation of VC downgradient of the landfill.

Concentrations of chlorinated ethenes demonstrate that this predicted behavior is in fact observed (Fig. 13.12). Near the landfill, concentrations of PCE decrease rapidly, with a concomitant increase in TCE concentrations. TCE, in turn, decrease with an increase in *cis*-DCE and VC. Finally, in the downgradient Fe(III)-reducing portion of the aquifer, concentrations of VC decrease rapidly. This site is an example of highly efficient biodegradation of chlorinated ethenes driven at least in part by the natural organic matter present in the aquifer.

The three examples given in this section illustrate those conditions that lead to either efficient or inefficient biodegradation of chlorinated ethenes in ground-water systems. In fully oxic aquifers, biodegradation is limited by the lack of reductive dechlorination. In anoxic aquifers, however, reductive dechlorination under methanogenic or sulfate-reducing redox conditions, followed by oxidation of the dechlorination products under Fe(III) or oxic conditions, leads to highly efficient degradation. These patterns are consistently observed, and show that an accurate delineation of redox processes is central to understanding the biodegradation of chlorinated ethenes in ground-water systems.

13.3.2 Cometabolic Degradation of Chlorinated Ethenes

Chlorinated aliphatic compounds such as TCA and TCE present special technical challenges to in situ methods of bioremediation. In the case of petroleum hydrocarbons, degradation is typically limited by the availability of appropriate electron acceptors or trace nutrients. However, under the aerobic conditions commonly encountered in shallow water-table aquifers, the degradation of alkyl halides is limited by the availability of particular degradative enzyme systems. Inducing indigenous microorganisms to produce the enzyme systems that are capable of actively degrading alkyl halides, and using this as the basis for in situ bioremediation, has received considerable attention.

One bioremediation strategy, and one that has attracted considerable attention in recent years, is the stimulation of indigenous populations of methanotrophic bacteria with consequent induction of their methane monooxygenase (MMO) enzyme systems. As MMOs are capable of gratuitously oxidizing alkyl halides, such stimulation could result in the large-scale degradation of these compounds in ground-water systems. The microbiologic basis for this has been well documented (Wilson and Wilson, 1985; Fogel et al., 1986); however, scaling up this process so that it can be applied to ground-water systems creates special challenges.

A field-scale evaluation of in situ bioremediation of chlorinated ethenes, which tested the feasibility of MMO induction to degrade alkyl halides, was conducted at the Moffett Naval Air Station in California (Roberts et al., 1990; Semprini et al., 1990; Semprini et al., 1991). The hydrologic system selected for study was a shallow (4–6-meter), highly permeable (K: 200 ft/d) sand-gravel aquifer that was confined (S approximately 0.00012) by overlying and underlying silts and clays.

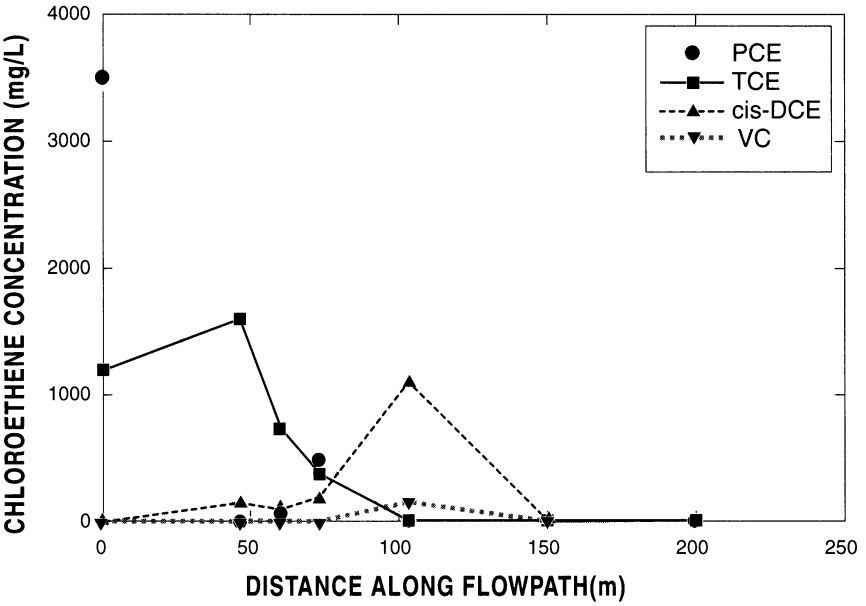


Figure 13.12 Concentrations of perchloroethene (PCE), trichloroethene (TCE), cis-dichloroethene (cis-DCE), and vinyl chloride (VC) versus distance along the flowpath of at the Kings Bay site. (Modified from Chapelle and Bradley, 1998.)

Hydrologic control of the system was maintained by a series of injection- extraction wells, and the system was monitored by a series of sampling wells.

In a series of tests that were conducted over a three-year period, the feasibility of stimulating indigenous methanotrophic bacteria by injecting oxygen and methane into the aquifer, and the efficiency of TCE, C-DCE, and T-DCE degradation were evaluated. In order to stimulate indigenous populations of methanotrophic bacteria, alternating pulses of first oxygen (−20 mg/L), followed by methane (−5 mg/L), were introduced at a rate of 1 L/min into the injection well. Water was withdrawn continuously from the extraction well at a rate of 10 L/min, and concentrations monitored at the intervening sampling wells.

The results of the first biostimulation experiment performed at this site are shown in Figure 13.13. Initially, concentrations of both methane and dissolved oxygen increased in an intermediate sampling well (S2) in response to the pulsed additions. After a lag time of about 200 hours, concentrations of both DO and methane began to decrease. This indicated the growth of methanotrophic bacteria and consequent methane oxidation. The decrease of methane concentrations to near zero after 400 hours of injection at sampling well S2 indicated that methane oxidation was most active near the injection well. This created concern that the injection well might become biofouled, so the pulse cycle was increased to 12 hours. This, in turn, increased DO and methane concentrations at well S2 at about 500 hours (Fig. 13.13A).

Once an active population of methanotrophs was established, the next phase of the experiment was to evaluate the efficiency of chlorinated ethene removal. This was accomplished by injecting TCE, *trans*-DCE, *cis*-DCE, and VC into the aquifer

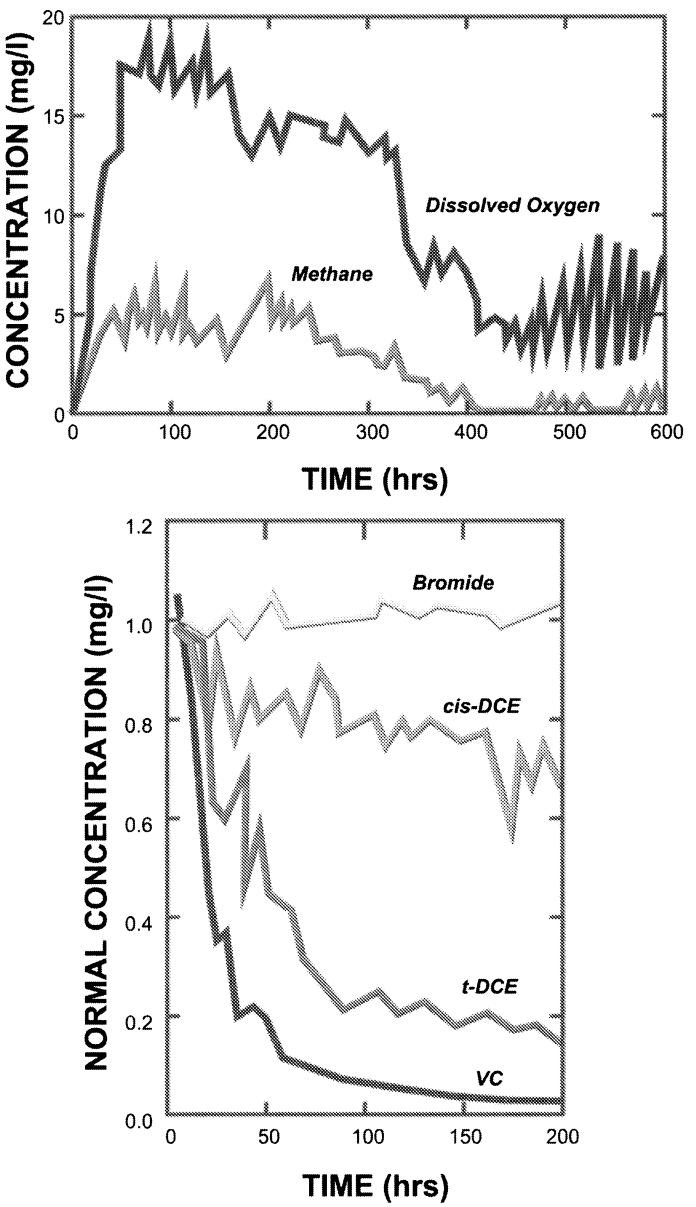


Figure 13.13 (*a*) Concentrations of dissolved oxygen and methane, and (*b*) concentrations of bromide, cis-dichloroethene (cis-DCE), trans-dichloroethene (t-DCE), and vinyl chloride (VC) in a sampling well during cometabolic in situ bioremediation of a shallow ground-water system. (Modified from Semprini et al., 1991, with permission courtesy of the National Ground Water Association.)

superimposed on the alternating pulses of DO and methane. Some results of this experiment are shown in Figure 13.13B. Concentrations of TCE decreased the least (data not shown), with measured concentrations relative to injected concentrations (C/C.) near 0.9 at the sampling point. *cis*-DCE was also transformed relatively slowly. However, *trans*-DCE and VC exhibited relatively rapid rates of transformation. In addition to demonstrating *trans*-DCE removal, it was also shown that the epoxide intermediate of *trans*-DCE oxidation, *trans*-dichloroethene oxide, appeared during these experiments.

The results of these experiments demonstrate that some degree of chlorinated ethene degradation could be attained by stimulating indigenous populations of methanotrophic bacteria. In addition, these experiments demonstrate that the more highly chlorinated compounds (TCE) are degraded less efficiently than lightly chlorinated compounds (VC). This is in general agreement with the results of laboratory experiments. The maximum TCE degradation observed was between 20% and 30% over a flowpath distance of 2 meters and a residence time of one to two days. The maximum degradation of VC, however, was 90–95% over the same flowpath segment.

Given the results of this field experiment, it becomes possible to evaluate the efficiency of methanotrophic stimulation as an in situ bioremediation strategy. Careful consideration of these results suggests several limitations of this technology. First of all, it is apparent that primary, highly halogenated compounds such as perchloroethene and trichloroethene are the least susceptible to this kind of remediation. If, under even when closely controlled, removal efficiencies are only on the order of 20–30 %, it becomes questionable whether this strategy is suitable for the less favorable conditions achievable under normal field conditions.

The second consideration is economic practicality. It is clear that the efficiency of treatment will be based on extensive hydrologic site characterization, construction of elaborate networks of injection-withdrawal wells, and construction of technologically sophisticated injection equipment. Furthermore, as not all contaminants are likely to be removed at withdrawal wells, some provisions for either effluent treatment and/or reinjection of the waste water must be made. Given the level of technology involved in this kind of strategy, there may be no clear economic advantage of in situ treatment over conventional pump-and-treat or vacuum-extraction technologies. In any case, the economic pluses and minuses of this possible technology must be carefully considered when selecting appropriate treatment methodologies.

Savannah River Site Demonstration Project The Savannah River Site in South Carolina is owned by the Department of Energy (DOE) and has been involved in nuclear weapons manufacture since it was built in the early 1940s. For many years, large amounts of degreasers and solvents, predominantly TCE and TCA, were disposed of by percolation into the ground from unlined settling ponds. These waste-disposal practices, which have since been discontinued, have extensively contaminated the Tertiary and Cretaceous aquifers that underlie the site.

Because TCE contamination is so prevalent at many DOE sites due to past waste disposal practices, DOE has invested considerable resources into investigating innovative technologies for remediating this problem. One of the technologies currently being tested is bioremediation by methanotrophic bacteria.

The bioremedation system, which was tested at the SRS by Terry C. Hazen and his associates, differs from the Moffett Naval Air Station experiments in several respects. The scale of the hydrologic system is much larger, for example. The Tertiary aquifers that have been contaminated are about 140 feet below land surface, and the zone of contamination is several hundred feet in diameter. The larger scale of this system created several technical problems. The most serious of these was to design a system of wells that could efficiently deliver oxygen and methane to the zone of contamination. Rather than drilling a large number of standard vertical wells to deliver nutrients and remove contaminated water, two long horizontal wells were constructed instead. The deeper of the two wells was used to inject air and methane into the contaminated aquifer, and the shallow one was used to extract air and contaminants from the unsaturated zone overlying the aquifer. A network of vertical monitoring wells was installed in order to assess the effectiveness of the system.

The bioremediation system was started up in the spring of 1992 with the injection of air into the saturated zone surrounding the deeper well, and the withdrawal of air from the shallower well in the unsaturated zone. Two months later, methane was added to the stream of injected air. It was quickly realized that a major challenge was to assess the relative effectiveness of biologic and nonbiologic processes in remediating the contamination. Because of the volatility of TCE, simply injecting and retrieving air will effectively remove contamination. Thus, simply documenting decreasing concentrations of TCE in the aquifer is not sufficient to show effective bioremediation.

This difficulty of assessing the relative importance of biologic from nonbiologic effects in contamination removal is common to all in situ bioremediation systems. Hazen's group addressed this problem by instituting a comprehensive microbiologic and hydrologic sampling program. The results of this sampling program showed that concentrations of TCE and numbers of methanotrophic bacteria were fairly constant during the air injection campaign. However, with the addition of methane, numbers of methano- tropic bacteria increased and concentrations of TCE declined noticeably.

These data suggest that methane addition contributed to a buildup of methanotrophic bacteria with a consequent increase in TCE biodegradation rates. However, the evidence is indirect and inconclusive. This difficulty of clearly establishing the effectiveness of biologic relative to nonbiologic processes is an unfortunate characteristic of many bioremediation systems.

13.3.3 Degradation Patterns of Alkyl Halide Insecticides

Particular alkyl halides, such as 1,2-dibromo-3-chloropropane (DBCP) and ethylene dibromide (EDB), are widely utilized as fumigants for controlling nematodes in agricultural areas. In addition, compounds such as 1,2,3-trichloropropane (TCP) (a compound more widely used as paint remover) are present as contaminants in soil fumigant preparations. Since these compounds are applied directly to the soil, it is possible for them to be leached by vertically percolating recharge and delivered to ground-water systems. Because of the relatively high solubility of EDB and DBCP relative to other chlorinated insecticides such as DDT, these compounds are more commonly found in ground water.

EDB Contamination, Hawaii One particularly well-documented case of alkyl halide contamination of ground water occurred on the island of Oahu in Hawaii (Oki and Giarnbelluca, 1987). DBCP and EDB were used extensively for nematode control in pineapple agriculture. In addition, TCP was present as a contaminant of the fumigant DD (dichloropropane-dichloropropene) and applied as well. Concentrations of EDB appear to have increased between 1983 and 1985, whereas concentrations of TCP appear to have decreased. Since these compounds are considered to be readily degradable by aerobic microorganisms, their presence might not be expected. However, a combination of geologic, hydrologic, and horticultural factors appears to speed delivery of these chemicals to the ground-water system and to depress microbial degradation once the chemicals have entered the ground water.

Pineapples must have a high water content for them to be marketable. In order to produce such high-quality pineapples, pests such as nematodes must be effectively suppressed prior to planting and the fields must be liberally irrigated. To control nematodes, the soils are fumigated once every three to five years. Because of the high rates of irrigation, however, these fairly soluble compounds are readily leached to the underlying aquifer. This aquifer consists of unweathered basalts that are highly permeable. Because of the high temperature of lava that formed the basaits (1200 °C), the rocks contain virtually no organic carbon and microbiologic activity is probably correspondingly low. Thus, even though EDB is readily degradable, degradation rates are less than ground-water flow rates. The net effect is the rapid transport of these chemicals in the subsurface and their appearance in ground-water supplies.

Pesticide Contamination, Long Island Although DDT is relatively insoluble, the large amounts of this compound applied to agricultural areas have resulted in its being found in ground-water supplies. On Long Island, New York, for example, DDT, DDD, and DDE have been reported in ground water sampled in Suffolk County. Reported concentrations, however, were very low, in the parts per billion range. The most significant aspect of these data is that they demonstrate that production and accumulation of the DDD and DDE, which would be expected based on laboratory studies of DDT degradation, are actually observed in field studies.

13.3.4 Degradation Patterns of Chlorobenzenes

Chlorobenzenes can be delivered to ground-water systems by a number of different avenues and are readily degraded under aerobic conditions (via hydroxylation to chlorocatechol, with subsequent ortho cleavage) and less readily degraded anaerobically via reductive dechlorination.

The behavior of chlorobenzenes in aerobic aquifers seems to be consistent with what would be expected based on laboratory studies. The classic example of this was given by Schwarzenbach et al. (1983), who studied the infiltration of contaminated surface water into a shallow ground-water system in Switzerland. A series of observation wells were placed so that concentration changes of trichloroethylene (TCE) and 1,4-dichlorobenzene (DCB) could be monitored as ground water flowed downgradient. Concentrations of TCE changed very little along the flowpath of this aerobic aquifer, which is consistent with the general observation that nonmethan-

otrophic aerobic metabolism does not appreciably transform TCE. Concentrations of DCB, however, showed a consistent and significant trend of decrease along the flowpath. Since DCB is chemically stable and not strongly sorbed on aquifer material, these patterns were attributed to aerobic microbial degradation. It is likely that degradation of chlorobenzenes is widespread in aerobic aquifers.

The behavior of chlorobenzenes in anaerobic aquifers, however, appears to be much different. In one study of a sewage effluent–contaminated anaerobic aquifer, DCB was shown to persist for a minimum of 20 years (Barber, 1988). However, because denitrification was the predominant terminal electron-accepting process in this system, it is difficult to generalize these results for other anaerobic redox processes, such as FE(III) reduction or methanogenesis. It may simply be that microbially mediated reductive dehalogenation of DCB is less efficient under denitrifying conditions. However, this question has yet to be systematically studied in the laboratory.

A more recent study of anaerobic chlorinated benzene biodegradation in ground water was carried out at a waste-water treatment plant at Naval Air Station Pensacola. The contaminant source at this site was a series of unlined drying beds used for sludge generated by industrial wastes. Chlorinated benzenes were an important component of the waste stream, and were present in the sludge generated by the wastewater treatment plant. As the sludges dewatered in the drying beds, significant amounts of chlorinated benzenes were delivered to the underlying shallow water table aquifer. This shallow aquifer was highly anaerobic due to a combination of natural organic matter present in the aquifer, and due to dissolved organic carbon that leached from the sludge.

Concentrations of chlorinated benzenes plotted versus distance along the flowpath in the core of the contaminant plume are shown in Figure 13.14A. This shows that, although concentrations of tri- and di-chlorobenzene decrease along the flowpath, this concentration decrease is approximately what would be expected due solely to hydrodynamic dispersion. The only suggestion that reductive dechlorination of tri- or dichlorobenzene is occurring is that concentrations of monochlorobenzene apparently increase slightly along the flowpath (Fig. 13.14A). The observed behavior of chlorinated benzenes in this system is in marked contrast to the behavior of chlorinated ethenes, which rapidly dechlorinate along aquifer flowpaths (Fig. 13.14B). Thus, while it is possible that reductive dechlorination or anaerobic oxidation of chlorinated benzenes occurs in this system, these processes are much less efficient than reductive dechlorination and anaerobic oxidation of chlorinated ethenes.

13.3.5 Degradation of Chlorinated Herbicides

Atrazine is one of the most commonly detected contaminants of ground water in agricultural areas. For example, in a study of 2,263 wells in Nebraska, 12.5% were found to contain atrazine concentrations ranging from 10 to 0.01 tLg/L (Spalding, Burbach, and Exner, 1989). The occurrence of atrazine in ground water reflects both high rates of usage (about 20 million lbs/yr in Nebraska alone) and its relatively high solubility (32 mg/L).

In spite of the tremendous usage of atrazine as a preemergent herbicide for corn production during the 1980s, it is significant that average concentrations in in-

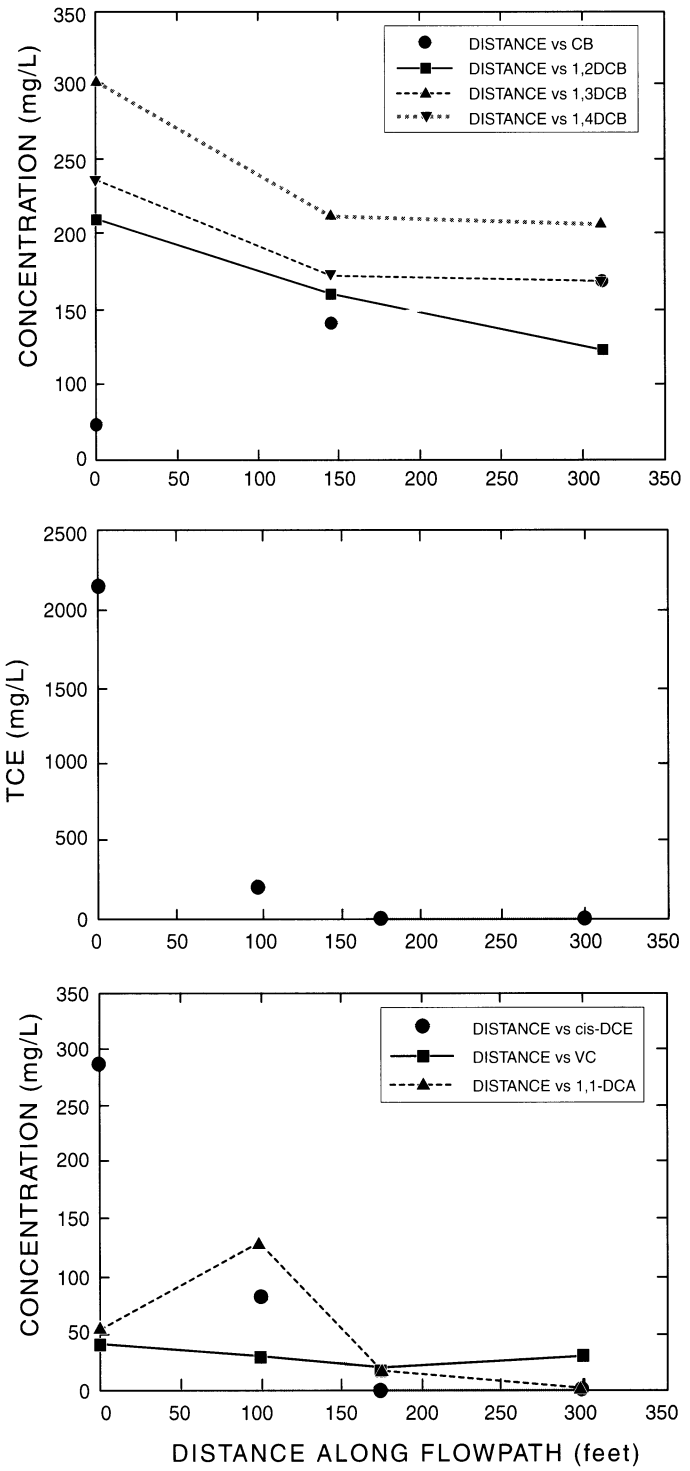


Figure 13.14 (*a*) Concentrations of chlorobenzenes, (*b*) trichloroethene (TCE), and (*c*) cis-dichloroethene (cis-DCE), vinyl chloride (VC) and 1,1-dichloroethane (1,1-DCA) versus distance along the flowpath in an anaerobic shallow aquifer, Pensacola, Florida. Note that chlorobenzene biodegradation is much less apparent than chloroethene biodegradation.

tensely irrigated areas do not seem to be increasing over time. This implies that atrazine transport to these ground-water systems is approximately at steady state with rates of degradation. Atrazine degradation in aquifer systems can be considered as the sum of both biologic and abiologic processes such as irreversible sorption. However, based on its relatively low octanol-water partition coefficient, it seems unlikely that sorption is the sole process leading to removal from aqueous solution. This raises the possibility that microbial degradation in ground-water systems may be significant.

The microbial degradation of atrazine in soils has been well-documented (Behki and Khan, 1986). The potential for atrazine degradation in aquifer sediments is less clear. The first reported study of atrazine degradation by subsurface sediments (Sinclair and Lee, 1990) showed little or no atrazine degradation in aquifer sediments from Oklahoma. Similarly, Konopka and Turco (1991) were unable to detect atrazine degradation in slurries of aquifer sediment from Indiana that were incubated for as long as 128 days.

The first laboratory evidence for microbial degradation of atrazine in aquifer sediments was provided by McMahon et al. (1992). In a series of experiments, it was shown that ^{14}C ethyl-labeled atrazine was slowly degraded to $^{14}CO_2$ under aerobic conditions, whereas ^{14}C ring-labeled atrazine was not significantly degraded. The aquifer sediments used in this study were cored from a coarse-grained alluvial aquifer underlying an active cornfield in Ohio. Despite the long-term heavy usage of atrazine on the cornfield, monitoring well data never reported measurable concentrations of atrazine. However, measurable concentrations of deethylatrazine were found. Thus, based on both laboratory and field evidence, it appears that atrazine in this ground-water system is subject to partial microbial deethylation. Complete degradation of the atrazine molecule, however, appears to be more sluggish.

Taken together, the available evidence from field studies (Spalding et al., 1989) and laboratory studies (Sinclair, 1990; McMahon et al., 1992) suggests that microbial processes in ground-water systems are partially capable of degrading atrazine at a finite but slow rate. Because of the apparently slow rates, steady-state concentrations of atrazine may be fairly high (1–10 gg/L) in aquifers receiving high rates of atrazine-laden recharge. However, for most aquifer systems, atrazine contamination of ground water is unlikely to persist due to microbially mediated deethylation reactions (Fig. 13.6).

13.3.6 Degradation of Chlorophenolic Compounds

The microbial degradation of phenolic compounds in ground-water systems was one of the first aspects of subsurface microbiology to be systematically investigated. Ehrlich et al. (1982) reported on the methanogenic degradation of phenols associated with coal tar wastes in a shallow aquifer system, and Godsy et al. (1983) investigated the methanogenic degradation of these compounds in laboratory digesters. Similarly, the degradation of chlorinated phenols associated with creosote wastes in a shallow sand and gravel aquifer was described by Troutman et al. (1984).

An interesting aspect of these early studies, particularly that of Troutman et al. (1984), is that consideration of ground-water chemistry and hydrology clearly in-

dicated the importance of microbial processes in degrading the phenolic and chlorophenolic compounds prior to laboratory evidence to that effect. The site studied by these investigators was the American Creosote Works (ACW) site in Pensacola, Florida. At this site, telephone poles were treated with wood preservatives. Prior to 1950, creosote was used exclusively for this purpose. After 1950, pentachlorophenol (PCP) was increasingly utilized and reached about 25,000 gallons per month by the time the plant was closed in 1981.

After being used to treat logs, the creosote and PCP were collected in a recirculation impoundment. An overflow impoundment served to provide additional storage when needed. Leakage from these impoundments resulted in extensive contamination of ground water at this site (Fig. 13.15). Near the impoundments, concentrations of total phenols exceeded 20 mg/L, and concentrations of PCP were in the 1 mg/L range.

Troutman et al. (1984) closely examined the ground-water hydrology of the ACW plant and its vicinity. They determined that the mean horizontal groundwater flow velocities ranged from a maximum of 700 ft/yr in the shallow parts of the system to 200 ft/yr in the deeper parts. Using the minimum flow velocities and

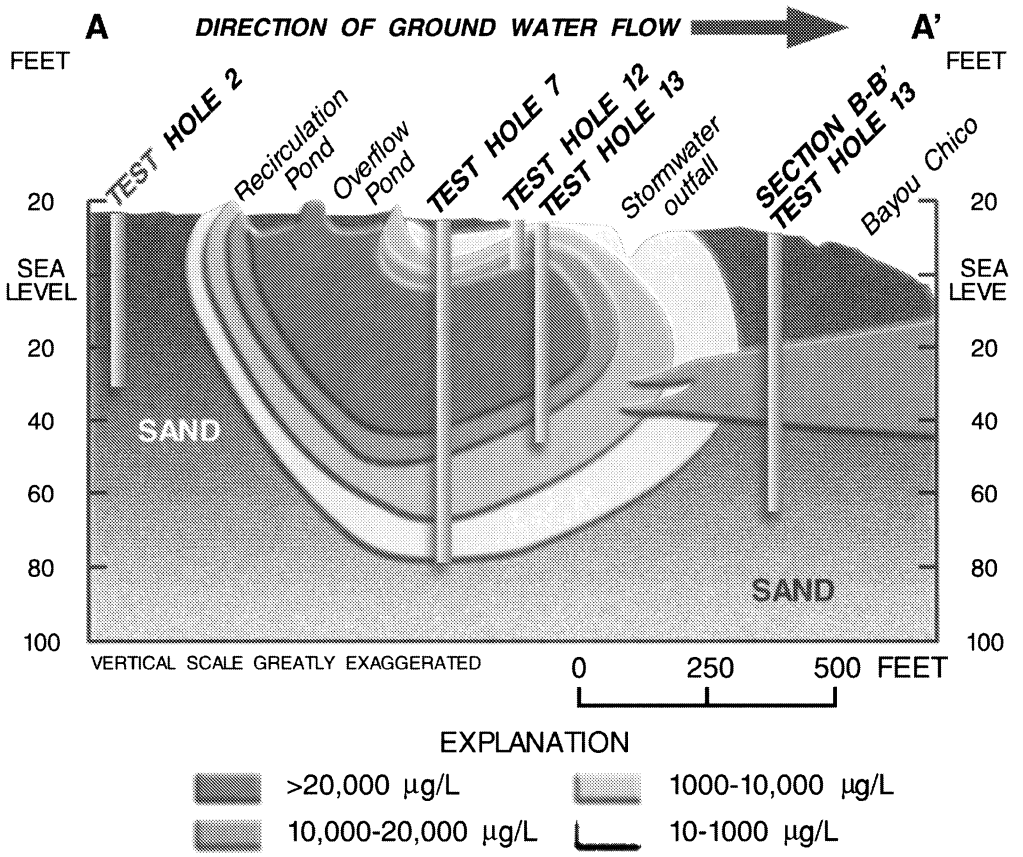


Figure 13.15 Phenol contamination at the American Cresote Works Plant, Pensacola, Forida. (Modified from Troutman et al., 1984.)

assuming that waste disposal had continued for 80 years, then a plume of phenolic compounds up to 16,000 feet long could be expected downgradient of the impoundments. Instead, however, extensive ground-water monitoring documented that phenolic compounds were undetectable in ground water more than 1,000 feet downgradient (Fig. 13.15). This considerable discrepancy could not be explained by either hydrologic or geochemical mechanisms. It was concluded, therefore, that microbial degradation of phenolic compounds was the principal attenuation mechanism.

In support of this hypothesis, Troutman et al. (1984) showed that ground water from downgradient of the impoundments contained elevated counts of methanogenic bacteria. In addition, the ground water contained relatively high concentrations of methane, suggesting that methanogenesis was the predominant terminal electron accepting process at this site. Finally, it was shown that phenols were degraded in laboratory digesters maintained under methanogenic conditions. Interestingly, however, PCP was not degraded in the digesters, and it was suggested that PCP inhibited phenol degradation when present in concentrations greater than 0.25 mg/L.

Additional work by Mueller et al. (1991) reached similar conclusions regarding the fate of PCP at the ACW site. These investigators incubated samples of ground water in shake flasks and monitored the degradation of a number of organic compounds over time. The results of these studies were largely consistent with the results of Troutman et al. (1984). Whereas phenols, polyaromatic hydrocarbons (PAH; groups 1, 2, and 3 PAHs contain 2, 3, and 4 fused benzene rings, respectively), and N-, S-, and O-bearing heterocyclic compounds were actively degraded, there was no measurable degradation of PCP. The degradation of PCP under anaerobic conditions in ground-water systems has, therefore, yet to be demonstrated in the laboratory. Circumstantial evidence, such as PCP not extending beyond the phenolic plume, suggests that degradation does occur.

13.4 SUMMARY

Chlorinated organic compounds are widely used for industrial and agricultural purposes. Alkyl halides such as trichloroethylene (TCE) are used as solvents and cleaning agents. Aryl halides such as dichlorobenzene are used for household and industrial deodorizing. Other chlorinated organic compounds are used as insecticides, herbicides, antifungal fumigants, dielectric agents, lubricants, and a host of other applications. It is fairly safe to assert that modern industrial society could not function without the use of these chemicals. As a result of their wide usage, however, these compounds have contaminated a large number of ground-water systems and represent a significant health hazard in some cases.

Microorganisms are capable of transforming chlorinated organic compounds under a wide range of environmental conditions. However, the mechanisms, pathways, and rates of degradation vary widely and depend largely on the structure of the chemical. Alkyl halides such as TCE are not significantly degraded by aerobic heterotrophic bacteria. Consequently, alkyl halides tend to persist in aerobic ground-water systems. These compounds are, however, degraded by the methane monooxygenase enzyme present in methanotrophic bacteria. Methanotrophs cannot

obtain energy for growth by TCE oxidation, and methane competitively inhibits this reaction. Nevertheless, stimulation of methanotrophic activity is presently being evaluated as a potential in situ and ex situ bioremediation strategy. Under anaerobic conditions, reductive dehalogenation reactions are the most important alkyl halide-degrading processes. In electron-donor-poor ground-water systems, however, reductive dehalogenations tend to be incomplete, resulting in the accumulation of vinyl chloride.

Reductive dehalogenation reactions may also be important microbial processes involved in degrading aryl halides. Unlike alkyl halides, however, aryl halides are capable of supporting the growth of heterotrophic aerobic bacteria. Consequently, aryl halides tend to be rapidly degraded in aerobic ground-water systems. While reductive dehalogenation reactions have been shown in laboratory studies to degrade halogenated benzoates, the importance of reductive dehatogenation of aryl halides in anaerobic ground-water systems is not clear. Studies have reported that dichlorobenzenes persist for long periods of time in some anaerobic ground-water systems.

Chlorinated pesticides vary widely in their susceptibility to microbial attack in ground-water systems. Some insecticides such as DDT are recalcitrant and degrade only partially. As a result of this recalcitrance, DDT has been virtually banned from agricultural practice in the United States. Herbicides such as atrazine are widely used in agricultural practice and are biodegradable in the soil zone. However, degradation rates of atrazine in nutrient-poor aquifer systems is relatively slow and appear to be limited to deethylation reactions. Thus, if rates of atrazine delivery to shallow ground-water systems exceeds rates of degradation, atrazine can accumulate in solution.

Contamination of ground-water systems by phenolic compounds is fairly common and often accompanies wood-treatment operations. Creosote and, later, pentachlorophenol (PCP) were widely used as wood preservatives, and wastes from these operations often reach shallow ground-water systems. Phenolic compounds are rapidly degraded in ground-water systems under either aerobic or anaerobic conditions. However, the most common chlorinated phenol, pentachlorophenol, appears to be much more recalcitrant. Nevertheless, field evidence suggests that degradation does occur in some systems.

Available microbiologic and biochemical evidence is sufficient to explain many patterns of chlorinated hydrocarbon degradation observed in ground-water systems. However, because field conditions typically involve multiple contaminants, the presence of inhibiting chemicals or competing substrates, and other factors that make microbial processes difficult to evaluate, it is clear that much more field-oriented research is needed before a consistent picture of degradation in ground-water systems can emerge.

QUESTIONS TO CONSIDER

1. Trichloroethene was considered to be nonbiodegradable in the early 1970s. What observations from ground-water systems changed that thinking in the late 1970s?

2. Why was reductive dechlorination initially thought to be an exclusively cometabolic process? What evidence eventually changed that thinking?
3. Aerobic cometabolism of chlorinated ethenes involves what enzyme system commonly found in soil bacteria?
4. Why is reductive dechlorination of chlorinated ethenes observed to me more efficient under methanogenic conditions than under Fe(III)-reducing conditions?
5. It has been shown that vinyl chloride is subject to a net oxidation even under strongly reducing, methanogenic conditions. What class of fermentative microorganisms is involved with these degradation processes?
6. What succession of oxidation/reduction processes in ground-water systems facilitates the complete biodegradation of trichloroethene to carbon dioxide and chloride? How commonly encountered are these conditions?
7. Do chlorobenzenes exhibit the same biodegradation processes as chlorinated ethenes? What important differences are observed?
8. What electron donor has been shown to drive reductive dechlorination of chlorinated ethenes? Is this electron donor present at the same concentrations in all ground-water systems? How does this affect biodegradation efficiency?
9. What set of environmental conditions makes the natural attenuation of chlorinated ethenes highly efficient?
10. What set of environmental conditions makes the natural attenuation of chlorinated ethenes highly inefficient?

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