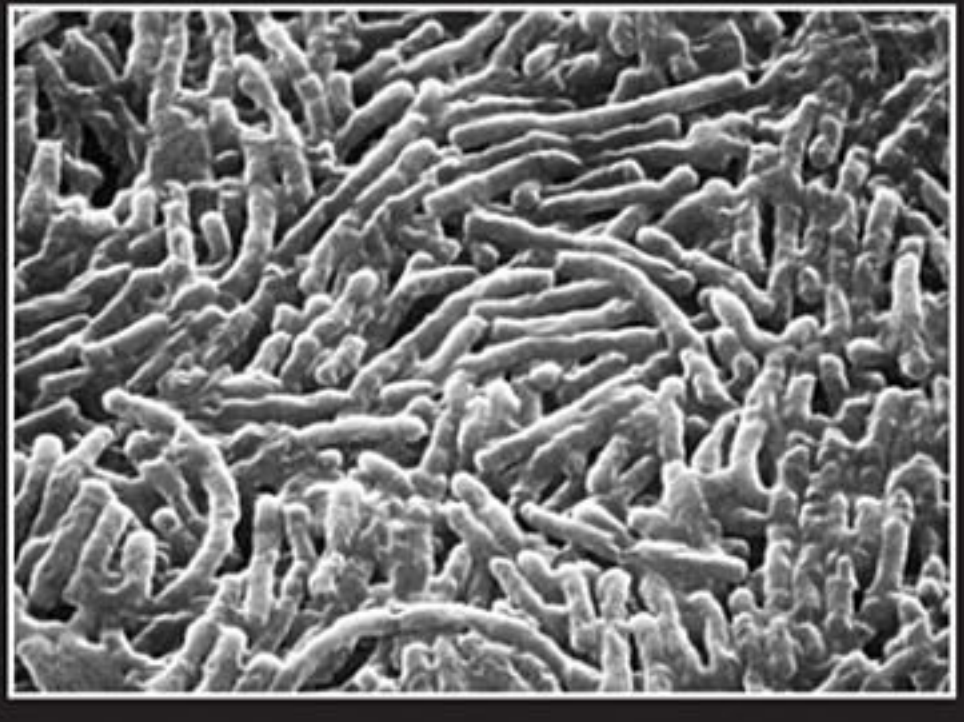
ENVIRONMENTAL MICROBIOLOGY

Second Edition



Edited by
RALPH MITCHELL AND JI-DONG GU

WILEY-BLACKWELL

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PREFACE

It is more than fifteen years since the publication of the last edition of *Environmental Microbiology*. During that time there have been momentous advances in this field both conceptually and experimentally. For example, we have become increasingly aware of the involvement of microbial processes in climate change. New molecular techniques now permit much more accurate identification of the microorganisms and processes involved in both environmental deterioration and remediation.

In this volume we focus on the role of microorganisms in a wide range of ecosystems and deterioration processes. We cover such diverse subjects as the role of microorganisms in the deterioration of cultural heritage materials and the effects of genetically modified crops on microbial processes. In addition to providing historical reviews of their subject, we have asked contributors to speculate on future trends. Our objective in the volume is to further our understanding of the essential role played by microorganisms in both environmental deterioration and the control of pollution. We hope that this book will be helpful to a wide range of scientists and engineers, and will stimulate students to new and original approaches to environmental challenges.

R.M. would like to thank Trinity College, Dublin, Ireland and, particularly, Robin Adams, the director of the college library. Much of the work for this book was carried out in the Trinity College library. Both the help of the librarians and the excellent electronic resources were an enormous help in the production of this book.

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CHAPTER 1

Bacteria in the Greenhouse: Marine Microbes and Climate Change

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1.1 INTRODUCTION: A MICROBIAL OCEAN IN A WARMING WORLD

The global ocean covers 70% of Earth's surface and comprises most of the volume of the biosphere (except the deep subsurface). It supports about half the annual net primary production (NPP) on the planet (Figure 1.1) (Field et al., 1998). This vast, interconnected network of marine ecosystems is warming in response to anthropogenic climate change, with uncertain consequences for human societies. In this chapter we address the possible responses of ocean warming on marine microbes (protists, phytoplankton, bacteria, and archaea, with emphasis on the bacteria). Other anthropogenic changes related to CO_2 accumulation in the atmosphere, such as ocean acidification (Orr et al., 2005), will also have uncertain effects on ocean microbes.

The ocean is, and always has been, dominated by microbes. Microscopic unicellular phytoplankton and cyanobacteria inhabiting the sunlit upper approximate 100 m of the water column carry out nearly all the photosynthesis on which oceanic life depends (Falkowski et al., 1998, 2000). NPP on land and in the oceans is the process dominating solar energy and CO_2 fixation into organic matter, thus driving the global carbon cycle (Houghton, 2007). Nearly all of the approximately 50 Pg (1 petagram = 1 Pg = 10^{15} g = 10^9 tons) of carbon fixed annually in marine photosynthesis is ultimately oxidized by bacterioplankton, protozoans, and zooplankton (Ducklow and Carlson, 1992) with a very small fraction (<0.1%) escaping heterotrophic metabolism in the deep water column to be buried in the sedimentary record and hydrocarbons. The myriad pathways by which marine organic matter is cycled through particulate and dissolved forms and

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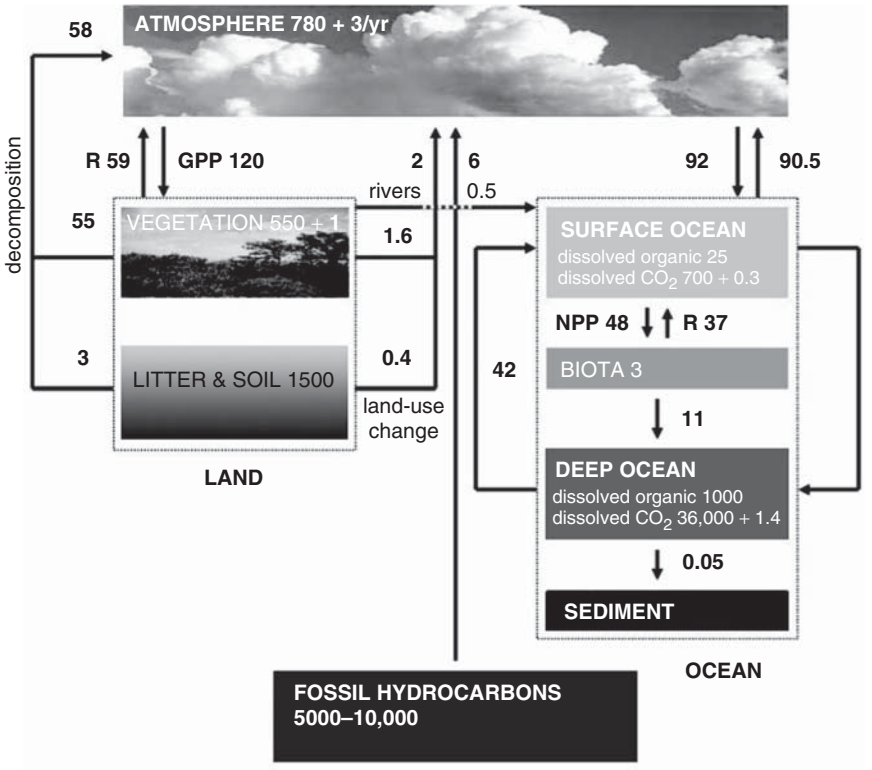

Figure 1.1 The global carbon cycle, including human perturbations in the 1990s. The quantities in the boxes are the size of the carbon reservoir in petagrams (Pg; 10^{15} g), with the annual growth, if any, due to the perturbations. Note that there is direct exchange between the atmosphere and terrestrial ecosystems, whereas exchange with the ocean is mediated by the physicochemical exchange across the air–sea interface. The downward transport of organic carbon, both particulate and dissolved, constitutes the biological pump. There is a riverine input of about 0.5 Pg from the land to ocean, balanced by outgassing and burial in sediments. Currently, the annual net land sink for atmospheric CO_2 is 1 Pg and the ocean sink is 2 Pg, leaving an annual net anthropogenic accumulation in the atmosphere of 3.2 Pg. (Modified from Houghton, 2007.) (See insert for color representation.)

back into CO_2 overwhelmingly involve microbial exchanges among organisms less than 1 to 5 μm in diameter—the microbial loop (Pomeroy, 1974; Azam et al., 1983; Azam and Worden, 2004; Azam and Malfatti, 2007). This large-scale view of global biogeochemistry makes the point that microbial ecology depends ultimately on the patterns and products of photosynthesis. In metabolizing the products of photosynthesis on land and in the sea, bacteria perform important ecosystem services, such as decomposition, nutrient cycling, regulating the composition of the atmosphere, enhancing soil fertility, and purifying water, on which human societies depend for healthy and sustainable existence (Ducklow, 2008). Here we review marine plankton and microbial ecology to understand how marine bacteria may respond to anthropogenic climate change, and suggest potential research directions for making more informed projections.

Bacteria respond directly to changes in environmental temperature, but these responses occur in complex communities with phytoplankton and zooplankton and in

a complex biogeochemical milieu. After reviewing the microbial loop to set the stage for a more detailed look at the connections between climate and plankton processes, we take two complementary approaches. First, we examine how bacterial activity varies as a function of temperature. Then we examine how marine phytoplankton respond to climate variability in the ocean and how these responses modulate the effects of climate change on bacterial and animal consumers. We have a reasonable understanding of the mechanisms by which phytoplankton (especially eukaryotes) will respond to climate change, based on physical theory and knowledge of past changes from the fossil record. But as Falkowski and Oliver (2007) stated in their review of phytoplankton and climate, "Whether this fundamental principle holds for marine prokaryotes remains to be seen, because the spatial and temporal distribution of prokaryotic taxa, as well as their relevant ecophysiological attributes, is not yet well characterized." Our knowledge of the mechanisms linking phytoplankton to bacterial variability is still fragmentary, but much new research is directed at this problem. We use this nascent understanding to suggest how bacterial carbon cycling may change and what knowledge is needed for better predictions of such changes. Finally, we synthesize these approaches and consider scenarios of how microbial communities will respond (or may already be responding) to climate change in the coastal seas, cold polar seas, and in warm oligotrophic subtropical gyres. First, we sketch briefly the projected physical changes to the global oceans in response to anthropogenic climate forcing.

1.1.1 Impact of Climate Change on the Oceans

The world is warming in response to climate change driven by the accumulation of anthropogenic greenhouse gases (Kerr, 2007). The 2007 IPCC Assessment projects that the mean global surface atmospheric temperature will rise by 1 to 6°C by 2099, depending on various assumptions or scenarios of population growth, economic and technological development, energy use, and greenhouse gas emissions (IPCC, 2007b). The projected warming is not uniform in space or time, with different rates forecast for various regions and seasons. Surface ocean temperatures may rise by 3 to 7°C in some regions, with the largest increases (although not necessarily the largest *effects*; see below) in polar seas (Figure 1.2). Climate warming will take longer to percolate into the deep ocean. The surface ocean west of the Antarctic Peninsula has already increased by 1°C since the 1950s (Meredith and King, 2005), and Arctic sea ice has declined alarmingly in the past few years (Serreze et al., 2007). Enhanced greenhouse warming is projected to cause impacts ranging from changes in winds, clouds, sea level, precipitation, storm frequency and intensity to more complex alterations in long-term climate modes (e.g., ENSO), ecosystems, biodiversity, and human well-being (Millennium Ecosystem Assessment, 2005; IPCC, 2007a). The impact of changes in surface temperatures, clouds, and wind on ocean stratification, mixing, and circulation has been examined using coupled atmosphere–ocean general circulation models (AOGCM) (Meehl et al., 2007). In addition to atmospheric warming, major changes predicted by many different AOGCMs include increases in ocean surface stratification in the tropics and subtropics; and reductions in mixed-layer depth in the middle to upper latitudes (Figure 1.3; Boyd and Doney, 2002), Other changes include loss of sea ice in both hemispheres (especially in the north), and increases in westerly winds and coastal upwelling (Sarmiento et al., 2004). As the temperature fields shown

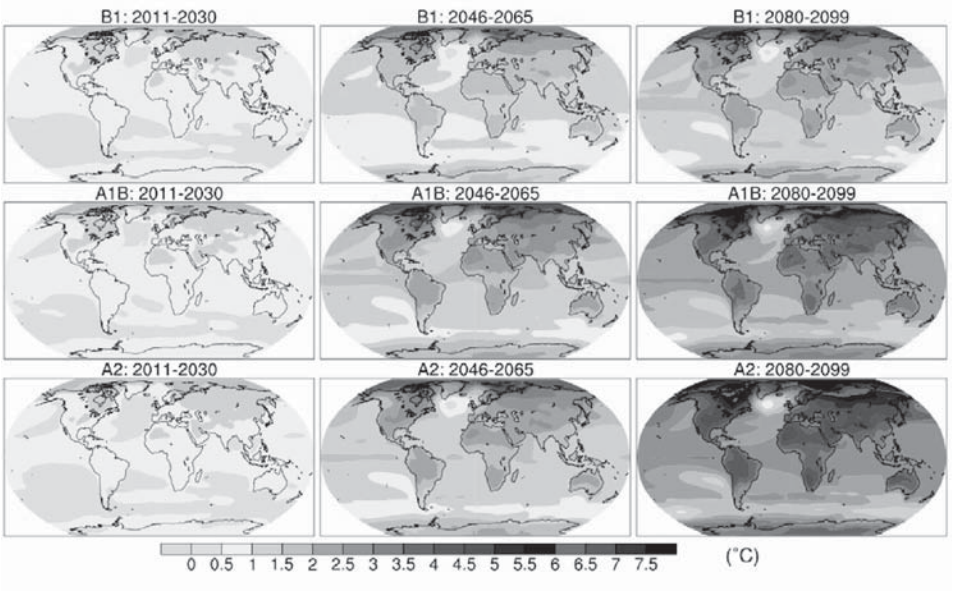

Figure 1.2 Multimodel mean of annual mean surface warming (surface air temperature change, °C) for scenarios B1, A1B, and A2, and three time periods, 2011 to 2030 (left), 2046 to 2065 (middle), and 2080 to 2099 (right). Stippling is omitted for clarity (see the text). Anomalies are relative to the average of the period 1980–1999. (From IPCC, 2007b, with permission of the IPCC, http://www.ipcc.ch/graphics/graphics.htm.) (See insert for color representation.)

in Figures 1.2 and 1.3 indicate, projected changes are far from uniform. Specification of the changes in any particular region is more uncertain than projections of the global means. Responses of ocean ecosystems and biogeochemistry are less certain than physical changes.

1.1.2 The Microbial Loop and Marine Bacterioplankton Communities

A typical milliliter of seawater harbors about 10^6 bacterial and archaeal cells, the great majority of which are revealed to be active based on fluorescent in situ hybridization and visualization of intact ribosomes (Church et al., 2003). This assemblage contains extraordinary genetic and metabolic diversity (Venter, 2004; Sogin et al., 2006), and with it the potential for adaptation to wide ranges and combinations of environmental conditions. The planktonic archaea make up a variable fraction of the prokaryote assemblage, comprising up to about 50% of the total abundance in deep-ocean waters, but less in the more active surface layer (Karner et al., 2001). Recent work has revealed new insights concerning the metabolism and ecological roles of planktonic GI marine crenarchaeota and GII marine euryarchaeota. Collective evidence suggests that perhaps a significant portion of the GI marine crenarchaeota are ammonia oxidizers (Francis et al., 2005; Konneke et al., 2005; Hallam et al., 2006; Wuchter et al., 2006), while the first environmental genomic sequence from a GII marine euryarchaeote identified a proteorhodopsin-encoding gene (Frigaard et al., 2006). Both of these metabolisms are consistent with the ecological distributions of these organisms in the ocean, where the GI marine crenarchaeota are typically found below the photic zone, and the GII

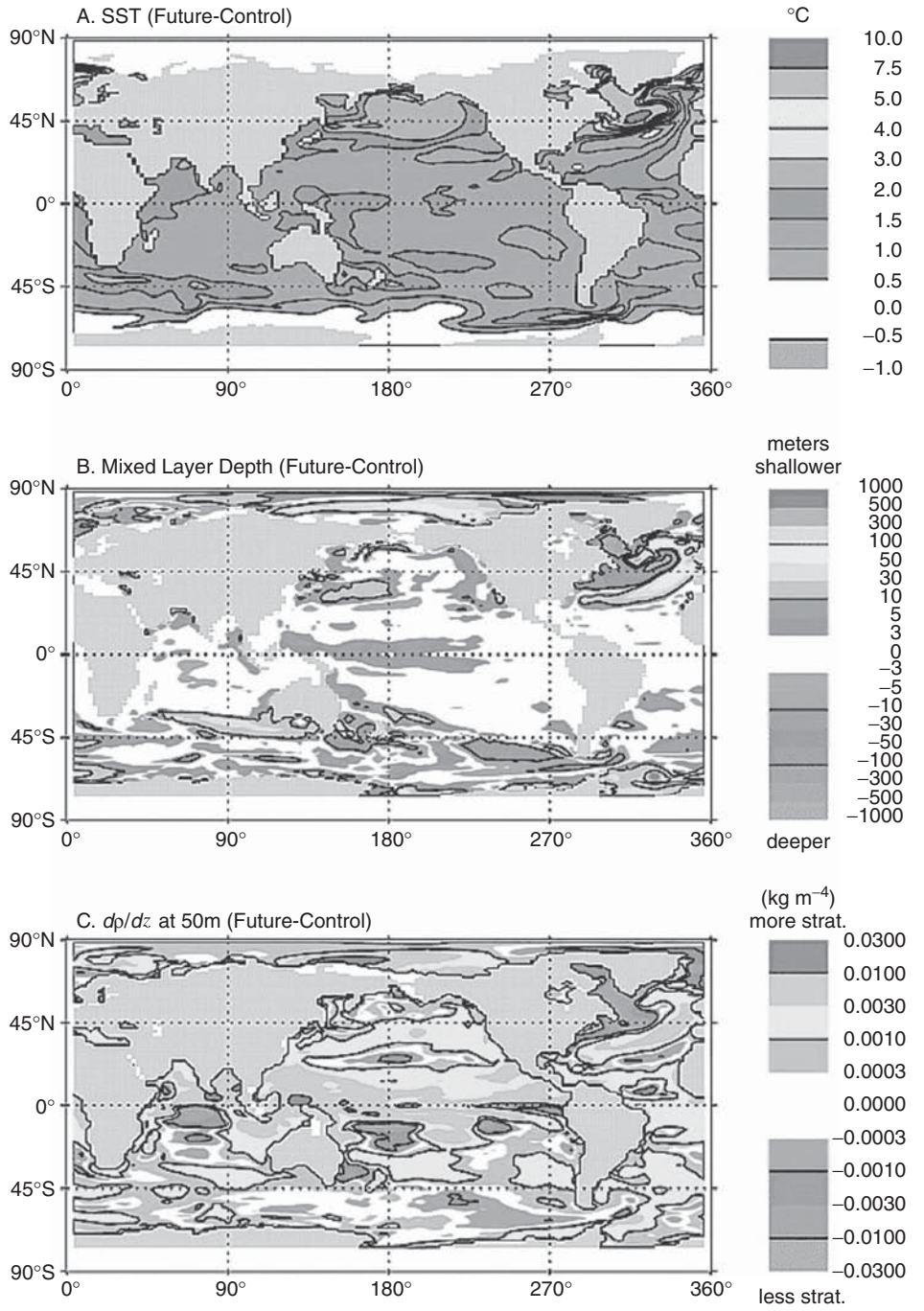

Figure 1.3 Projected climate-mediated changes in ocean physical forcing (future-control, i.e., 2060–2070 minus the present) from the NCAR Community Climate System Model for (A) sea surface temperature, (B) mixed-layer depth, and (C) upper ocean (50 m) stratification. (From Boyd and Doney, 2002, with permission of the American Geophysical Union.) (See insert for color representation.)

marine euryarchaeota are found in oceanic surface waters. Marine aerobic heterotrophic bacteria directly take up and metabolize low-molecular-weight (LMW, <500 MW) dissolved organic matter (DOM) such as easily metabolized mono- and oligosaccharides, free amino acids, and small peptides (Kirchman et al., 2001). Most bacterial cells in the ocean are free-living and thus dependent on DOM (Azam and Hodson, 1977). Free and attached bacteria hydrolyze polymeric substances and particulate matter into LMW compounds that can be passed through cell membranes (Hoppe et al., 1993). Bacterial metabolism in the surface ocean depends predominantly on uptake of labile LMW and HMW compounds with turnover times of minutes to days (Fuhrman, 1990). However bacterial metabolism may be supplemented by a variable contribution from semilabile DOM that turns over on approximately seasonal time scales (Kirchman et al., 1993; Carlson and Ducklow, 1996). Bacterial turnover of DOM and the associated remineralization of micro- and macronutrients such as iron, nitrogen, and phosphorus close the major biogeochemical cycles of these elements in the sea (Falkowski et al., 2008).

Marine net primary production is processed through a complex trophic network of consumers, with a global average 15 to 20% escaping microbial respiration in the euphotic surface layer (upper approximately 100 to 200 m) to be exported to the ocean interior. A change in this fraction would have a major impact on the global carbon cycle (Sarmiento and Toggweiler, 1984). In the open sea and in many coastal and shelf seas under stratified conditions, carbon and nitrogen flows are dominated by microbial food webs (Figure 1.4). The intensive recycling of the dissolved and particulate fractions of the NPP through consumers results in a large fraction (about 50%) of the NPP passing through bacteria and the dissolved pool (Pomeroy, 1974; Williams, 1981). In the open sea, most grazing is by microzooplankton (Landry and Kirchman, 2002; Calbet and Landry, 2004); thus most heterotrophic consumption and respiration is microbial. Microbial dominance of marine food webs appears to hold even for the Antarctic seas, previously thought to be the last bastion of the classical diatom–krill–whale food chain based on large plankton (Daniels et al., 2006).

As this discussion and Figure 1.4 suggest, a large fraction of the organic matter fixed in marine NPP is cycled through dissolved pools and metabolized by bacteria. All organisms leak DOM into the environment via passive diffusion, cell lysis and breakage, and active metabolic processes (Bjørnsen, 1988; Nagata, 2000). Phytoplankton actively exude dissolved organic carbon (DOC) to dispose of excess photosynthetically fixed carbon not combined with nitrogen and phosphorus into biomass in the approximate Redfield ratio 106 : 16 : 1 (C/N/P). Phytoplankton exudation is the process most directly coupling primary to bacterial production (Morán et al., 2002), but not the only process. Larger-celled phytoplankton such as diatoms may be broken between capture and ingestion by crustacean predators, spilling their fluid contents, in a process known as *sloppy feeding* (Lampert, 1978). According to Nagata (2000), egestion and excretion during feeding by protistan grazers may be the greatest relative contribution to photosynthetically derived DOC fluxes. Clearly, passive diffusion across phytoplankton cell membranes, active exudation, egestion, and sloppy feeding will produce DOM with different chemical composition and at different rates. Following ingestion and digestion, zooplankton grazers excrete DOM, and it also diffuses out of fresh fecal material (Jumars et al., 1989). Additional DOM may be released during viral lysis, abiotic dissolution, or bacterial hydrolysis of suspended and sinking detrital particles and marine snow. Figure 1.4 suggests one scenario for the relative

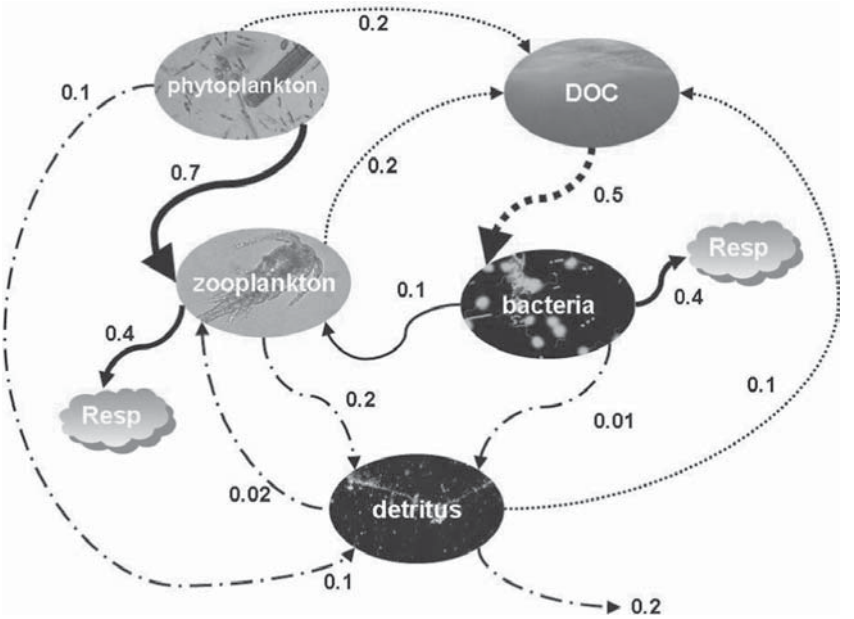

Figure 1.4 Microbial food-web diagram, showing exchanges of carbon in the oceanic surface layer. The flows are normalized to NPP = 1.0. The partitioning of flows among compartments is based on the physiological budget model given in Anderson and Ducklow (2001). Note that the carbon flows are dominated by zooplankton grazing (70% of NPP), DOC uptake by bacteria (50%), and heterotrophic respiration (80%). In this depiction the respiration is divided evenly among zooplankton and bacteria, but note that oceanic zooplankton may be dominated by protozoans smaller then 20 μm. Here the bacterial production is 12% of the particulate NPP, the fraction approximated by traditional ^{14}C assays, and a typical value for the open sea (Ducklow, 1999). Solid lines, biomass flows and respiration; dotted lines, dissolved flows; dashed–dotted lines, detrital flows and mortality. (See insert for color representation.)

magnitudes of DOC fluxes from these diverse sources. This model explains how the level of bacterial production (BP) is limited by how much DOC flows through the food web to bacteria, and by the bacterial allocation of the carbon ingested between production and respiration (bacterial growth efficiency; see below). Phytoplankton extracellular release (PER) may vary widely even in healthy cells, ranging from less than 5 to more than 50% of the total (dissolved plus particulate) primary production (Morán and Estrada, 2002). Anderson and Ducklow (2001) outlined the importance of PER vs. grazer-related sources in setting the overall level of bacterial metabolism in the sea. Williams (1981) hypothesized a larger role for PER, whereas Jumars et al. (1989) emphasized the primacy of grazers as DOM sources. Depending on the season, location, and particular environmental conditions of nutrient and light levels, grazing intensity, and other factors, many different scenarios are possible. Specifying how bacteria may respond to climate change requires better understanding of these rates, and the chemical composition of DOM released from a multitude of sources. For example, DOM released by grazing activities is probably of lower quality for bacterial nutrition, due to enzymatic attack, than that released by phytoplankton or viral

lysis (Nagata, 2000). Changes in the physical or ecological state of ocean ecosystems affecting herbivores may cascade to changes in bacterial activity or community composition.

Our understanding of microbial life in the oceans has escalated rapidly following the application of molecular tools (e.g., gene or whole genome sequencing, molecular profiling, fluorescent in situ hybridization), many of which have targeted the SSU rRNA gene, a phylogenetically informative molecule in which all forms of life can be compared. Marine microbial communities are diverse, although moreso at finer taxonomic scales than at gross phylum levels. Overall at the phylum level, there are some differences between ocean realms and between coastal vs. open ocean. Some distribution and biogeographically-based patterns are now emerging following extensive surveys of the world's ocean with these new tools. The upper oceans are dominated (25 to 33%) by SAR11-related bacteria members of the α-proteobacteria (Morris et al., 2002), and the deeper oceans below 150 m harbor abundant marine GI crenarchaeotal populations (20 to 40% of DAPI-stained cells; Karner et al., 2001; Teira et al., 2006). Another numerous group of α-proteobacteria, common to both coastal and open ocean regions, the Roseobacter clade is often abundant (about 15% of the community; Buchan et al., 2005). There is high phylogenetic and functional diversity of marine γ-proteobacteria, which comprise a significant fraction of the bacterioplankton. Interestingly, though, γ-proteobacteria are often the most commonly cultivated marine bacteria, yet the cultivated species are rarely detected in molecular surveys. The more commonly detected γ-proteobacteria in cultivation-independent surveys on global scale harbor diverse metabolisms, and are in a number of cases strictly oligotrophic (Cho and Giovannoni, 2004). Marine Bacteriodetes-related organisms are a third group that includes diverse members that are not well represented in culture collections but can be numerically dominant (Cottrell et al., 2000). Interestingly, we now also know that species in each of these major phylogenetic groups found in oceanic photic zones contain proteorhodopsin, a membrane-associated light-driven proton pump. How these organisms utilize proteorhodpsin to supplement growth is still not well understood, although it may be a theme for organisms living in the upper ocean. Campbell et al. (2008) estimated that at least 14% of organisms living in the photic zone contain proteorhodopsin homologs.

Biogeographic, temporal, and spatial surveys have also revealed considerable diversity and dynamics in community composition. For example, Pommier et al. (2007) compared the diversity of bacterioplankton assemblage at nine different sites around the world. Their findings suggest that despite the fact that the major phyla detected were similar, there were very few sequences that were detected repeatedly. Seasonal variation in coastal Antarctic waters is dramatic, with bacterial richness shifting significantly (Murray et al., 1998; Murray and Grzymski, 2007) and archaeal populations increasing in relative proportions in winter (Massana et al., 1998; Murray et al., 1998; Church et al., 2003). Vertical density stratification in the ocean (see below) has the most apparent impact on gradients of diversity in the ocean, however, as community diversity shifts most significantly with depth rather than with longitude or latitude. One of the caveats of nearly all studies is that the diversity is undersampled but new technologies in massively parallel DNA sequencing promise to reshape our views of species richness and our ability to compare in detail community composition from many sites using the same deep sequencing approach (Sogin et al., 2006; Huber et al., 2007).

1.2 BACTERIAL ACTIVITY AND TEMPERATURE

Temperature is a critical property affecting virtually all metabolic processes of organisms (Gillooly et al., 2001; Brown et al., 2004), especially those of microbes (Price and Sowers, 2004). However, by concentrating solely on temperature as a key control of bacterial properties, we are necessarily neglecting other biological interactions that shape bacterial communities in the oceans (Strom, 2008). Theoretically, with no other constraints and unlimited resources, increases in temperature should result through increased enzymatic activity in higher growth rates and eventually, larger abundances of cells. Thus, a first and indirect approach to the possible effects of climate warming on heterotrophic bacteria could be the examination of how their abundance or activity changes with temperature in present-day natural conditions (Figure 1.5). A corollary to this reasoning is that if resources (nutrients and organic matter) are limiting, increased temperature will not necessarily cause increased activity.

The major problem with trying to describe the response of bacteria to temperature is to separate it from other environmental factors that actually covary with temperature, such as nutrient concentration or primary productivity, which may themselves have a larger effect on bacterial properties (Lomas et al., 2002). In permanently warm regions or during summer in temperate waters, strong stratification of the upper layers of the water column precludes the input of inorganic nutrients from below the euphotic zone, thus severely limiting the photosynthetic fixation of CO_2 (Longhurst, 1998). A simple way around this complication is to correct by the other limiting factor, such as examining the relationship of the ratio of bacteria to phytoplankton biomass or activity versus temperature (Li et al., 2004; Apple et al., 2008). In Li's analysis, the bacteria/phytoplankton biomass ratio increases with increasing temperature up to an estimated upper limit of 1, consistent with the predictions of the food-web model of Laws et al. (2000).

Despite the complex nature of interactions between temperature, substrate availability, cell size and abundance in the ocean, strong apparent increases with temperature in the abundance of picoplankton, the smallest size class of planktonic organisms ($< 2\ \mu m$ in diameter) comprising all free-living heterotrophic bacteria, have consistently been reported for different aquatic ecosystems (Li, 1998; Rae and Vincent, 1998; Agawin et al., 2000; Li et al., 2006; Morán et al., 2008). Heterotrophic bacterial numbers generally peak in the summer months, while minima are frequently found during winter in most regions (Li, 1998; Steinberg et al., 2001; Calvo-Díaz and Morán, 2006; Alonso-Sáez et al., 2008; Garneau et al., 2008). This relationship is, however, not constant through the entire -2 to $30^{\circ}C$ temperature range. Usually, the initial increase in abundance with temperature in colder conditions is followed by more constant or even decreasing abundance (Hoch and Kirchman, 1993; Shiah and Ducklow, 1995), and with considerably more variation, as temperature rises above some value around $15^{\circ}C$ (Figure 1.5A). Bacterial growth rates calculated as production/biomass ratios also show a remarkably similar response to temperature, with no further significant increases at temperatures above $15^{\circ}C$ (Figure 1.5B). This figure implies that climate warming may alter the microbial ecology of both polar and tropical environments, representatives of extreme temperature conditions of Earth's marine ecosystems, in different ways. We use these environments as case examples of possible changes in carbon fluxes mediated by bacteria in a warmer ocean (see below).

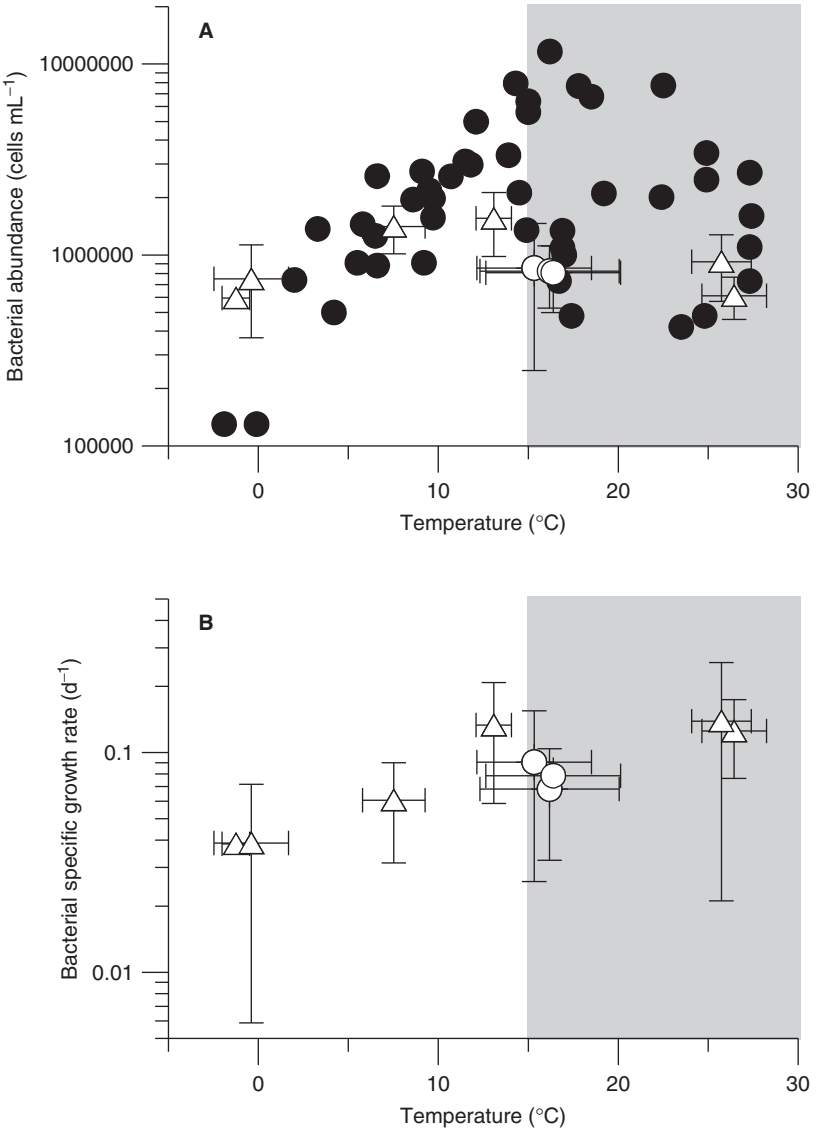

Figure 1.5 (**A**) Relationship between bacterial abundance and temperature. Closed circles are mean annual values at the surface of different regions of the world ocean. Open circles are mean ± SE annual values in 2006 at the surface of three stations in the southern Bay of Biscay. Open triangles are mean ± SE values averaged for the euphotic layer from studies that partially covered the annual cycle in the Antarctic, Arctic, subarctic Pacific, North Atlantic, Arabian, and equatorial Pacific. (**B**) Bacterial specific growth rates (BP/BB per day) vs. temperature of the two data sets added to Li's original figure. The shaded area represents the general change in response at temperatures at or above 15°C. (Modified from Li, 1998.)

Since bacterial abundance is the result of in situ growth minus losses due to predation and lysis, not necessarily involving the same temperature dependences (Sherr et al., 1988), we now focus on the intrinsic physiological processes of bacteria that are affected directly by temperature. The two fluxes mediated by bacteria that are most relevant from the perspective of biogeochemical cycling of organic matter are bacterial production (BP) and respiration (BR). BP and BR are related by bacterial growth efficiency (BGE), which is the fraction of the total organic carbon assimilated or bacterial carbon demand (BCD) that is used to build up biomass: $\mathrm{BGE} = \mathrm{BP}/\mathrm{BCD} = \mathrm{BP}/(\mathrm{BP} + \mathrm{BR})$. A more mechanistic approach involves the use of mathematical descriptions of the temperature dependence of BP and BR. The simplest is the temperature coefficient, Q_{10}, the factor by which a biological reaction changes with a temperature increase of 10°C. Although as for other planktonic groups, the mean Q_{10} for BP, BR, and growth rates clusters around 2 (Robinson and Williams, 1993; Kirchman et al., 1995; Lomas et al., 2002; Kirchman et al., 2005), actual values may vary considerably as much within as across regions (Pedros-Alió et al., 2002; Middelboe and Lundsgaard, 2003). This pattern argues against the use of a fixed Q_{10} value in models predicting biogeochemical responses to rising sea temperatures.

A more rigorous description of the temperature dependence of metabolic rates stems from the work of van't Hoff and Arrhenius in the late nineteenth century on chemical reactions, later applied to biological processes. The *van't Hoff–Arrhenius equation* (Arrhenius, 1915) ($Q = Q_0 e^{-Ea/kT}$), also called *Boltzmann's factor* (Gillooly et al., 2001; Brown et al., 2004; Allen et al., 2005), describing the exponential increase in metabolic rates with temperature was recently extended into the more general framework of the metabolic theory of ecology (MTE) (Brown et al., 2004). The MTE predicts a value of activation energy E_a of 0.65 eV for heterotrophic organisms (Brown et al., 2004; Allen et al., 2005). In a recent review of published data on temperature and bacterial metabolism, López-Urrutia and Morán (2007) showed that the temperature dependencies of cell-specific BP and BR rates were statistically indistinguishable, with E_a values close to the predicted 0.65 eV (0.42 to 0.59 eV) (Figure 1.6). In other words, although the absolute amount of organic carbon processed by bacteria may change in the future, they would essentially be allocating the same fraction of resources to growth (BP) and metabolism (BR) in a warmer scenario, contradicting previous claims of a strong inverse relationship of BGE with temperature (Rivkin and Legendre, 2001). Invariant BGE values with an increase of 2°C over in situ temperatures have been shown recently in an annual cycle in Mediterranean coastal waters (Vázquez-Dominguez et al., 2007). The relationship between BGE and temperature observed by Rivkin and Legendre (2001) probably reflects variations in regional ecology rather than a direct temperature effect.

However, resource limitation strongly affected the temperature-corrected BP, while BR remained unaltered (López-Urrutia and Morán, 2007). Hence under resource-limiting conditions, assimilated organic carbon (BCD) would decrease solely at the expense of BP. Although the response of BP to temperature was constant at three levels of resource availability using chlorophyll as a proxy for bacterial substrates (<0.5, 0.5 to 2, >2 mg of chlorophyll per cubic meter), the intercepts were significantly different (1.6). The implication of this result is that low-productivity areas (<0.5 mg chlorophyll per cubic meter) will have lower BP than that of eutrophic regions (>2 mg m^{-3}) but essentially the same BR. As a consequence, BGE would decrease with decreasing chlorophyll concentrations, from about 30% at values greater

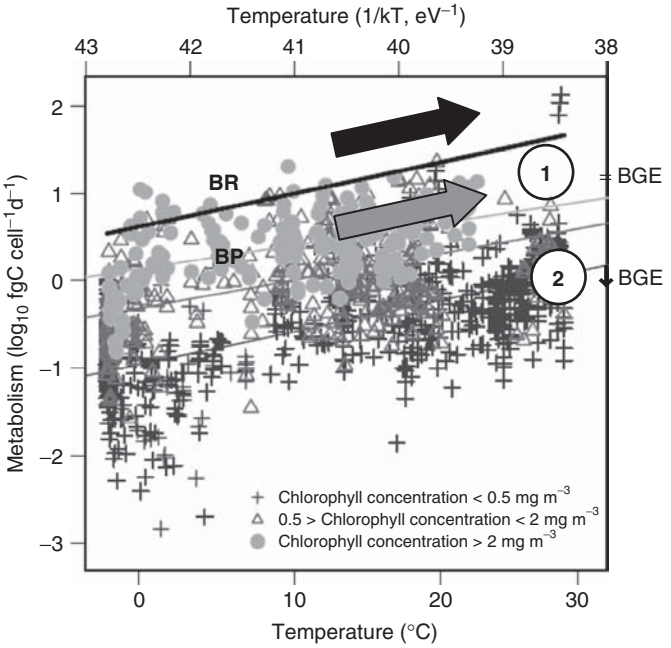

Figure 1.6 Individual rates of bacterial production (BP_i) and respiration (BR_i) versus temperature at three levels of chlorophyll concentration. The black line represents the relationship between ln BR_i and $1/kT$ ($y = 26.49 - 0.59x$) and the colored lines represent the ln BP_i–temperature relationships for each data subset (green, $y = 18.14 - 0.42x$; red, $20.54 - 0.50x$; blue, $22.58 - 0.58x$). An increase in temperature with no changes in resource availability would result in similar increases in BP_i and BR_i (i.e., the same BGE: case 1). Resource limitation would slow the rate of increase of BP_i with temperature compared to BR_i, thus lowering BGE (case 2). (Modified from López-Urrutia and Morán, 2007.) (See insert for color representation.)

than 5 μg/L to values about 5% at lower chlorophyll levels, confirming previous work on systematic changes of the BGE with resource availability (del Giorgio and Cole, 1998). We suggest that a climate-induced change toward lower phytoplankton biomass and production in vast areas of the oligotrophic ocean (Bopp et al., 2001; Behrenfeld et al., 2006) or a shift in composition to smaller organisms (Karl, 1999a) (see below) would affect BGE by changing the resource level at which the BP–temperature relationship operates. That is, if we collected data in a warmer ocean in 2100 at exactly the same locations as those used for plotting Figure 1.6, substantially more of the data would fall in the oligotrophic and mesotrophic categories, so the BGE would decrease—but due to resource limitation rather than to a direct temperature effect. To sum up, increases in temperature will undoubtedly increase BR and BP, but respiration would proceed unabated while production will probably level off. This is because BP is affected by resource availability, which is influenced by temperature. The obvious consequence is that proportionally more dissolved organic carbon would be released as CO_2 by bacteria than is made available as biomass to bacteriovores in marine food webs. This shift would have follow-on effects for nutrient limitation and upper trophic levels (Ducklow et al., 1986).

Some of the models aimed at predicting how biogeochemical cycles in the ocean will respond to global warming use the functional groups approach (e.g., Hood et al., 2006), where bacteria appear as the major respirers. This view has been held by most researchers (Rivkin and Legendre, 2001; del Giorgio and Duarte, 2002), although recent work suggests a slightly lower mean contribution of bacterial respiration to total values (Robinson, 2008). Yet since respiration at the community level would be largely bacterial, the consequences for the metabolic balance of the oceans of a differential regulation of BP and BR are potentially large. Using the MTE approach (see above) to model gross primary production and respiration of planktonic communities, López-Urrutia et al. (2006) predict a significant (21%) reduction in ocean uptake of atmospheric CO_2 as a result of changing metabolic balance.

An important issue regarding temperature and substrate availability interactions as factors determining bacterial activity is the claimed difference between permanently cold (<5°C) waters and elsewhere. The hypothesis developed by Pomeroy and co-workers (Pomeroy and Deibel, 1986; Pomeroy et al., 1990; Wiebe et al., 1992) states that polar bacteria need comparatively higher concentrations of DOM for efficient uptake than do those inhabiting lower latitudes, and hence would process a lower proportion of primary production (Pomeroy and Wiebe, 1988), with the remainder sedimenting into the benthic food web. Changes in the kinetics of DOM uptake have been observed (Nedwell, 1999; Yager and Deming, 1999) as predicted by Pomeroy and Wiebe (2001), and low bacterial production/primary production (BP/PP) ratios have been documented frequently in cold waters (Bird and Karl, 1999; Ducklow, 1999; Morán et al., 2001). However, there is no such clear pattern of systematic changes in BP/PP with temperature (Figure 1.7) as observed for abundance or growth rates (Figure 1.5). The lack of consistent occurrence of low BP/PP values in cold waters, together with contrary results reported for the Arctic (Kirchman et al., 2005) and the

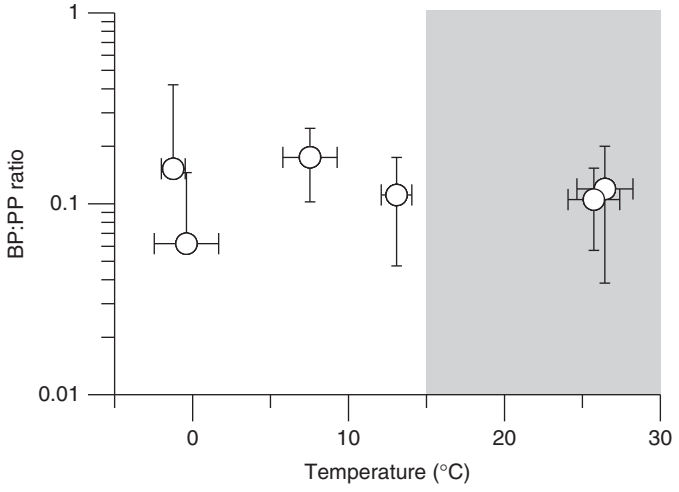

Figure 1.7 Relationship between the mean ± SE ratio of bacterial production to primary production (BP/PP) and temperature averaged for the euphotic layer from surveys conducted in the Antarctic, Arctic, subarctic Pacific, North Atlantic, Arabian, and equatorial Pacific. Shaded area as in Figure 1.5.

independence of temperature and resource effects in the analysis shown in Figure 1.5, all argue against the universal validity of Pomeroy's hypothesis.

Another way of approaching the consequences of global warming on bacterial-mediated carbon fluxes is by conducting perturbation experiments in which experimental warming is applied over short- to medium-term-duration incubations with natural samples to capture local responses (e.g., Kirchman et al., 2005). The small number of realistic warming perturbation experiments performed to date have shown differences in the respective responses of phytoplankton and bacteria (Morán et al., 2006; Hoppe et al., 2008), suggesting the possibility of disruptions in their trophic relationship at higher temperatures. This type of experiment, together with examination of patterns across large temperature ranges, can provide us with clues about the possible directions of change. However, they obviously fail to incorporate the possibility of adaptations of extant communities over the longer term, or of shifts in community composition (see below). Interestingly, agreement in the absence of direct warming-driven changes in BGE between the results of experimental perturbations and theoretically based predictions linking metabolism and temperature (cf. López-Urrutia and Morán, 2007; Vázquez-Domínguez et al., 2007) would still encourage their use as hypothesis-generating tools.

In conclusion, although temperature is only one of the many factors affecting bacterial metabolism, current knowledge suggests that changes are expected in the amount of organic carbon being processed by bacteria in a warmer ocean. Whether these changes will be a larger amount of DOM being channeled through bacterial communities with unaltered bacterial physiology (BGE; Figure 1.6, case 1) or, more likely, a general slowdown of system metabolism, as implied by decreasing phytoplankton biomass and primary production in vast areas of the world ocean, lowering BGE (Figure 1.6, case 2) remains to be tested.

1.3 CLIMATE AND PLANKTON ECOLOGY

As we stated above, bacterial growth in the sea is regulated by the complex interplay of resource limitation (bottom-up forcing; Billen et al. 1990), temperature, and removal processes (top-down controls; Strom, 2008). Bacterial abundance in many marine environments is correlated with bacterial production rates, a proxy for resource supply, suggesting that resource limitation is the primary control on bacterial stocks (Ducklow, 1992). But the abundance seldom reaches the saturating level predicted from physiological models of growth and resource supply (Wright and Coffin, 1984; Ducklow and Hill, 1985). This effect means that predators and viruses must suppress bacterial accumulation below the saturation level set by resource supply. Together, these observations suggest that bacterial abundance is set by a dynamic balance between resource supply and removal processes, a combination of bottom-up and top-down controls. Changes in resource supply cause changes in abundance, but some of the resources are funneled to bacteriovores, and thence into the food web. Bacteria may be limited by inorganic nutrients such as phosphorus (Thingstad et al., 2005), organic matter (Carlson and Ducklow, 1996), or combinations of organic and inorganic forms, such as DOM and iron (Church et al., 2000; Kirchman et al., 2000). Similarly, removal is some combination of grazing by bacteriovores (Strom, 2000) and lysis by bacteriophages (Suttle, 2007). The balance among supplies of different limiting resources for bacteria,

and between different bacterial consumers is a function of the composition of the entire plankton community. Thus, we need to look in more detail at plankton communities to get a better understanding of how bacteria might respond to climate-driven changes in the physical environment.

Delivery of solar energy to the ocean surface determines two critical processes that structure the plankton ecosystem: vertical density stratification of the upper ocean water column; and the maximum rate of photosynthesis under ambient temperature, nutrient, and grazing conditions (Miller, 2004). Phytoplankton growth is proportional to light intensity and depletes nutrients from the surface layer, creating a vertical nutrient concentration gradient opposite to the profile of light intensity. Heating the ocean surface results in vertical stratification because warmer water is less dense and more buoyant than the colder layers below. Hence, global warming is predicted to lead to more stratification. The vertical temperature–density gradient is determined both by heating at the surface and by the temperature of deep-ocean water, which reflects temperatures at the poles. In coastal and polar regions, inputs of fresh water also contribute to enhanced stratification. The stratification, or contrast in density between shallow and deeper layers, is a barrier to turbulent mixing generated by winds. Turbulent mixing has two opposing impacts on phytoplankton growth: It mixes nutrients from deep into shallow water, and mixes phytoplankton from the illuminated surface down into darker subsurface waters. Coastal, polar, and high-latitude regions experience higher winds and more turbulence; in contrast, tropical, subtropical, and offshore waters receive more heating and tend to be more stratified. Thus, very generally, phytoplankton in higher latitudes and coastal waters with more nutrient supply are relatively more light-limited, and those in warm, stratified seas are more nutrient-limited.

These geographic patterns are set by the climate and have important implications for phytoplankton community composition (Falkowski and Oliver, 2007). Thermal gradients between the tropics and polar regions, and between the land and ocean, generate large-scale wind patterns, causing regional differences in mixing and turbulence. During periods with stronger equator-to-pole thermal contrasts, such as glacial epochs, winds are stronger and storms are more frequent, enhancing ocean turbulence. During warm periods the winds are lower and there is greater stratification, with a lower turbulence regime. In past geological history, the temperature difference between the surface and deep ocean has ranged from about 10°C, a relatively well-mixed condition 65 million years ago (65 Ma), to 25°C, a more stratified condition today. We live today in a cryosphere-influenced climate still largely set by glacial conditions with polar ice caps, sea ice, strong thermal gradients, and high winds and turbulence. Sixty-five million years ago, the world was warmer, ocean bottom temperatures were higher, and the ocean was less well stratified. It took less wind energy to mix nutrients up from deep water in the pan-tropical era of 65 Ma.

Plankton community composition is set ultimately by environmental selection of phytoplankton cell size (Cullen et al., 2002; Katz et al., 2004). In nutrient-depleted tropical seas, and in the seasonally stratified surface layer in midlatitudes in summer, small cells with high surface/volume ratios and superior ability to compete for scarce nutrients dominate the phytoplankton community (Agawin et al., 2000). The classic example of this condition is the oligotrophic North Pacific subtropical gyre (NPSG) to the north of Hawaii, a vast region dominated by *Prochlorococcus* and *Synecchococcus*, photosynthetic prokaryotes about 1 μm in diameter (Karl, 1999a). Diatoms are favored under turbulent conditions because they have internal nutrient storage vacuoles and can

"stock up" during transient mixing events caused by storms and other turbulent events. In addition, because they have dense and heavy silicified shells (frustules), diatoms sink into deep water under stratified, quiescent conditions, but stay suspended in the upper water column in more turbulent conditions. There has been a long, steady increase in diatom diversity over the last 65 million years during the transition from tropical to glacial conditions, reflecting the increase in the turbulence climate over that time span (Falkowski and Oliver, 2007). Other groups, including dinoflagellates and coccolithophorids, predominate in regions and time periods with conditions intermediate between these oligotrophic and well-mixed regimes. The grazer community adapts to the size of its prey: In tropical seas, the predominant herbivores are protozoans specialized to feed efficiently on small phytoplankton (Landry and Kirchman, 2002). Where phytoplankton are larger, copepods and krill are the major grazers (Miller, 2004).

To summarize at this point: Heterotrophic bacteria depend on, and respond to, the flux of organic matter and nutrients produced by phytoplankton and processed by grazers in marine food webs. Climate sets the physical regime of wind mixing, turbulence, and stratification in marine environments. The large-scale biogeography of marine plankton ecosystems follows the global gradients of temperature and wind. The physical regime, in turn, determines the characteristic size and community composition of planktonic phytoplankton (and by extension, zooplankton) in different marine ecological provinces or biomes (Longhurst, 1998).

The projected responses of ocean phytoplankton to changes in physical properties as outlined above have been studied by adding models of plankton food webs to AOGCMs (Bopp et al., 2001; Boyd and Doney, 2002; Sarmiento et al., 2004; Boyd et al., 2008). In the early cases, the models were very simple, including only single phytoplankton, zooplankton, and bacterial compartments (e.g., Figure 1.4), using only nitrogen as a model currency (Sarmiento et al., 1993). The underlying concept embodied in these models is that plankton ecosystem response to climate variability is modulated through direct phytoplankton responses to ocean mixing, light, and nutrient supply. These simple models have a critical limitation: that only crude bulk-rate changes such as the total primary production can be modeled. Importantly, however, climate change will also affect the species and functional group composition of phytoplankton communities (Falkowski et al., 1998). For example, changes in stratification and mixing may lead to shifts in the relative importance of diatoms and other forms. Such changes have already been reported, with shifts from diatoms to cryptophytes in Antarctica (Moline et al., 2004), large blooms of coccolithophorids in the Bering Sea (Napp and Hunt, 2001; Broerse et al., 2003), and shifts toward the smallest phytoplankton in the NPSG (Karl, 1999b). To capture such ecosystem shifts, modelers have had to construct more complex models with several different classes of phytoplankton rather than a single aggregate compartment (Ducklow and Fasham, 1991; Doney et al., 2004; Follows et al., 2007).

One approach to greater articulation in model community structure and forecasting acuity has been to incorporate representations of planktonic biogeochemical functional groups: plankton groups that mediate specific chemical transformations such as diatoms (fixing silica), coccolithophorids (calcium carbonate), and diazotrophs (nitrogen fixation). In this context, heterotrophic bacteria can be thought of as a functional group that decomposes organic matter and respires CO_2 back into the water. Indeed, "bacteria," first added to a plankton system model by Fasham et al. (1990), are included in one of the most complex plankton functional group models currently in use (Le Quéré

et al., 2005). Boyd and Doney (2002) coupled diatoms, coccolithophorids, and diazotrophs in the NCAR Community Climate System AOGCM and looked at how the abundance of these groups changed in different ocean regions under a global warming scenario with increased stratification (as shown in Figure 1.3). In such an approach, all the functional forms are potentially present in any region and may become dominant, depending on their model-defined environmental preferences ("universal distribution, local selection").

Boyd and Doney's model predicted increases in diazotrophs in the tropics in response to decreased mixed layers and increased stratification (Figure 1.8), both of which favor the buoyant, colonial *Trichodesmium* with its high light requirement (Karl et al., 1997). Other projected changes include reductions in diatoms and increases in picoplankton in the central gyres, increases in diatoms in coastal regions (in response to increased upwelling), and increases in coccolithophorids. A caveat is that few such model studies have been performed, and the results are still speculative (Falkowski and Oliver, 2008; Peters, 2008). But they represent the current state of our understanding, with the best tools we possess. To date, models have not included different microbial functional or taxonomic groups, such as photoheterotrophs or archaea.

1.3.1 Linking Bacteria to Climate Changes

Bacteria may respond directly to increasing temperature, but their responses to other changes in the ocean climate will be modulated through the plankton community. Current knowledge about the trophic interactions between phytoplankton and bacteria, called *phytoplankton–bacterioplankton coupling* (Morán et al., 2002), may allow us to make predictions based on predicted changes in primary producer assemblages and their production rates and physiology. The composition of phytoplankton assemblages is known to affect the quality and quantity of released photosynthate (Lancelot, 1979,1983) and the fraction of primary production directly available for bacterial uptake (Cole et al., 1982,1988; Baines and Pace, 1991). Shifts toward smaller phytoplankton cell sizes (see above) can be accompanied by a relatively higher proportion of photosynthate being released extracellularly. Teira et al. (2001) and Morán et al. (2002) have reported consistent increases in the percentage of extracellular release when phytoplankton assemblages are dominated increasingly by the picoplankton size class in subtropical and temperate regions, respectively. Thus, although lower primary production rates are anticipated in vast areas of the ocean (Behrenfeld et al., 2006), the amount of substrates readily available to bacterial assemblages might be somewhat compensated by increased extracellular release. In general, the coupling between phytoplankton growth and removal by grazers is tighter in plankton communities dominated by smaller cells. Shifts in cell size may alter the balance between DOM supplied by exudation and that originating from grazers and their feeding behavior, changing the rates and composition of the DOM supply. Below we focus on the changes predicted in phytoplankton in polar, tropical, and coastal oceans, and the potential implications for bacteria.

1.3.2 Polar Regions

In the Antarctic peninsula, one of the fastest-warming regions of the Earth (Vaughan et al., 2003), experimental 2°C warming of incubated samples collected in summer

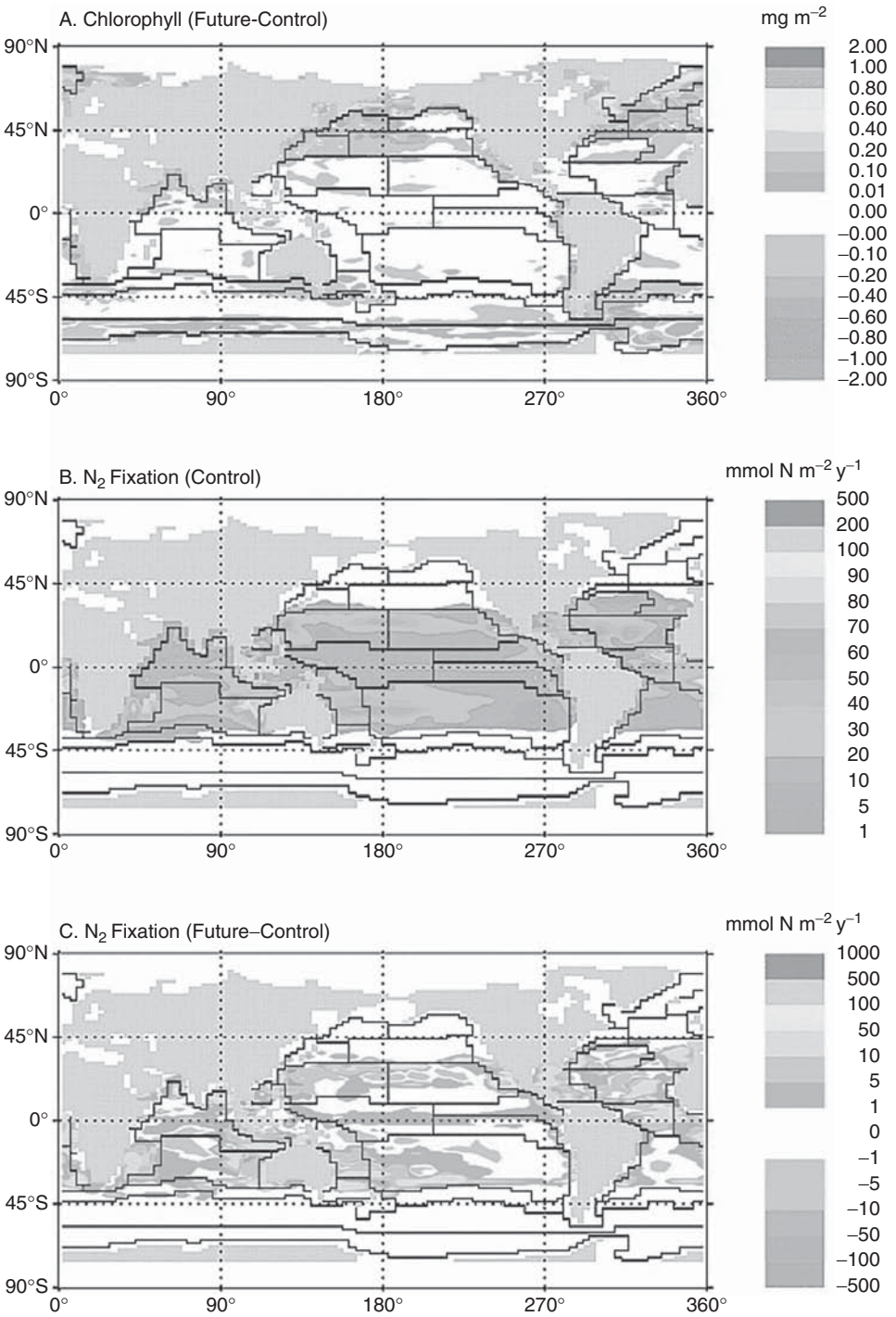

Figure 1.8 Numerical model simulations of ocean ecosystems using the CSSM (see Figure 1.3) with an off-line, multispecies pelagic ecosystem model for (A) the difference between predicted chlorophyll with warming and a control run (future-control), (B) N_2 fixation (control), and (C) N_2 fixation (future-control). (From Boyd and Doney, 2002, with permission of the American Geophysical Union.) (See insert for color representation.)

significantly changed the partitioning of primary production between the particulate and dissolved phase, but a clear effect on bacterial demand for carbon could not be demonstrated at the time scale of the experiments (Morán et al., 2006). Models predict an increase in primary production rates during the growth season due to enhanced stratification and abundance of macronutrients in both polar regions (see above). The expected impact of higher temperatures and larger amounts of fresh DOM provided to bacterial communities is an increase in bacterial abundance and consequently, in activity (Figure 1.5). Below we discuss potential shifts in bacterial species composition as well. The role of micronutrients such as iron in the response of bacteria is still uncertain (Pakulski et al., 1996; Church et al., 2000; Arrieta et al., 2004; Oliver et al., 2004), as are such changes in phytoplankton communities as the replacement of diatoms by cryptophytes already observed (Moline et al., 2000, 2004). However, the predicted lack of substrate limitation suggests that a greater DOM flux would be processed by a larger number of bacteria with essentially the same growth efficiency. According to the model of Anderson and Ducklow (2001), partitioning of food web flows in favor of extracellular release by phytoplankton would result in higher BP/PP ratios, even without concomitant alteration in the BGE. Increasing BP/PP may constitute a positive feedback, with higher bacterial biomass enhancing viral lysis, more available DOM, and so on.

The existence of strong hydrographic fronts that serve as faunal boundaries in the Southern Ocean is a singular feature of Antarctic polar waters (Tréguer and Jacques, 1992). Despite claims of unlimited dispersal across such biogeographical boundaries (Finlay, 2002), scenarios of a mean warming higher than 5°C may entail loss of Southern Ocean bacterial and archaeal species without immediate replacement by lower-latitude species (Papke and Ward, 2004; Newsham and Garstecki, 2007), due to endemism in extreme environments. However, the greatest effect of warming is likely to be loss of sea ice. The microbial communities in sea ice probably have a higher incidence of endemism than that of the pelagic community.

1.3.3 Tropical Regions

Tropical and subtropical ecosystems correspond to the right end of the bacterial vs. temperature relationship depicted in Figure 1.5, thus implying that factors other than temperature are more critical for regulating bacterial standing stocks and metabolism. We generally lack specific formulations for the temperature dependence of bacterial grazers (see Sherr et al., 1988) and how they may change in a warmer ocean, but even leaving top-down controls aside, a fundamental difference exists between most tropical–subtropical and higher-latitude environments. With the exception of the equatorial provinces, waters with a mean temperature of 25°C and higher are oligotrophic, characterized by chlorophyll values below 0.2 mg m^{-3} (Longhurst, 1998; Ducklow, 2003). Moreover, they cover the vast majority of the ocean's surface, so changes in their functioning will greatly affect biogeochemical cycles.

Inorganic nutrients not only limit phytoplankton growth, but nitrate and phosphate can also directly affect bacterial growth (Mills et al., 2008). Coupled models of ocean circulation and ecology agree strongly in predicting a general decrease in the supply of new nutrients from below the thermocline due to increased stratification (see above). The obvious consequence is that phytoplankton primary production will decrease, as already observed in the satellite record over the past decade (Behrenfeld et al., 2006).

Decreases in phytoplankton size at increased temperatures (Morán et al., 2008) could be associated with an increase in PER, as discussed previously, but it may be insufficient to support a larger bacterial demand driven by increased temperature. Although considerable scatter is seen in at temperatures higher than 15°C (Figure 1.5), the constraints exerted by lower primary productivity will probably result in lower bacterial standing stocks. Our hypothesis is that a greater proportion of the organic carbon processed by bacteria (from increased PER) would be respired rather than used to build up biomass (Figure 1.6). The decrease in BGE and increase in respiration could have an impact on global carbon cycling by enhancing ocean-to-atmosphere CO_2 fluxes.

Changes in stratification may also affect the oxygen balance in deeper waters: for example, the extensive oxygen minimum zones (OMZs) in the eastern tropical Pacific and Arabian Sea. These are habitats for chemolithotrophic bacteria that would respond to expansion or contraction of the OMZs. The key question concerning these vast areas of the global oceans is whether we can extrapolate the general patterns described so far to predict future behavior of heterotrophic bacteria in a warmer ocean. A question that remains unsolved is what will happen if, as models predict, oceanic primary production decreases in the huge oligotrophic oceanic regions as much as to severely limit the amount of substrate provided. Abundances will decrease to accommodate the supply of substrates or, alternatively, bacterial populations may adapt to using more of the large, seasonally varying pool of semilabile DOM (Hansell et al., 1995; Carlson and Ducklow, 1996; Church et al., 2002). An alternative hypothesis is that these conditions will favor organisms that utilize dissolved inorganic carbon chemolithotrophically.

1.3.4 Coastal Oceans

In the open sea, remote from external inputs of organic matter and nutrients, heterotrophic bacterial metabolism must be sustained on the flow of carbon from the primary producers, either directly, via exudation, or indirectly after passage through grazers and the detrital pool. The coastal oceans are influenced by multiple inputs from terrestrial ecosystems, and on diverse anthropogenic perturbations, in addition to, and interacting with, climate change (Robinson et al., 2004). Inputs of sediments change the local light regime. Nutrient inputs cause enrichment and eutrophication (Rabelais, 2004). Inputs of terrestrial organic matter are respired by bacteria, possibly leading to an excess of respiration (R) over production (P) (Ducklow and McCallister, 2004) and local to regional-scale hypoxia. The future P/R balance will depend on the relative amounts of inorganic nutrients and organic matter exported from land (Ver et al., 1999) and on the relative temperature sensitivities of phytoplankton and bacterial processes as the ocean warms. Recently, Hoppe et al. (2008) conducted a wintertime mesocosm experiment in which water from the Kiel Fjord (Baltic Sea) and its plankton community was warmed experimentally over a two-month period to simulate the effects of regional warming on phytoplankton–bacterial coupling. They found that the timing of the phytoplankton bloom was unchanged by temperature increases of 2 to 6°C, although the bloom magnitude declined with increasing temperature, as observed in a multiyear analysis in Chesapeake Bay (Lomas et al., 2002). In contrast, the lag period between the phytoplankton and peak bacterial production declined with increasing warming, apparently tightening the coupling between organic matter production and consumption. But they also found that the ratio of bacterial to primary production in the experimental mesocosms increased from 37% to 63% as the warming increment

was increased from $+2^{\circ}$C to $+6^{\circ}$C. The higher BP/PP values indicate increased utilization of allochthonous DOM in the coastal water rather than use of recent products of phytoplankton bloom. This experiment illustrates that climate warming could have a major impact on the metabolic balance of coastal oceans.

1.3.5 Potential Impacts of Climate Change on Bacterioplankton Diversity and Structure

Our discussion so far has concentrated on bulk fluxes of organic matter through an undifferentiated bacterial community. Upon consideration of the scenarios developed in this chapter and by others (e.g., Falkowski and Oliver, 2007) which suggest that climate change may result in increased stratification, strengthened links between primary and secondary producers and increased DOC and bacterial respiration, it is also likely that microbial diversity and/or community structure will shift to accommodate these changes. Species competition and resource utilization in complex microbial communities may result in three possible outcomes in a situation with increased resources (such as DOC) (1) the functional complexity and diversity will increase (complementarity), (2) the resource will act as a factor of selection favoring reduced complexity and a less diverse assemblage (bloom situation), and (3) the community will absorb the increased resource, resulting in an increased growth rate and/or respiration, although diversity will remain unchanged. This last case may be the result of marine bacterial species being generalists rather than specialists (Mou et al., 2008). Unfortunately, for marine bacterial and archaeal diversity, our understanding of these interactions is still not well developed.

We can, however, propose a few rather simple examples of impacts on species diversity resulting from climate change. First is the consideration of habitat loss on marine microbial diversity. Antarctic and Arctic sea ice microbial communities are distinct from plankton communities (Bowman et al., 1997; Brinkmeyer et al., 2003), tend to be dominated by true psychrophiles and have a higher degree of endemism than that of many marine planktonic organisms. Loss of sea ice will not only result in habitat loss for marine psychrophiles, but will also affect sea ice-melt-related oceanic processes like seeding phytoplankton blooms (especially diatoms) and stratification of surface waters. A second bacterial habitat that may be at risk are large phytoplankton themselves. Species-specific relationships exist between bacteria and the extracellular zones surrounding algal cells (Jasti et al., 2005). Somewhat related to this is the particulate organic carbon that accumulates as marine snow. Large phytoplankton (e.g., diatoms) are thought to contribute most significantly to marine snow particles. These particles are home to unique microbial consortia which differ from their counterparts in the surrounding seawater (e.g., DeLong et al., 1993).

Another potential effect results from a fueling of the microbial loop (discussed previously). With increases in bacterial abundance, respiration, and growth, it is possible that viral control and lysis will result in a positive feedback to this loop, resulting in additional DOC and additional top-down control. Current concepts suggest that viruses play roles not only in control of dominant species ("kill the winner," Thingstad and Lignel, 1997) but that they also influence bacterial diversity through population control of less dominant populations in addition to being resources of lateral gene transfer (Weinbauer and Rassoulzadegan, 2004; Suttle, 2007). As resources in the ocean shift toward higher DOC, inevitably this will influence the competitive abilities of organisms in the upper ocean for carbon utilization, and likely species composition.

1.4 SUMMARY

The projections we have reviewed suggest a geographic redistribution of plankton rate processes with climate change. Primary production rates will increase in polar waters and decrease in the tropics, concentrating more of the global ocean productivity at higher latitudes. In both regimes, more primary production may be routed through the dissolved pool, resulting in a greater flux of photosynthate through bacteria over much of the world ocean, and possibly increases in bacterial stocks at higher latitudes. This may lead to alterations in food web structure as more biomass at the base of food webs is concentrated in the microbial loop, and may increase the recycling rate of carbon through the loop. In general, however, the changes we discuss would not lead to large-scale alterations of the ocean carbon balance (Falkowski and Oliver, 2007). The total amount of ocean production that is respired would ultimately change little, although the location and timing of the respiration could be affected. For example, if warming increases the recycling rate of locally produced, labile organic matter in the surface layer, less carbon will be exported to the deep sea. But a change in the bacterial use of oceanic semilabile DOC could represent a major alteration of the marine carbon cycle. A similarly large change would be seen if a change in trophic structure of marine bacteria favored chemolithotrophic organisms under these conditions, The total amount of DOC in the global ocean is about equal to the amount of CO_2 in the atmosphere (Houghton, 2007), and repartitioning of carbon in the ocean–atmosphere system is a major control on the climate system (Sarmiento and Toggweiler, 1984). Climate warming is already causing increased soil respiration in the Arctic, in which soil microbes are decomposing organic matter stored in the tundra since the last glaciation (Oechel et al., 1993). This process is potentially a major positive feedback in the climate system (Luo, 2007). Increased bacterial utilization and respiration of DOM, the "soil" of the oceans, could alter the metabolic balance of ocean ecosystems, which are currently finely balanced, with equal rates of production and respiration (Robinson, 2008). Such a change may depend on temperature selection of bacterial populations capable of decomposing the more refractory components of marine DOC, as well as temperature-mediated increases in production and respiration rates.

When it comes to marine microbial diversity, we are still knowledge limited, particularly with regard to the links between phylogenetic diversity and functional capacities and the repertoire of growth strategies characteristic of marine bacterioplankton. These limitations affect our ability to predict response of the community to increased sea surface temperature and resulting changes in the flow of organic carbon through the microbial community. Marine microbial genome (Moran et al., 2004; Giovannoni et al., 2005) and metagenome (Venter, 2004; DeLong and Karl, 2005) studies have begun to crack the door open on these processes, but we are still at the beginning of this knowledge curve. We can look to certain regions of the world's ocean to gain further understanding of processes that may inform our predictive capabilities for understanding climate change. Two such regions are the oxygen minimum zones of the tropics, where conditions resulting from increased respiration can be studied, and the Southern Ocean, where annual primary production varies tremendously, and the responses of the marine microbial communities to a highly dynamic and predictable change in dissolved organic carbon resources can be studied. Further, improvements are needed to develop predictive coupled physical–biological models which incorporate both process (function) and species diversity information in the photic zone (Follows et al., 2007;

Moran et al., 2007) as well as inner ocean, since it is likely that changes in the upper water column, particularly with loss of larger phytoplankton that are responsible for a significant fraction of export production, will have an impact on carbon respiration in the deep sea. We are only now beginning to understand the relationships between diversity and functional capabilities to inform these models, but with the rapid advances in this field and large-scale oceanographic studies (Rusch et al., 2007) we will be poised to understand emergent properties and predict vital impacts on the marine ecosystem following anthropogenic climate change. Disentangling the multiple effects of climate warming on ocean ecosystems—changes in the physical environment, phytoplankton community composition, and bacterial processes—will be a major challenge for marine ecologists in the coming decade.

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CHAPTER 2

Control of Waterborne Pathogens in Developing Countries

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2.1 WATERBORNE DISEASE IN PERSPECTIVE

There is no such thing as a "developing country" disease. Many diseases, however, may be far more prevalent in developing countries, due to the multifactorial and interacting conditions of poor hygiene and sanitation; lack of access to clean water and food; lack of education; poor economic status; refugee status; political instability; droughts, floods, and other natural disasters; and so on. In addition, many of the less developed countries are in the tropics, providing conditions that favor many pathogens, parasites, and vectors of disease.

No one can accurately measure the true burden of waterborne disease. There are multiple routes of transmission for most infectious agents that can be transmitted directly from water, including direct ingestion, ingestion of contaminated food, and secondary transmission through the fecal–oral or oral–oral route. However, it is increasingly apparent that many waterborne agents can also be transmitted through aerosols and dermal contact. The greatest problem to determining the burden of waterborne disease is the lack of surveillance data. The majority of cases of gastrointestinal disease are never reported, and this includes deaths from many of the diarrheal diseases, particularly in the poorest regions of the world. Even in the relatively limited number of cases where health care is sought, most disease remains undiagnosed, misdiagnosed, and/or never reported to a centralized surveillance system.

The World Health Organization (WHO) report that on a global scale, 88% of cases of diarrhea are the result of poor hygiene and sanitation, including unsafe water, and are therefore preventable (Prüss-Üstün et al., 2008). They also estimate that these cases of diarrheal disease result in 1.5 million deaths per year, primarily among children. The

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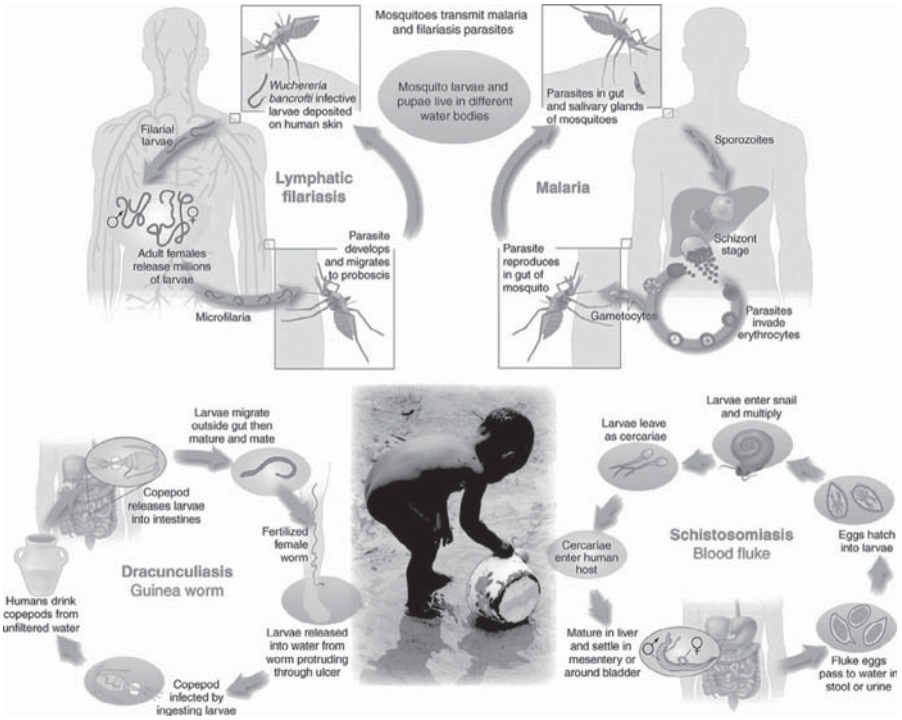


Figure 2.1 Transmission cycles of lymphatic filariasis, malaria, dracunculiasis, and schistosomiasis. (From Fenwick, A. 2006. Waterborne infectious diseases- could they be consigned to history? *Science* 313, 1077-1081. Reprinted with permission from AAAS.) (See insert for color representations.)

authors' analysis suggests that approximately 10% of the global burden of all diseases is related to water and is therefore preventable.

Much of the global burden of disease is water-related rather than simply waterborne. In other words, water is the critical habitat for the entire life cycle of the vector of the pathogen [e.g., the water flea (copepod) involved in transmission of dracunculiasis, and the water snail involved in the transmission cycle of schistosomiasis]. Water may also be critical for one or two stages of the life cycle of the vector, as for mosquitos, which transmit malaria and lymphatic filiariasis, and the black fly, which transmits onchocerciasis (Figure 2.1).

There is also an enormous burden of disease from the intestinal soil-transmitted helminthes. According to the WHO report, these parasites infect one-third of the world's population (Prüss-Üstün et al., 2008), or at least one-third of the population in the developing world (Hotez et al., 2008). The discrepancy in numbers reported may be due in part to the fact that co-infection is common. These nematode worms are transferred from person to person by contaminated soil as a result of poor sanitation; hence, control of these diseases should also be part of an integrated water management strategy. The burden of disease from these parasites should not be underestimated—mortality alone accounts for about 500,000 deaths per year. Additionally, co-infection frequently occurs with malaria and HIV, both increasing a person's susceptibility to these diseases and exacerbating disease progression (Hotez et al., 2008).

2.2 DISEASE CONTROL

In 1796, Edward Jenner found that serum from a cow infected with cowpox could protect a human being against smallpox (orthopoxviruses). This landmark discovery paved the way for some of the most important global advances in public health in the twentieth century. By 1980, the WHO declared that smallpox had been eradicated. Frozen lab strains still exist and could still pose a future risk to human health; however, they could also be critical in developing new treatments or vaccines should smallpox ever reemerge (Painter and Hostetler, 2004). The potential success of vaccination programs led the WHO to focus on eradication of disease in the latter part of the twentieth century. This is a strategy that has met with mixed success. Polio, a disease caused by poliovirus (family Picornaviridae), can be transmitted readily through contaminated water and has been eradicated successfully from many countries through aggressive vaccination programs. However, polio remains endemic in some areas, particularly in the northern Indian states of Uttar Pradesh and Bihar, where thousands of cases still occur each year despite massive efforts at eradication (NPSP, 2008).

Vaccination programs are not the only approach that has been used to eradicate global disease. Although not directly waterborne, malaria is water-related in that it is transmitted by the mosquito vector (*Anopheles* spp.), which depends on the presence of water for the egg and larval stages of its life cycle. Eradication programs have focused on elimination of breeding sites and use of insecticides (primarily DDT) to eliminate the vector rather than the protozoan pathogen (*Plasmodium* spp.). As such, on a global level they have been spectacularly unsuccessful, and in fact created immunologically naive populations that were devastated by the disease when mosquito populations resurged. According to the U.S. Centers for Disease Control and Prevention (CDC), there are 350 to 500 million (clinical) cases of malaria each year, with over 1 million deaths, primarily among young children (CDC, 2007). Failure of mosquito eradication programs targeting malaria is in part due to the complexities of the life cycle of the mosquito vector.

Eradication programs that target dracunculiasis have been more successful, although the disease is still a serious problem in rural African communities without access to clean drinking water (WHO, 2008a). What is unique about dracunculiasis eradication is that it has been brought about at relatively little cost by changing people's behavior rather than through development of vaccines or medicines (Barry, 2007). The disease is transmitted through ingestion of larvae-infected copepods in drinking water. The copepods and larvae can be killed by application of insecticides to open ponds, but behavioral changes have really been the key to successes so far. Thousands of rural village volunteers have been involved in educating communities to (1) filter drinking water though finely woven cloth, (2) prevent infected people from entering drinking water sources, and/or (3) identify other uncontaminated drinking water sources, such as wells.

The successes have been remarkable. Since the program was established by the CDC in 1980, and later led by the Carter Center with assistance from other agencies, the estimated incidence of dracunculiasis has fallen from 3.5 million in 1986 to less than 10,000 in 2008, localized to Sudan, Ghana, Nigeria, Niger, and Mali (Carter Center, 2008). The key to a successful control program is to interrupt the cycle of transmission by (1) preventing new infections through vaccination, vector control (habitat change, insecticide use, etc.), improved hygiene and sanitation, and behavioral change,

and (2) rigorous treatment of disease to prevent infected persons from transmitting or shedding the pathogen. As has happened in many countries with dracunculiasis, infection rates drop to levels that are no longer sustainable and the pathogen population crashes to the point where disease is considered eliminated. Unfortunately, for many of our pathogens, the life cycles are complex and may include many different vertebrate hosts, essentially making the second step almost impossible (e.g., *E. coli* O157, *Giardia*), or they can rapidly become resistant to treatment (e.g., *Plasmodium*).

2.3 PATHOGENS OF INTEREST

There are a large number of human pathogens that can be transmitted directly by the water route or are associated with water through vector-borne transmission. Although not exhaustive, Table 2.1 lists many of the known pathogens, their infectious doses, the diseases they cause, estimated mortality rates, and current methods of prevention and treatment. It should be remembered that in most cases, infectious dose and mortality rates are crude estimates. *Infectious dose* is defined as the number of infectious agents that produce infection (asymptomatic or symptomatic) in 50% of volunteers tested. In many cases this information does not exist, and in others the "young, healthy volunteers" do not represent a cross section of today's society, where the majority of persons may be far more susceptible to infection (e.g., the elderly or the immunocompromised). Mortality rates also vary widely in the literature and cannot take into account the additive effects of multiple infections or the contribution of an individual pathogen to those infections. A limited number of examples are explored in more detail below.

2.3.1 Old and Reemerging Pathogens

***V. cholerae* Highlighted** Although not at the forefront of public health concerns in more developed, resource-rich countries, *Vibrio cholerae* continues to be an important cause of life-threatening diarrheal disease in many parts of the world. Over the past two centuries, *V. cholerae* has been the agent of epidemic and pandemic cholera on the Indian subcontinent, in Central and South America, in Africa, and in Eastern Europe (Colwell, 1996). Cholera infection leads to rapid and serious dehydration and is often fatal if not treated promptly. Additionally, nonepidemic *V. cholerae* and members of other *Vibrio* species can cause milder forms of gastroenteritis.

V. cholerae reside in aquatic ecosystems. To cause disease, *V. cholerae* must acquire genes for virulence factors. Among the most important of the cholera virulence factors are cholera toxin (CT) and toxin-coregulated pilus (TCP). Fully virulent *V. cholerae* are believed to originate from nontoxigenic strains present in the environment (Lipp et al., 2002). For this to be possible, there must be an aquatic, environmental reservoir of bacteria that are nontoxigenic but nonetheless harbor virulence factor genes such as the TCP gene. The persistence of *V. cholerae* in aquatic environments has been linked to association with chitin, a ubiquitous component of the cell walls of fungi and the cell walls of arthropods (Pruzzo et al., 2008). Chitin serves as a carbon and nitrogen source for vibrios and other aquatic bacteria. *V. cholerae* attachment to chitinaceous surfaces leads to biofilm formation, which in turn can serve a protective role, enhancing the survival of the bacteria. *V. cholerae* cells growing on chitin are stimulated to take

Table 2.1 Pathogens in Drinking Water, Their Infectious Doses, Diseases, and Additional Comments

Pathogen	ID	Disease	Mortality	Methods of Prevention	Treatment
			Bacterial		
Vibrio cholerae	10^8	Cholera	< 1% with treatment	Public sanitation; potential for vaccine	Oral or intravenous rehydration, antibiotics
Salmonella typhae	10^{6-7}	Typhoid	~600, 000 deaths annually	Public sanitation; vaccine	Antibiotics
Salmonella spp.	10^{6-7}	Salmonellosis	10–30% of cases in parts of Asia and Africa	Food-related hygiene	Rehydration, antibiotics
Toxigenic/diarrheagenic *E. coli*	10^{1-9}	Diarrheal diseases	?	Public sanitation, hygiene	Rehydration, antibiotics
E. coli O157:H7	10^1	Diarrhea, hemorrhagic colitis, hemolytic–uremic syndrome (HUS)	<1% overall; 3–5% with HUS	Food-related hygiene	Rehydration; antibiotics are contra-indicated
Shigella spp.	10^2	Shigellosis	~1.1 million deaths/year	Sanitation, hygiene	Rehydration, antibiotics
Campylobacter spp.	10^6	Campylobacteriosis; Guillain-Barré syndrome	?	Food hygiene	Rehydration
Leptospira spp.	3	Leptospirosis	?	Sanitation, vaccine	Rehydration, antibiotics
Francisella tularensis	10	Tularemia	?	Hygiene, sanitation, insect repellents, vaccine	Antibiotics
Yersinia enterocolitica	10^9	Yersiniosis	Uncommon, except with bacteremia	Food-related hygiene	Rehydration, antibiotics for bacteremia
Aeromonas spp.	10^8	Skin and respiratory infections	?	Avoid exposure; boil water if imunocompromised	Antibiotics

(*Continued*)

Table 2.1 (*Continued*)

Pathogen	ID	Disease	Mortality	Methods of Prevention	Treatment
Helicobacter pylori	?	Gastric ulcers and cancer	?	Personal hygiene, reduce crowding	Antibiotics
Legionella pneumophila	>10	Legionellosis, Pontiac fever	?	Maintenance of water systems	Antibiotics
Mycobacterium avium	?	Disseminated infections, pulmonary disease	?	Avoid exposure, boil water if imunocompromised	Antibiotics
Burkholdaria pseudomallei	~10	Melioidosis	As high as 90% with septicemia	Experimental vaccine	High-intensity intravenous antibiotics, followed by maintenance dosage of oral antibiotics
			Protozoal		
Giardia lamblia	1–10	Giardiasis	Rarely fatal	Sanitation, hygiene	Metronidazole
Cryprosporidium spp.	10^{1-3}	Cryptosporidiosis	Rarely fatal	Sanitation, hygiene	Nitazoxanide, rehydration
Naegleria fowleri	High	Primary amoebic meningoencephalitis	Of ~200 known cases, >95% mortality	Avoid exposure in surface waters	Amphotericin B
Acanthamoeba spp.	?	Granulomatous amebic encephalitis, keratitis and others	~100% for encephalitis	Avoid exposure, boil water if imunocompromised	Not well established
Entamoeba histolytica	10^{1-2}	Amoebiasis, including amoebic dysentery	40,000–110,000 deaths annually	Food-related hygiene, sanitation	Metronidazole, tinidazole
Cyclospora cayetanensis	?	Cyclosporidiosis (cyclosporiasis)	Not fatal	Sanitation	Rehydration, trimetho-primsulfamethoxazole

Isospora belli	?	Isosporiasis	Not fatal; can be fatal for AIDS patients	Sanitation	Rehydration, trimethoprimsulfamethoxazole
Microsporidia	?	Microsporidiosis	Often fatal for immunosuppressed patients	Counseling for immunosuppressed patients	Rehydration, albendazole
Balantidium coli	$25-10^2$	Balantidiasis	Up to 30% mortality for untreated, immunosuppressed patients	Avoiding contact with pigs (primary reservoir)	Rehydration, tetracycline, metronidazole
Toxoplasma gondii	?	Toxoplasmosis	Can be fatal for fetus or immunosuppressed	Hygiene, vaccine may be close	Pyrimethamine
			Viral[a]		
Norovirus	10	Viral gastroenteritis	Very rarely fatal	Vaccine may be close	Rehydration
Hepatitis A	10^{1-2}	Fever, malaise, nausea, anorexia, and abdominal discomfort, jaundice	<0.4% overall, higher in elderly	Hygiene, vaccine	Immune globulin; symptom relief
Hepatitis E	?	Fever, vomiting malaise, nausea, anorexia, hepatomegaly, abdominal pain and tenderness, jaundice	As high as 20% in pregnant women	Vaccine may be close	Rest
Rotavirus	10^{1-2}	Acute gastroenteritis	~600, 000 children/year	Vaccine	Rehydration

(*Continued*)

Table 2.1 (*Continued*)

Pathogen	ID	Disease	Mortality	Methods of Prevention	Treatment
			Major Parasitic Diseases Related to Water		
Plasmodium spp.	?	Malaria	>1 million deaths/year	Impregnated bednets	Artemisinin combination therapy
Dracunculus medinensis	?	Dracunculiasis	Rarely fatal, high morbidity with pain and second-degree infections common	Behavioral change, insecticides	Careful removal of worms
Schistosoma spp.	?	Schistosomiasis	0.4% (can be much higher in specific areas)	Interrupt transmission though annual mass drug administration (MDA); behavioral change	Praziquantel
Wuchereria spp. and *Brugia* spp.	?	Lymphatic filiariasis	Rarely fatal, permanent and long-term disability	MDA; insecticides (larvicides)	Diethylcarbamazine citrate plus albendazole
Onchocerca volvulus	?	Onchocerciasis	Rarely fatal, permanent and long-term disability, blindness	MDA; insecticides (larvicides)	Ivermectin

Source: Adapted in part from Ford [1999, 2004]. Data compiled from WHO (1993), Hazen and Toranzos (1990), and Geldreich (1996); the Web site "eMedicine" (http://www.emedicine.com/) is an invaluable source of information on the etiology of infectious diseases.

[a] Also, poliovirus, coxsachievirus, echovirus, reovirus, adenovirus, astrovirus, coronavirus, and others yet to be identified.

up free DNA present in the environment (Meibom et al., 2005). Growth on chitin stimulates an array of gene expression, including a type IV pilus assembly complex that has been associated with competence in a variety of other bacteria.

A notable feature of *V. cholerae* survival in between outbreaks of disease is the ability to persist in a viable but nonculturable state as defined by testing under standard laboratory growth conditions. This model has been refined through identification of *conditionally viable environmental cells* (CVECs), which exist as aggregates or biofilms in aquatic environments (Faruque et al., 2006). Cycling between planktonic and biofilm growth states appears to be related both to environmental survival and to expression of pathogenicity. After *V. cholerae* begins colonization of chitinaceous surfaces, biofilm growth and differentiation are mediated by expression of TCP (Reguera and Kolter, 2005), which itself is the specific receptor for phage carrying the CT gene. Biofilm-induced selection for TCP expression and concomitant uptake of phage harboring the CT gene may accordingly play a major role in driving the appearance of virulent *V. cholerae*.

The genome of *V. cholerae* is remarkedly diverse, exhibiting a high degree of clonal diversity (Thompson et al., 2004). There are over 200 O-antigen serogroups, but only two of these, O1 and O139, are known to cause cholera. The activity of integrons within *Vibrio* and other bacteria appears to play a major role in genetic evolution of bacteria, including the emergence of newly pathogenic and even pandemic forms of *V. cholerae* (Faruque et al., 1998). Integrons are genomic elements that can capture open reading frames (ORFs) lacking promoters and convert them into genes through association with an integron-linked promoter (Mazel et al., 1998). *Vibrio* integrons, in particular, are capable of harboring a large number of integrated genes (Rowe-Magnus et al., 2003). These superintegrons allow bacteria to take in and "try out" a large array of "new" genes that may confer on the bacteria a selective advantage to survive and thrive under changing environmental conditions or express new virulence traits during the course of disease evolution.

In many regions, cholera outbreaks occur in a cyclic seasonal pattern. In the Ganges delta region, outbreaks generally occur twice a year, during the spring (March through May) and during the post-monsoon period (September through December). In noting that cholera outbreaks occur in a seasonal pattern, Jensen and co-workers (Jensen et al., 2006) have also observed a cyclic correlation between cholera outbreaks and decreased numbers of cholera phage. During cholera outbreaks, pathogenic *V. cholerae* were readily detected in water samples, while cholera phages appeared absent. Conversely, during periods of no disease, cholera phages were present but viable bacteria were absent in water samples.

Cholera outbreaks continue to affect the most vulnerable members of society, with the word *vulnerable* alluding to a host of interrelated socioeconomic conditions, including poverty, low educational level, poor nutritional status, and restricted access to medical care, safe drinking water, and sanitation. Children are particularly prone to infectious outbreaks of cholera. The challenge of cholera control includes the elements of prevention, preparation, and response (WHO, 2007a). An open and transparent surveillance system involving international cooperation is crucial for improving cholera control and in allaying both public panic and human suffering when outbreaks occur.

Rotaviruses Highlighted Rotaviruses are arguably the most common cause of severe diarrhea in children worldwide and are thought to result in more

than 600,000 deaths per year (CDC, 2008a), which is about one-third of all diarrhea-related mortality. Rotavirus infection can lead to rapid dehydration, which can be life-threatening if not managed quickly. As is the case for diarrheal disease in general in developing countries, it is a lack of access to rehydration therapy and general medical care that accounts for most rotavirus-associated mortality. The virus is stable in the environment, allowing for transmission through contaminated food and water and by contact with contaminated surfaces. A first vaccine for rotavirus was approved in the United States in 1998, but use of the vaccine was suspended in 1999 due to an increased incidence of intussusception associated with vaccine administration (Glass and Parashar, 2006). Although a second vaccine is now licensed for use in the United States, there is still a pressing need to develop vaccines effective for a wide variety of serotypes for use in more resource-limited countries.

2.3.2 More Recently Recognized Pathogens

E. coli 0157 Highlighted *Escherichia coli* is a normal inhabitant of the digestive tract of humans and other warm-blooded animals. For this reason, public health monitoring of source waters used for recreation and drinking often includes testing for *E. coli* as an indication of recent fecal contamination. Although most *E. coli* do not cause disease, several pathogenic variants exist that can cause serious diarrhea and associated disease in humans. Several diarrhea-associated pathotypes of *E. coli* have been described based on host clinical symptoms and serotyping (Nataro and Kaper, 1998). These pathotypes (with disease description) include:

- Enterohemorrhagic *E. coli* (EHEC): bloody diarrhea, hemorrhagic colitis, and hemolytic–uremic syndrome
- Enteropathogenic *E. coli* (EPEC): diarrhea in children and animals
- Enterotoxigenic *E. coli* (ETEC): traveler's diarrhea, porcine and bovine diarrhea
- Enteroaggregative *E. coli* (EAEC): persistent diarrhea in humans
- Enteroinvasive *E. coli* (EIEC): watery diarrhea and dysentery

Serotype O157:H7 is considered to be the prototypic EHEC and has received considerable attention, due to being the cause of several foodborne and waterborne outbreaks of disease (Feng, 1995). *E. coli* O157:H7 was first recognized in 1982 as the result of an outbreak of abdominal cramps and bloody diarrhea caused by contaminated, undercooked hamburger meat served at fast-food restaurants in the United States (Riley et al., 1983). Cattle can harbor O157:H7 asymtomatically and are a main reservoir for the bacteria in the United States. In addition to tainted meat, O157:H7 outbreaks have also been associated with vegetables and with unpasteurized milk and juices. Waterborne transmission has been linked to contaminated recreational and drinking water.

Genetic and molecular biology studies of O157:H7 and related bacteria highlight the challenges inherent in developing prevention and treatment strategies for pathogens that are continuously evolving and adapting. Like *V. cholerae*, the *E. coli* genome is also diverse, showing evidence of a rapid rate of genetic evolution, and having genes

with a high degree of polymorphism (Donnenberg and Whittam, 2001). Comparative sequence analysis has revealed that O157:H7 has over 1300 "new" genes not found in nonpathogenic K12. Pathogenic *E. coli* strains appear to gain and lose genes quickly as they adapt to rapidly changing environmental conditions. A striking example of this genetic fluidity has been demonstrated in variant strains of EHEC O26, which readily gain and lose shiga toxin genes during human infection, and like O157:H7 strains, possess multiple "hot spots" for Stx-encoding bacteriophage insertion and excision (Bielaszewska et al., 2007).

The determinants of pathogenicity for O157:H7 are incompletely understood. The major virulence factor genes first described for O157:H7 include *stx1* and *stx2* encoding the Shiga-like toxins 1 and 2 (Nataro and Kaper, 1998), and the 30 or so genes contained within the five operons of the LEE (locus of enterocyte effacement) pathogenicity island (Garmendia et al., 2005). The LEE genes include *eae*, which codes for intimin, an adherence factor contributing to intimate adherence of the bacteria to host cells, and several genes coding for elements of a type III secretion system involved in the host cell disease process. It has long been known that O157:H7 strains that cause disease in humans can colonize cattle yet not cause disease, and that *eae*-negative mutants can still cause disease in humans. Adherence factors other than intimin have recently been characterized as mediating adherence of O157:H7 to host intestinal epithelium (Torres and Kaper, 2003), yet other genes and factors are being implicated in O157:H7 adherence to and colonization of plant tissues (Torres et al., 2005). These findings have important implications in the control of O157:H7 contamination of human food sources.

Attempts to prevent O157:H7 outbreaks have proven to be problematic. The O157:H7 outbreak of September 2006 in the United States caused by contaminated spinach led to investigations of the management practices used on organic spinach farms as well as on neighboring cattle ranches. Genetically identical isolates of the outbreak strain were subsequently identified in soil samples from one farm, as well as in surface water, cattle, and feral swine (Jay et al., 2007). Investigations showed no evidence of the spinach being fertilized with cattle manure and no incidence of flooding or runoff that may have carried manure from nearby cattle ranches to the affected spinach fields. Freely roaming feral pigs were found to have ready access to both cattle ranches and spinach fields, however, and may represent a previously unrecognized threat to food safety.

Interestingly, while a great deal of attention has been focused on O157:H7 incidence and prevalence in developed countries such as the United States, far less attention has been paid to O157:H7 in developing countries. For example, O157:H7 has until recently been virtually unrecognized as a public health threat in India. In studies started by our laboratory group in 2004, we were able to readily isolate O157:H7 during sampling of the Ganges River in Varanasi, India. These were the first environmental isolates of the bacteria to be documented in India (Hamner et al., 2007). More recently, multiantimicrobial-resistant EHEC have been found in other river source water as well as in potable water distribution systems in India (Ram et al., 2008). With the prevalence of O157:H7 only recently being documented in India, it may be that O157:H7 infections and outbreaks have gone unrecognized and been overshadowed by an already high incidence of diarrheal disease.

2.3.3 Pathogens to Watch

Burkholderia pseudomallei Highlighted *B. pseudomallei* is a major cause of water- and soilborne disease in tropical countries. The CDC lists Africa, the northern part of Australia, Cambodia, India, Laos, Malaysia, the Middle East, Myanmar, the South Pacific, Thailand, and Vietnam as areas with endemic melioidosis, the disease caused by this pathogen. Sporadic cases have also occurred in Brazil, Ecuador, Guyana, Haiti, Panama, Peru, and the United States (Hawaii and Georgia) (CDC, 2008b).

The ease of environmental transmission, through direct contact with contaminated soils or water, has made this pathogen a potential candidate for bioterrorism. Infection can occur through exposure of open skin wounds, ingestion, or inhalation of aerosolized bacteria. Disease manifestations range from localized skin infections to acute pulmonary and bloodstream infections that, in turn, can lead to chronic suppurative infections of major organs. Although seldom fatal, a widespread epidemic of melioidosis has the potential to disrupt health care services on a major scale and incapacitate a large proportion of a population.

Like *V. cholerae* and *E. coli* O157:H7, *B. pseudomallei* are genetically diverse and appear to be evolving rapidly (Holden et al., 2004). The *B. pseudomallei* genome is relatively large, 7.2 megabase pairs in size, and consists of two chromosomes. The genome contains a large collection of genes, allowing for survival in a variety of different niches. The genetic fluidity of *B. pseudomallei* potentially poses challenges for study and future control of pathogenesis of the organism as well as for vaccine development.

In terms of control, to date there are no available vaccines and the ease of exposure would make prevention extremely difficult without significant behavioral changes, for example, in agricultural practices. The infections are, however, generally treatable with antibiotics. Treatment is somewhat complicated by the fact that *B. pseudomallei* are inherently resistant to certain antibiotics (Cheng and Currie, 2005). For severe melioidosis, current treatment and management protocols include an initial, intensive course of intravenous antibiotic administration followed by an eradication course of oral antibiotics. Relapse is sometimes observed even with aggressive treatment, however.

2.3.4 New World Diseases

In the more developed countries, considerable interest has revolved around the protozoan pathogen *Cryptosporidium parvum* and the calicivirus group of viruses, most specifically norovirus. Are they a major cause of waterborne disease in developing countries? This is not a trivial issue in relation to “old world” diseases such as cholera, due to the exceptional disinfection resistance of *C. parvum* and the high environmental stability of the norovirus (Poschetto et al., 2007). *C. parvum* are clearly present in water supplies of developing countries and can be isolated from stool samples of patients presenting with diarrhea (Snelling et al., 2007). There is less information on the potential burden of norovirus infections, although traveler’s diarrhea is often caused by norovirus that has clearly been contracted in developing countries (Chapin et al., 2005). As water treatment improves in any given region, we should anticipate seeing greater morbidity from such organisms as *C. parvum* and norovirus. Today, these infections are seldom diagnosed in developing countries but are undoubtedly present and contributing

to the overall burden of waterborne disease. Given that the source of exposure is often untreated or poorly treated water that is subject to both human and animal wastes, it is likely that exposed individuals are infected with multiple pathogens (Albert et al., 1999). In these cases, identifying the contribution to morbidity (and mortality) from *C. parvum* and norovirus would be extremely difficult. Both infectious agents can cause severe diarrhea and can therefore contribute significantly to dehydration and potentially mortality, particularly among the immunocompromized, the very young, the pregnant, or the elderly.

A similar argument can probably be used for the environmental pathogens that are of concern in treated drinking water: the *Mycobacterium avium* complex of organisms, *Legionella pneumophila*, and *Helicobacter pylori*. The burden of the diseases caused by these bacteria will probably never be fully assessed for developing countries (Kumar et al., 2006).

2.4 CASE STUDIES

Flooding is the most frequent form of catastrophic disaster, happening relatively predictably in countries such as Bangladesh (e.g., Hashizume et al., 2008) as the result of monsoon rains, and less predictably in other countries, due to unanticipated heavy rains, storm surges, tsunamis, snowmelt, and perhaps in the future, sea levels rise from melting polar caps (IPCC, 2007).

In all cases of flooding, waterborne disease outbreaks on different scales are almost inevitable. The most common illnesses associated with floods described in the literature are diarrhea, cholera and typhoid, hepatitis (jaundice), and leptospirosis. Unusual illnesses such as tetanus have also been reported. Cholera in particular has been associated directly with flooding in Africa. It is instructive to examine two different scenarios and the approaches to disease prevention, control, and mitigation. Almost every monsoon season in Bangladesh is accompanied by outbreaks of waterborne disease; however, the severity of flooding and the consequent severity of the outbreaks is not easily predicted. Major flooding events occurred in 1988, 1998, 2004, and 2007, accompanied by mass outbreaks of waterborne disease (Schwartz et al., 2006; ICDDR,B, 2007). The most commonly isolated pathogen is *V. cholera* (estimated 35% of cases in August 2007) followed by rotavirus (estimated 12% of cases in August 2007). Infections with enterotoxigenic *E. coli, Shigella*, and *Salmonella* species also increase during floods in Bangladesh, but the burden of identified disease is clearly dominated by cholera.

Flooding and disease have long been a part of Bangladeshi life, and aggressive rehydration therapy, even during mass waterborne disease events, is credited with saving large numbers of lives—no deaths from diarrhea or dehydration occurred among patients admitted to the Dhaka hospital in August 2007 (21,401 patients). However, tens of millions of people are thought to have been displaced in the 2007 floods (ICDDR,B, 2007), and it would be almost impossible to estimate the true morbidity and mortality from these events.

The International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), established in 1978 by the government of Bangladesh, is a world leader in diarrheal disease research. Through promotion of household treatment, vaccination programs, and support for aggressive rehydration therapies, the center has undoubtedly

achieved a remarkable reduction in mortality from waterborne diseases. However, the reality in Bangladesh is that although diarrheal disease is a preventable disease, until sanitation and safe drinking water are available to all the poor of Bangladesh, morbidity and mortality rates from these diseases will remain high. During the 1988, 1998, and 2004 monsoon seasons, 25 to 50% of Bangladesh was reportedly submerged (Schwartz et al., 2006). Widespread destruction of infrastructure, where it exists, and massive contamination of water supplies is inevitable. Added to the contaminated water supplies and lack of sanitation is the problem of millions of displaced peoples, often forced to try and survive in crowded "refugee camps." Global warming models suggest a future rise in sea level that can only exacerbate coastline flooding, displacing more people and contributing further to contamination of water and soils though increased salinity (IPCC, 2007).

2.4.1 The Asian Tsunami (December 2004)

One of the world's greatest disasters occurred on December 26, 2004, when an earthquake off the western coast of Sumatra in Indonesia caused a series of tsunamis that killed between 200,000 and 300,000 people in South and Southeast Asia. In one of the hardest-hit areas, Aceh Province in Sumatra, some towns lost more than two-thirds of the population at the time of impact, almost 100% homes were destroyed, and 100% lacked access to clean water and sanitation (Brennan and Rimba, 2005). At the time, many communities reported diarrhea as the main cause of morbidity (85% children under 5 years), but no increases in mortality and no outbreaks of cholera or other potentially epidemic diseases. Given the massive scale of the disaster, it seems surprising that major outbreaks, epidemics, and even pandemic diseases did not emerge, as were predicted in many media reports and by international aid organizations, including WHO (Morgan et al., 2005). To a large extent, the Australian army and other groups are to be credited with rapidly deploying environmental health teams to swiftly implement public health measures, including provision of safe drinking water, proper sanitary facilities, and mosquito control measures (Jamieson, 2005). Widespread fecal pollution of the surface waters was shown, yet the saltiness of the potable water supply after the disaster made much of the water unpalatable. Wells were vulnerable, perhaps to other etiological agents of fecal origin, including viruses and *Shigella*, with greater probability of infection than *Vibrio*, thus leading to widespread diarrhea. The WHO activated the Global Outbreak Alert and Response Network, posting "over 120 of the world's best disease surveillance and response experts ... to tsunami-affected communities within days" (WHO, 2005).

This raises an interesting issue: When so many nations can respond rapidly to a disaster on the scale of the Asian tsunami and prevent epidemic disease, why can we not respond successfully to smaller-scale droughts and floods? The answers are not difficult to understand and are largely political. In many countries the international community is not welcome, and of course the sovereignty of those nations should be respected; however, this speaks directly to the need to educate both communities and policymakers.

2.5 TREATMENT OPTIONS

The World Health Organization states that "narrow dispensers are key." Studies show that the use of containers with narrow openings for filling, and dispensing devices such as spouts or taps/spigots, protect the water collected during storage and household use. Improved containers protect stored household water from the introduction of microbial contaminants via contact with hands, dippers, other fecally contaminated vehicles, or the intrusion of vectors (WHO, 2008b).

This concept is, of course, not new; the problem of contamination of water during in-home storage has been recognized for several decades. In the early 1990s, the CDC and the Pan American Health Organization led the way to providing design criteria for safe water storage the major requirement being a single opening that could be covered easily and would not allow immersion of hands or utensils (Mintz et al., 1995). This is perhaps the simplest intervention approach. At the other end of the scale, in the 1970 s, UNICEF and other international relief agencies embarked on an ambitious plan to provide clean drinking water to Bangladesh and West Bengal by the installation of tube wells. Although initially successful in reducing morbidity and mortality from diarrheal diseases, it became apparent in the 1980s and 1990s that many of the groundwaters tapped by tube wells were contaminated with high levels of arsenic. It is now estimated that tens of millions of people are at risk from arsenicosis.

A wide variety of "simple" water treatment options have been proposed for developing countries and transitional economies (Table 2.2), and no doubt many other technologies will emerge in the future. Good sources of information are the Center for Affordable Water and Sanitation Technology (CAWST, 2008a) and the WHO drinking water page (WHO, 2008c). In terms of filtration technologies, two of the more popular approaches are use of ceramic and biosand filters. There are many different types, but examples of filter systems that have been widely deployed in the developing world are the Potters for Peace filters, which are locally made ceramic "buckets" coated with colloidal silver. Biosand filters are essentially slow sand filtration devices adapted for household use. Water is poured through layers of sand and gravel, usually in a locally made concrete vessel. With all water treatment techniques, operation and maintenance are critically important (Baumgartner et al., 2007).

Unfortunately, in many cases the water source is a shared resource between wildlife and humans, subject to frequent contamination from both animal and human wastes (Figure 2.2). As such, these environments are invariably turbid, which provides a challenge to all filtration techniques (filter pores rapidly become blocked) and decreases the effectiveness of even chemical disinfectants (pathogens adsorbed to particles). Disinfection is particularly problematic for ultraviolet (UV)-based technologies, as UV light has limited penetration in turbid water. Ideally, water is first treated to remove turbidity, followed by filtration and disinfection as in a municipal water treatment plant. This can be achieved on a household scale using the recommendations of organizations such as CAWST. Fine filter material can be effective in removing some particulate material, but simple sedimentation using the three-pot treatment system promoted by Loughborough University in the UK can also be effective (raw water is poured or strained into pot 1, water is then slowly decanted into pot 2, and then into pot 3; drinking water is taken from pot 3 after storage for at least two days, Skinner and Shaw 1999). Far better still than simple sedimentation is flocculation sedimentation. In municipal treatment systems, flocculants such as aluminum sulfate are used, but

Table 2.2 Examples of Treatment Options

Treatment Option	Mechanism of Pathogen Removal	Diarrhea Reduction	Recent Sources
Safe water system, bleach, vessel, and behavior change	Sodium hypochlorite	22–84%	CDC (2008c)
Tube wells	Supposedly "clean" groundwater	Significant	
Flocculant/disinfectant PUR	Calcium hypochlorite	16–>90%	PGHSI (2008)
Ceramic water filters	Variety of types, colloidal silver, also copper	60–70%	Clasen et al. (2005)
Biosand filter	Adsorption/competition	30–44% (unpublished data quoted on the CAWST Web site)	CAWST (2008b)
Boiling	Temperature	Potentially significant	
Solar water disinfection	UV and temperature	9–86%	SODIS (2008)
UV bucket	UV	Potentially significant	Niparajá (2008)
Life straw	Iodine and silver	Potentially significant	Vestergaard Frandsen (2008)
Sari cloth	Prefilter for particles and pathogen hosts (e.g., copepods)	48% reduction in cholera	Colwell et al. (2003)

Figure 2.2 Multiple uses of the Ganges River, Varanasi, India, in which *E. coli* O157:H7 has been detected. Clockwise, beginning from upper left: religious bathing, commercial laundry washing, washing and watering of cattle, milkmen washing pails in a "least polluted" section of the river, where the fecal coliform count exceeds 10^4 CPU/1000 mL (From Hamner et al., 2006, 2007.) (See insert for color representations.)

there are many locally available products that can achieve similar results. The CAWST Web site lists the following as examples of plant-derived coagulants:

- Seeds from the Indian Nirmali tree (*Strychnos potatorum*)
- Seeds of the trees of the Moringaceae family: *Moringa oleifera*, occurring in India, Guatemala, Mexico, Senegal, and Sudan (Behenus tree), and *Moringa stenopetala* in Kenya
- Sap from the tuna or raquette cactus (*Opuntia ficus indica*) in Peru, Chile, Dominican Republic and, Haiti
- Bark of the South American tree *Schinopsis Quebracho-Colorado*
- Potato starch
- Dried beans (*Vicia fava*)
- Peach seeds (*Percica vulgaris*) in Bolivia

Whatever technology is recommended to a community, it will essentially be ineffective at reducing disease without education and behavioral change. There is no point in giving narrow dispensers to a household if the top of the container will be cut

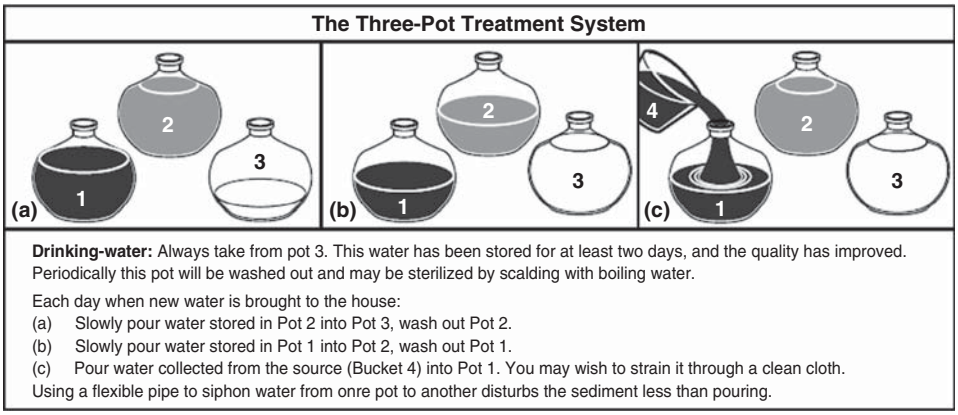


Figure 2.3 Three pot system. (From Skinner, B., and Shaw, R. 1999. Household water treatment 1. Technical Brief No. 58, Loughborough, UK: WEDC. Reprinted with permission from WEDC.)

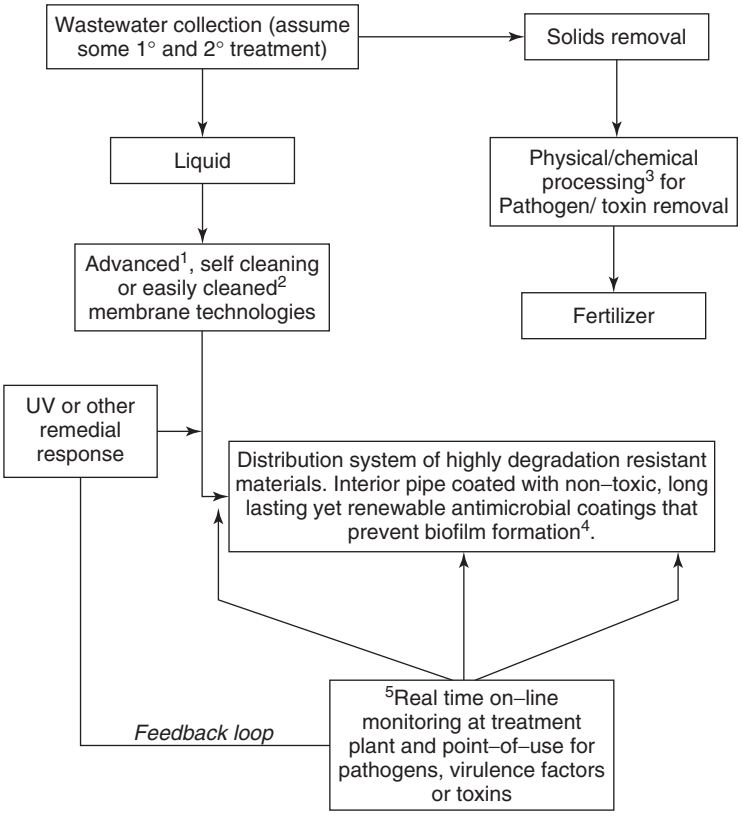
off after a few months to increase access! Similarly, operation and maintenance are critical and a technology is essentially useless if replacement parts are not available. Education can be achieved through many different avenues, from theater to workshops (Ford and Colwell, 1996). Presenting ideas through pictures is often far easier than the written word. For example, it is far easier to visualize the three-pot system promoted by Loughborough University (Figure 2.3) than to read a description.

It would be wrong to dismiss the technological advances of the developed world as options for control of waterborne disease in developing countries. The nanoparticle revolution may provide low-cost solutions that have yet to be developed. The idealized scheme for future provision of safe drinking water depicted in Figure 2.4 remains an ideal for both developed and developing nations. The key issue here is that water scarcity is linked to water quality in many countries, particularly in sub-Saharan Africa, where the greatest burden of many diseases remains. A clean water supply is simply not realistic in many of these areas, where water supplies may be transient and, when present, highly contaminated.

2.6 SURVEILLANCE, PREDICTION, AND MODELING

Important elements of public health preparation for prevention of infectious disease outbreaks include reporting and surveillance. Unfortunately, diarrheal diseases such as cholera are seriously underreported in developing countries, for a variety of reasons. Vulnerable elements of society are less likely to seek or have access to medical treatment and have their cases diagnosed and reported. Governments in developing countries may not be willing to allocate resources for preventive public health measures and surveillance. Fear of travel and trade restrictions may also lead to reluctance on the part of governments to acknowledge and report cholera outbreaks (WHO, 2007a).

Control of waterborne disease is perhaps best facilitated by accurate surveillance and prediction. Surveillance has been improved significantly by the introduction of ProMED Mail in the early 1990s. Today, an emerging area of research is the use of



[1]Many companies now invest heavily in micro–filtration, ultra–filtration, nano–filtration and Reverse Osmosis membrane technologies. Hollow–fiber filtration technologies, for example, are allowing filtration capacities adequate for municipal water systems.
[2]For example, patented gas backwash sytems for USFilter's Memcor® microfiltration systems.
[3]There is currently considerable debate on appropriate criteria for land application of sewage sludges. Known as biosolids, treatment ranges from production of Class B biosolids, with a consequent risk to surface waters, to Class A biosolids, which are essentially pathogen free.
[4]For example, the substituted furanones discussed in the text.
[5]The ideal monitoring tool depicted in the recent American Academy of Microbiology Report (Rose and Grimes, 2001).

Figure 2.4 Idealized scheme for future provision of safe drinking water. (Reprinted from Ford, T.E. 2004. Future needs and priorities. In Cloete, T.E., Rose, J., Nel, L.H., and Ford, T. (eds). *Microbial Waterborne Pathogens*, IWA Publishing, London. pp. 184–204, with permission from the copyright holders, IWA.)

satellite imagery in disease prediction—using satellite data to track the environmental conditions that lead to disease outbreaks. Although developed initially for tracking vectorborne diseases such as hantavirus and malaria through monitoring land-use patterns and climate change, recent conferences and publications have highlighted the predictive power of satellite data for diseases such as cholera. Colwell and Calkins (unpublished data) have used a composite environmental model to relate cholera outbreaks to sea surface temperature, sea surface height, and chlorophyll *a* (using NASA remote imaging technologies).

Cholera epidemics are known to have strong ties to changes in climatic conditions, and it has been suggested that cholera outbreaks can be predicted by monitoring changes in season, sea surface temperature, water temperature, water depth, rainfall, conductivity, pH, sunlight, and the abundance of phytoplankton, zooplankton, and copepods (summarized by Huq et al., 2005). Predictive cholera models are still in the developmental phase (WHO, 2004). An early warning system will need to include pathogen, host, and environmental components (Koelle et al., 2005). The ecological parameters of environmental change that influence the evolution and transmission of pathogens are still poorly understood (Sack et al., 2003). Knowing when an outbreak is likely to occur can inform public health workers to stress basic hygiene and sanitation and to implement simple mitigation efforts such as Sari cloth filtration, which in some areas is credited with reducing cholera mortality by more than 50% (Colwell et al., 2003).

2.7 CONCLUDING THOUGHTS

Most waterborne disease is preventable given basic hygiene and sanitation, but these two factors are critical and possible only through education. Over the past two decades, the mantra of the international agencies has become "education and empowerment of women." Although critical, it is not enough. Many developing countries are primarily male-dominated at the government level, and it is governments that decide resource allocations—new government offices or a new waste treatment plant? Governments are also responsible for aid distribution and for accelerating international conflict. No human behavior exacerbates infectious diseases more than international conflict. The inevitable refugee populations and destruction of infrastructure create enormous opportunities for disease transmission (Mbabazi et al., 2006), and the psychological stressors that must inevitably increase population susceptibility to all disease (Glaser et al., 1999).

Can we control waterborne disease in developing countries? From the Asian tsunami event, it is clearly possible, but a strong political will is necessary, as is cooperation between people and governments. For this to happen, education of politicians, and particularly people in power, becomes pivotal and will involve a dramatic cultural shift for most societies. The potential gains in global health are, however, significant. Prüss-Üstün et al. (2008) suggest that about 10% of the "global burden of infectious disease could be prevented by improving water supply, sanitation, hygiene and management of water resources." The authors suggest the percentage may be higher if we could include such currently unquantifiable diseases as legionellosis, leptospirosis, and others. In addition, the contribution of waterborne disease to morbidity and mortality from diseases such as HIV and malaria is as yet unknown, but is likely to be high.

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CHAPTER 3

New Molecular Methods for Detection of Waterborne Pathogens

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3.1 DISCOVERY, CHARACTERIZATION, AND MONITORING OF WATERBORNE PATHOGENS

The testing of waters for pathogens has been undertaken since waterborne diseases were first recognized. In 1884, after discovery of culture media and microscopy, Robert Koch first isolated a pure culture of *Vibrio*, and Georg Gaffky isolated the typhoid bacillus (Beck, 2000), the known major causes of waterborne disease in the nineteenth century: cholera and typhoid, respectively. In the 1950s, poliovirus was first isolated from water (Gerba and Rose, 1989) and the field of environmental virology began, with the first isolation of viruses from treated drinking water in 1961 in France. The examination of protozoan parasites in water occurred in the 1940s for *Entamoeba* and began in the 1960s and 1980s, respectively, for *Giardia* and *Cryptosporidium*, shortly after these parasites were described as human pathogens and waterborne disease outbreaks had been reported.

The discovery of new pathogens, especially those that can be spread to a multitude of people through water, has always sparked the development of methods for their isolation and characterization from water; this includes, for example, *Legionella* (McDade et al., 1977), *Helicobacter* (Goodwin et al., 1989; Nayak and Rose, 2007), norovirus (Kapidian et al., 1972), and the toxins of cyanobacteria in the 1980s (Sivonen and Borner, 2008). So while fecal indicator bacteria such as coliforms, *Escherichia coli*, and enterococci have been used for routine daily monitoring and for regulatory control, advancements in new tools and techniques have opened the door for evaluating and characterizing novel waterborne pathogens.

Direct waterborne pathogen monitoring can now be undertaken for any microorganism of concern or interest. Molecular tools since polymerase chain reaction (PCR)

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emerged on the scene (1984) have been used for every waterborne pathogen; in fact, multiplexing, the detection of many pathogens simultaneously, is an approach that is now being explored rapidly. The study of pathogens in waters has become imperative: in particular, for applications toward discovery and environmental epidemiology, outbreak investigation, and risk assessment and control.

Yet while advances are being made, it is clear that further considerations must be given to the complete process for pathogen testing of waters. Pathogen detection in water must be considered as a search for a rare biological entity in a soup of biomes. This means that larger volumes of water must be collected, and generally, filtration has been used and remains the primary method for concentration. Extraction and purification and in many cases further concentration are needed, inhibition then becomes a big problem. Both quantification and determination of the infectious nature of the pathogen are of extreme interest. To undertake adequate risk assessment sensitivity, specificity along with potential viability must be determined.

The methods described in this chapter can be used for the detection and characterization of all waterborne pathogens and should be used to better understand the geographical distribution of these microorganisms in our global waters, given the threat of waterborne disease. To improve knowledge of waterborne pathogen fate and transport, concentrations and accumulation, and finally, spatial and temporal occurrences, these novel methods can now be used in water quality studies worldwide.

The latest methods used for the detection of viruses, bacteria, and protozoans in water samples are primarily molecular. Conventional methods are presented briefly in Table 3.1, and the newest detection methods for waterborne pathogens are described in Table 3.2. Effective detection of waterborne pathogens includes a series of steps, such as sample concentration, nucleic acid extraction, inhibition control, design of

Table 3.1 Waterborne Pathogen Detection: Conventional Detection Methods

Pathogen	Method	Outcome	Characteristics
Viruses	Cell culture and infectivity	Presence/absence, infectivity determination	Infected cell cultures undergo morphological changes called cytopathic effects (CPEs) that are observed microscopically. The method is labor intensive and time consuming. Some viruses do not show CPEs.
Bacteria	Bacterial culture	Presence/absence quantification	Based on selected media, various groups of bacteria can be detected.
Protozoans	Microscopic methods along with fluorescent antibody tags	Counts of oo(cysts)	Antibody specific to the cell wall of the oo(cysts) tagged to fluorescence is used to stain the sample. Using morphometrics and fluorescence, the organisms are identified. No infectivity can be determined, and volumes that can be processed under the microscope are relatively small.

Table 3.2 Waterborne Pathogen Detection: New Detection Methods

Method	Outcome	Characteristics
PCR	Presence/absence	Able to detect nonculturable pathogens. High sensitivity and specificity. Requires design of primers that amplify specified DNA regions. Prone to environmental inhibition. Gel electrophoresis is required for visualization of results.
RT-PCR	Presence/absence	A reverse transcription step (RT) is required before PCR amplification, for the conversion of RNA to cDNA. Following RT, the same steps are followed as with conventional PCR.
Nested PCR	Presence/absence	Requires two sets of primers. Inner primers amplify the target sequence within the amplicon generated by outer primers. This technique has a higher sensitivity than that of conventional PCR.
Multiplex PCR	Presence/absence	Uses multiple primer sets in a single PCR reaction to detect multiple targets simultaneously. It is time efficient and reduces the cost of reagents. The design and optimization of multiplex assays can be more challenging than those of conventional PCR assays.
Real-time PCR	Quantitative	This is the only quantitative method (except for the MPN dilution method). No post-PCR handling step is required to view the results. It is a very target-specific and sensitive method. The costs of a thermocycler and reagents are higher than those used in conventional PCR.
ICC-PCR	Presence/absence, infectivity determination	This is a combination of the traditional cell culture/cell infectivity method with PCR. PCR is performed on cell culture supernatant. The method has higher sensitivity than that of conventional PCR.
Microarrays	Presence/absence	Able to detect multiple microbial agents simultaneously. Microarrays provide rapid results and are an appropriate screening method.

appropriate primers, and confirmation of results. A flow schematic for the detection of waterborne viruses, indicating all these steps and processes, is also shown (Figure 3.1).

Inhibition is one of the most common problems encountered environmental molecular laboratories. Wilson (1997) reviewed inhibition and facilitation of nucleic acid amplification. He grouped the mechanisms of inhibition into three categories: failure of lysis, nucleic acid degradation and capture, and polymerase inhibition. The inhibitor materials can be organic or inorganic compounds naturally present in the environment. All of these compounds may inhibit the enzymes involved in PCR. It is important to note that during sample collection and concentration, inhibition materials are also concentrated. Humic substances are the natural organic substances that have been most generally identified as inhibitors in environmental samples. Other organic compounds that may cause inhibition include fulvic acid, tannic acid, proteins, polysaccharides, and glycoproteins. Inhibitory inorganic compounds could be metals. Many studies have investigated the removal of PCR inhibitors. However, there is no single method that could remove all inhibitors. The humic acid removal techniques summarized by Wilson

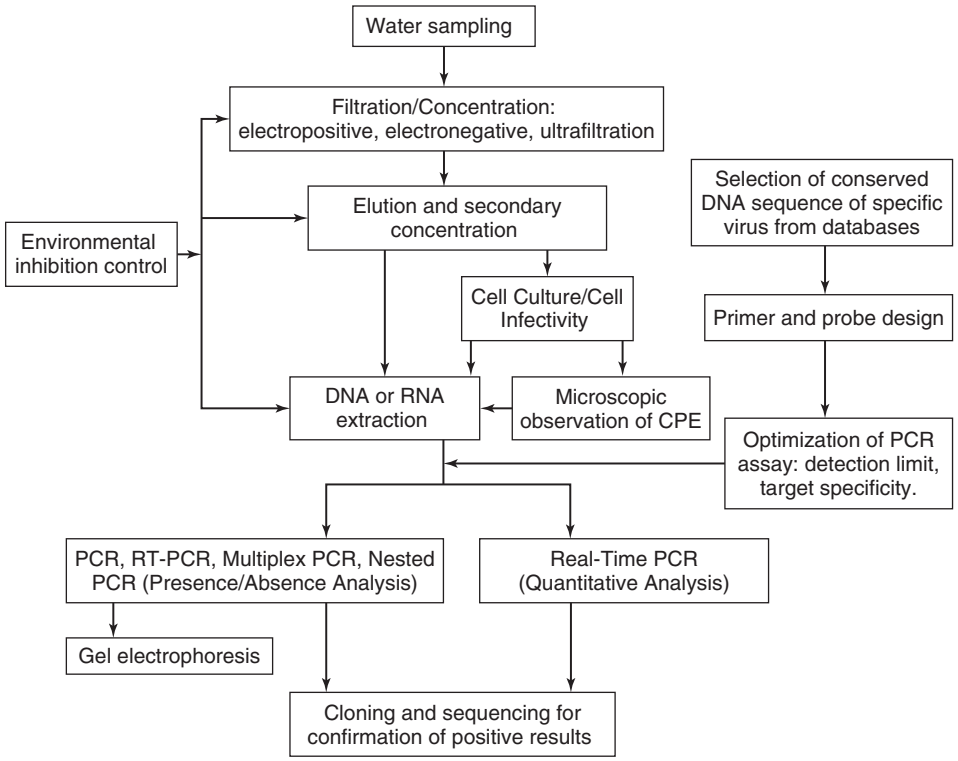

Figure 3.1 Overview of a virus detection procedure.

(1997) include Pro-Cipitate, polyethylene glycol, antibody capture, gel filtration, dilution, physicochemical extraction, lysozyme–hot phenol extraction, ion-exchange chromatography, percoll-sucrose centrifugation, and vortex flow filtration.

The design of primers and probes is a critical step in any molecular assay since it defines the specificity of the PCR reaction. The general procedure for primer and probe design begins with retrieving the nucleotide sequences of specific target organisms from the gene bank. Sequences are then aligned using software such as ClustalW. Primers and probes can be designed in a consensus or variable region of the genes, depending on the specificity and purpose of the PCR assay. Numerous software is available for different types of PCR assay designs. Some software is available online (e.g., Primer3, Input 0.4.0). Other software can be purchased (e.g., Primer Express 2.0, Lightcycler Probe Design 2). After the assay is designed, a BLAST search is recommended to make sure that the assay is specific for the targets. Assay optimization for increasing sensitivity is usually achieved by adjusting the annealing temperature, primer and probe concentration, and magnesium concentration. Sequencing of PCR products is commonly used to confirm the results of molecular assays.

3.2 NOVEL DETECTION METHODS FOR WATERBORNE BACTERIA

As with viruses and protozoans, the detection of bacterial pathogens in water is critical to the continued safety of drinking water supplies. The list of possible microbial

contaminants is long (e.g., *E. coli, Vibrio cholerae, Salmonella typhi, Campylobacter jejuni, Legionella pneumophila, Shigella sonnei, Helicobacter pylori*), as is the list of methods (e.g., colony counts, enzyme immunoassays, PCR, fluorescent In situ hybridization) to detect these microorganisms.

For samples with low bacterial counts, the most common way to process and concentrate the sample is by membrane filtration (MF). Nitrocellulose membranes with pore size 0.45 μm are commonly used for MF. During filtration, bacteria in the water sample are trapped on membrane filters. Then the filters are placed on the agar and are incubated at appropriate temperatures. Recovery of bacteria using MF is high. For example, the method enabled the recovery of more than 90% of *E. coli* from marine, estuarine, and freshwater samples (Dufour et al., 1981). Similarly, 102% of enterococci was recovered in marine water using the MF technique (Levin et al., 1975). The time required to process samples depends on sample turbidity.

Here we limit our discussion to novel and emerging detection methods, using *E. coli* as our example bacterial pathogen. *E. coli* remains one of the most important organisms in water and is also one of the most diverse genetically (both pathogenic and nonpathogenic types) as well as the best studied. *E. coli* exists as part of the normal intestinal flora and is typically harmless; however, some strains can cause illness. Emerging technologies to detect these bacteria typically target virulence genes; therefore, we first provide an introduction to the virulence factors for each *E. coli* category. Diarrheagenic *E. coli* have been divided into five categories (Nataro and Kaper, 1998): enteropathogenic (EPEC), enteroaggregative (EAEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and Shiga toxin–producing (STEC) *E. coli*. In addition, extraintestinal *E. coli* (ExPEC) can be an important human pathogen (Russo and Johnson, 2000).

The Shiga toxin (Stx1 and Stx2)–producing *E. coli* (STEC) can cause bloody diarrhea as well as potentially fatal diseases in humans, including hemolytic–uremic syndrome (HUS) and hemorrhagic colitis (Nataro and Kaper, 1998; Paton and Paton, 1998b; Kaper et al., 2004; Mainil and Daube, 2005). Many STEC carry additional virulence factors, including enterohemolysin (*ehxA*) (Brunder et al., 1999, 2006), extracellular serine protease (*espP*) (Brunder et al., 1997), and/or catalase-peroxidase (*katP*) (Brunder et al., 1996). Some pathogenic STEC strains also contain a pathogenicity island, known as the locus of enterocyte effacement (LEE), and these strains are often called enterohemorrhagic *E. coli* (EHEC) (Kaper et al., 2004). Factors encoded by LEE known to be contribute to pathogenicity include intimin (*eaeA*) and a host epithelial receptor for intimin called Tir (translocated intimin receptor) (LeBlanc, 2003; Welinder-Olsson and Kaijser, 2005). Diarrheagenic *E. coli* strains containing LEE but not producing Shiga toxins are called enteropathogenic *E. coli* (EPEC) and are a leading cause of infantile diarrhea, especially in developing countries (Nataro and Kaper, 1998; Trabulsi et al., 2002; Kaper et al., 2004). Intimin is a key virulence factor in EPEC (Nataro and Kaper, 1998). Some EPEC strains, called *typical EPEC*, also harbor an EPEC adherence factor (EAF) plasmid, whereas EPEC strains lacking this are called *atypical EPEC* (Trabulsi et al., 2002). Although the plasmid is not necessary to cause human diseases, some plasmid genes, such as regulatory genes (*perABC*) and genes encoding bundle-forming pili (*bfp*), enhance virulence (Gunzburg et al., 1995; Nataro and Kaper, 1998; Trabulsi et al., 2002). The other categories include enterotoxigenic *E. coli* (ETEC), which produce heat-labile (LT) and/or heat-stable (ST) enterotoxins;

enteroinvasive *E. coli* (EIEC), which invade the colonic epithelium; and enteroaggregative *E. coli* (EAEC), which are characterized by an aggregative adherence (AA) pattern on cultured epithelial cells and by the production of fimbrial colonization factors called *aggregative adherence factors* (AAFs) (Nataro and Kaper, 1998; Aranda et al., 2007).

Although traditional microbiological methods such as colony counts or enzyme immunoassays are clearly useful, methods based on specific nucleic acids have significant additional advantages for *E. coli* detection. Here we review several of these techniques (multiplex PCR, reverse transcription PCR, and microarrays), the advantages of these methods, and their use in investigating waterborne *E. coli*.

3.2.1 *E. coli* Detection Methods

Multiplex PCR (mPCR) A key advantage of mPCR involves increased specificity because more than one gene is targeted. Further, when the method is combined with real-time PCR, target quantification is possible. Many mPCR assays have been developed to detect and/or quantify *E. coli*, and the key details of a subset of these are provided (Table 3.3).

A particularly useful mPCR method to differentiate between the *E. coli* categories was recently reported. These researchers used seven primer pairs to differentiate EPEC (typical and atypical), EAEC, ETEC, EIEC, and STEC (Aranda et al., 2007). The method involved targeting *eae* (structural gene for intimin of EPEC and EHEC) and *bfpA* (structural gene for the bilus-forming pili of typical EPEC) for EPEC, *aggR* (transcriptional activator for the AAFs of EAEC) for EAEC, *elt* and *est* for ETEC (heat-labile and heat-stable enterotoxins of ETEC), *ipaH* (invasion plasmid antigen H found in EIEC and *Shigella*) for EIEC, and *stx* (Shiga toxins 1 and 2 and variants) for STEC. The assay was tested for specificity with reference strains and clinical isolates and used to detect *E. coli* in stool samples of children with and without diarrhea. They found that atypical EPEC was the most commonly isolated category of diarrheagenic *E. coli*.

A multitude of mPCR methods have also been developed to identify specific groups of *E. coli*. A particularly large number of techniques have been reported for EHEC and STEC *E. coli*, typically targeting two or more of the following: genes encoding for Shiga toxins 1 and 2 (*stx1* and *stx2*), intimin (*eaeA*), enterohemolysin (*ehxA*), a megaplasmid-encoded adhesion, Saa (*saa*), and/or a novel toxin A subunit gene (*subA*) (Paton and Paton, 1998b, 2002, 2005; Fagan et al., 1999; Feng and Monday, 2000; Bellin et al., 2001; Osek, 2002; Reischl et al., 2002). Other mPCR assays have enabled identification of the *E. coli* serotypes O157, O26, O111, O103, O121, and O145 (Paton and Paton, 1998a; Osek, 2002; Monday et al., 2007; Murphy et al., 2007b), *E. coli* O157:H7 (Hu et al., 1999; Wang et al., 2002; Jinneman et al., 2003; Osek, 2003; Sharma and Dean-Nystrom, 2003; Childs et al., 2006), enteroaggregative *E. coli* (Cerna et al., 2003; Jenkins et al., 2006a,b; Ruttler et al., 2006; Cordeiro et al., 2008), *E. coli* safety and laboratory strain lineages (K-12, B, C, and W) (Bauer et al., 2007), uropathogenic *E. coli* (Arisoy et al., 2006), and enterotoxigenic *E. coli* (Grant et al., 2006).

Methods Addressing Viability: Targeting E. coli mRNA The inability of DNA-based molecular methods to distinguish between live and dead cells is a significant limitation toward monitoring possible pathogens in water samples. To

address this, researchers have investigated the potential for mRNA as an alternative target for detecting viable *E. coli* (Sheridan et al., 1998; McIngvale et al., 2002; Yaron and Matthews, 2002) Early studies targeted *E. coli* mRNA for two genes (*groEL* and *rpoH*) involved in stress response, as well as a gene (*tufA*) for an abundant cellular housekeeping protein, and concluded that mRNA was indeed an effective indicator of viability (Sheridan et al., 1998). Since then, others have developed reverse transcription PCR methods to specifically target viable STEC cells via detection of mRNA associated with the production of Shiga toxin 2 (McIngvale et al., 2002). Another study investigated a number of target genes as indicators of viability and concluded that the *rfbE* gene (responsible for O side-chain synthesis) was the most appropriate target to detect viable *E. coli* O157:H7 (Yaron and Matthews, 2002).

The specificity of such mRNA detection methods has been advanced further by combining reverse transcription with multiplex PCR (RT mPCR). For example, viable *E. coli* O157:H7 was detected using RT mPCR targeting the lipopolysaccharide gene (*rfbE*) and the H7 flagellin gene (*fliC*) (Morin et al., 2004). Reverse transcription has also been combined with real-time multiplex PCR technologies (RT mRTPCR). For example, techniques have been developed to detect mRNA encoded by *rfbE* and *eae* genes of *E. coli* O157:H7 in pure cultures and bovine feces (Sharma, 2006). Others have developed RT mRTPCR methods (targeting *eae*, rfb_{O157}, and *stx2*) to detect *E. coli* (EHEC) O157 in pure culture, food, and stool samples (Tsai et al., 2006). The researchers investigated viability and found the method that was able to distinguish viable and nonviable cells (Tsai et al., 2006).

Microarrays Microarrays probably represent the future for detecting waterborne pathogens because of the tremendous investigative power provided by the parallel detection of many genes. For instance, one microarray was able to detect 189 *E. coli* virulence and virulence-related genes as well as 30 antimicrobial resistance genes (Bruant et al., 2006). A large number of other studies have developed microarray technology for *E. coli*, targeting virulence genes (Bekal et al., 2003; Bruant et al., 2006; Dowd and Ishizaki, 2006; Hamelin et al., 2006; Kon et al., 2007), O-antigen gene clusters (Liu and Fratamico, 2006), or antibiotic resistance genes (Chen et al., 2005; Bruant et al., 2006; Hamelin et al., 2006; Kon et al., 2007). Microarray technology has also been developed to detect other bacterial pathogens, targeting virulence genes (Chizhikov et al., 2001; Lee et al., 2006), 23S rRNA gene (Lee et al., 2006), 16S rRNA gene (Maynard et al., 2005), *cpn60* gene (GroEL, a chaperonin protein) (Maynard et al., 2005), *wecE* gene (enterobacterial common antigen biosynthesis) (Maynard et al., 2005), and *gyrB* gene (subunit B of bacterial gyrase)(Kostic et al., 2007).

3.2.2 Detecting *E. coli* in Water

Numerous mPCR methods have been used to detect *E. coli* in water. For example, mPCR techniques have been developed to detect ETEC and STEC *E. coli* in water through targeting the genes associated with the heat-labile toxin I and heat-stable toxin II (ETEC) as well as Shiga-like toxins 1 and 2 (STEC) (Tsen and Jian, 1998). Others have established mPCR methods to specifically target *E. coli* O157:H7 in water and soil, targeting genes associated with O157, H7, intimin, Shiga-like toxins 1 and 2 of *E. coli* O157:H7 (Campbell et al., 2001; Hu et al., 1999). Further, through combining mPCR (*stx1, stx2*, and *eae*) with real-time PCR, quantification of *E. coli* O157:H7 in dairy wastewater wetlands was possible (Ibekwe et al., 2002).

Table 3.3 Multiplex PCR Methods to Detect *E. coli*

Target	Methodology and Target	Reference
Urinary tract *E. coli*	Multiplex PCR (mPCR) for genotyping: *chuA* and *yjaA* genes and TspE4.C2 (anon. DNA fragment)	Piatti et al. (2008)
E. coli O157:H7	Real-time mPCR: *stx1*, *stx2*	Grant (2008)
EAEC	Real-time mPCR: three EAEC: *aggR, aap, aatA*	Cordeiro et al. (2008)
O-serogroups of STEC	Real-time mPCR: O-antigen of *E. coli* O26, *E. coli* O111, *E. coli* O157, *eae* of *E. coli* O103, O-island 29 of *E. coli* O145	Perelle et al. (2007)
EPEC, EAEC, ETEC, EIEC, STEC	mPCR: *eae* and *bfpA* (EPEC), *aggR* (EAEC), *elt* and *est* (ETEC), *ipaH* (EIEC), *stx* (STEC)	Aranda et al. (2007)
Four safety strains	mPCR: five primer pairs for lineage specific DNA fragments	Bauer et al. (2007)
Pathogenic *E. coli*	mPCR: *stx1*, *stx2*, *eaeA, ehxA*	Ishii et al. (2007)
EHEC serotypes	mPCR: *stx, eae*, 16S sequences in *wzx* genes, specific for O157, O26, O111, O103, O121, and O145	Monday et al. (2007)
Intestinal pathogenic *E. coli*	mPCR: *uidA, escV, bfpB, stx1*, *stx2*, *elt, estIa, estIb, invE, astA, aggR, pic*	Muller et al. (2007)
E. coli O26, O111, O157	Triplex PCR: *rfb* genes of *E. coli* O157 and O111 (two primer pairs, *E. coli* O26 *wzx*)	Murphy et al. [2007a,2007b]
ETEC, EPEC, EIEC, EHEC	Real-time mPCR: *lt, st, stx1*, *stx2*, *eae, ipaH*	Yang et al. (2007)
Pathogenic *E. coli*	Real-time mPCR: fimbriae K88, K99, and F41, LT, STa, CDT IV, fimbriae F17, F18, and 987p	West et al. (2007)
E. coli O157:H7	Three mPCR procedures: *stx1*, *stx2*, *hlyA, rfbE*$_{O157}$, *fliC*$_{H7}$, *eaeA*$_{O157}$	Childs et al. (2006)
ETEC	Real-time mPCR: heat-labile and heat-stable toxin genes	Grant et al. (2006)
EAEC	mPCR: *aat, aai, astA*	Jenkins et al. (2006b)
EPEC, atypical EPEC, STEC	mPCR: *escV, bfpB, stx1*, *stx2*	Muller et al. (2006)
EAEC	mPCR: *aggR, astA*	Ruttler et al. (2006)
E. coli O157:H7	mPCR—real-time reverse transcription: *rfbE*$_{O157}$, *eae*$_{O157:H7}$ genes	Sharma (2006)
	mPCR—real-time reverse transcription: *eae, rfb*$_{O157,}$ *stx2*	Tsai et al. (2006)

Uropathogenic *E. coli*	mPCR: *pap, sfa, afaI, hly, cnfI, aer*	Arisoy et al. (2006)
Diarrheagenic *E. coli*	Two mPCR reactions: *st, lt, vt, invE, aggR, eaeA, astA*	Bii et al. (2005)
	mPCR: *stx1, stx2, eaeA, bfpA, invE, elt, estp, esth, astA, aggR*, EAF, CVD432	Kimata et al. (2005)
	mPCR: *eaeA, bfpA, vt1, vt2, eltB, estA, ial*, pCVD	Nguyen et al. (2005)
STEC isolates	mPCR: *subA, stx1* , *stx2*	Paton and Paton (2005)
Diarrheagenic *E. coli*	mPCR: *stx1* , *stx2* , *eae, bfp, stII, lt, virF, ipaH, aafII, daaE*	Vidal et al. (2005)
VTEC/EHEC, ETEC, EIEC, EPEC	mPCR: *stxI, stxII, st, lt, eaf* , IAL	Watterworth et al. (2005)
EHEC, EPEC, ETEC	mPCR: *stx1* , *stx2* , *eae, bfp, stII, lt*	Vidal et al. (2004)
EPEC, ETEC, verocytotoxin *E. coli*	mPCR: *hlya, st, eaeA, lt, vt1, vt2*	Bottero et al. (2004)
STEC	Duplex TaqMan PCR: *stx1* , *stx2*	Perelle et al. (2004)
EHEC *E. coli* O157:H7	Real-time mPCR: $eae_{O157:H7}$, *stx1* , *stx2*	Sharma and Dean-Nystrom (2003)
EAEC	mPCR: aggregative adherence (AA), *aap, aggR*	Cerna et al. (2003)
E. coli O157:H7/H$^-$	Real-time mPCR: *stx1* , *stx2* , SNP in *E. coli* O157:H7/H$^-$ *uidA*	Jinneman et al. (2003)
EPEC, EIEC, ETEC, EAEC, STEC	mPCR: *eae, stx, elt, est, ipaH, aggR*	Toma et al. (2003)
STEC	mPCR: *stx1, stx2, stx2c, stx2d, stx2e, stx2f, eaeA, ehlyA, rfb* O157, *fliC* H7	Osek (2003)
	mPCR: *stx1, stx2, eaeA, rfb* O157	Osek (2002)
	mPCR: *stx1* , *stx2* , *eae, ehxA, saa*	Paton and Paton (2002)
	Real-time PCR: *stx1* , *stx2* , *eae*, E-*hly*	Reischl et al. (2002)
	mPCR assays: *stx1* , *stx2* , *stx2c*, *stx2d*, *stx2e*, *stx2f* , EHEC *hlyA, eaeA, rfbE, fliC, E. coli* 16S rRNA	Wang et al. (2002)
	Real-time PCR: *stx1* , *stx2* ,	Bellin et al. (2001)
Enterovirulent *E. coli*	mPCR: *stx1* , *stx2* , *eae* (X2), *bfpA, ipaH* , LT, ST, *aggC, east-1*	Rich et al. (2001)
EHEC serotypes	mPCR: *uidA, eaeA, stx1, stx2*, both variants of *ehxA*	Feng and Monday (2000)
E. coli virulence genes	mPCR: VT1, VT2, VT2e, *eaeA*, CNF1, CNF2, LTI, STI, STII, Einv, Eagg	Pass et al. (2000)

Other mPCR assays have enabled detection of certain *E. coli* serogroups (O157, O111, and O113) in water. Here, researchers applied two previously developed mPCR methods (China et al., 1996; Paton and Paton, 1999) to detect these organisms in water sampled from dairy farms in Brazil (Vicente et al., 2005). They first targeted the virulence genes *stx1*, *stx2*, and *eae* (China et al., 1996); then for positive samples, a second mPCR assay was utilized targeting *rfb* O157, *rfb* O113, and *rfb* O111 (Paton and Paton, 1999). Others have used mPCR to identify potentially pathogenic *E. coli* from a beach environment (Lake Superior) frequently closed because of high *E. coli* counts (Ishii et al., 2007). In this study, four virulence genes [*stx1*, *stx2*, *eaeA*, and *ehxA* (Paton and Paton, 1998a)] were targeted. The researchers found that from 3557 *E. coli* strains obtained from lake water, sediment, and sand, only one could be classified as a potential human pathogen, an enteropathogenic *E. coli* (EPEC).

Microarrays have been especially valuable for investigating *E. coli* pathotypes in water samples (Hamelin et al., 2006, 2007). In one study, researchers found that a significant percentage (29%) of beach isolates carried a pathotype set of virulence-related genes, and a smaller percentage (14%) carried antimicrobial resistance genes (Hamelin et al., 2006). Another microarray-based study found that the distribution of *E. coli* pathotypes differed significantly between sampling sites (surface water at six locations), with ExPEC being the most commonly encountered pathotypes (Hamelin et al., 2007).

A recent article illustrates the impressive potential of microarray technology for detecting a large number of pathogens (Miller et al., 2008). These researchers developed and validated an *In situ*–synthesized biochip for the detection of 12 microbial pathogens relevant to clinical diagnostics as well as water and food safety (Miller et al., 2008). The method involved probes designed for multiple virulence and marker genes (VMGs) for each pathogen, with each VMG being targeted by an average of 17 probes. A split multiplex PCR design was used to amplify target genes simultaneously, providing a detection limit of 0.1 to 0.01% relative abundance, depending on the VMG and the pathogen. In addition, the biochip was validated using DNA obtained from three different types of water samples spiked with pathogen genomic DNA. The ability to detect multiple pathogens in parallel and in complex matrixes indicates that microarrays will probably play a growing role for monitoring water quality.

Molecular methods offer both advantages and disadvantages over traditional methods for detecting and identifying *E. coli* populations in water. Both sides are nicely illustrated by a recent investigation of ETEC in household and environmental water samples from Bangladesh, involving both a quantitative real-time PCR method and the toxin GM1 ganglioside-enzyme-linked immunosorbent assay (GM1-ELISA) (Lothigius et al., 2008). The real-time PCR method, which quantifies the ETEC enterotoxin genes for the production of heat-labile (LT) and heat-stable (ST) enterotoxins (*estA, estB*, and *eltB*), found that 26 of the 39 samples (67%) were positive for ETEC, but only six samples (15%) were positive in the GM1-ELISA. The study highlights the common advantage of molecular-based methods, increased sensitivity, and thus reduced risk for false negatives. However, this advantage also comes with the concern of overestimating the number of infectious cells, as the method does not discriminate between live and dead cells. In addition, the real-time method does not facilitate differentiation between different strains in the same sample. For example, the method does discriminate between samples with a double positive strain (ST/LT) or with separate ST-positive and LT-positive strains. In contrast, in GM1-ELISA, separate colonies can be tested for ST and LT enterotoxin production.

Although molecular methods provide a powerful platform for pathogen detection, it appears that in some cases, more refinement is needed for the detection of specific pathogenic *E. coli* in water samples. For example, researchers involved in a 30-month surface water monitoring study concluded that current immunological and PCR assays (real-time PCR targeting stx_1, stx_2, and *eae* genes (Higgins et al., 2005) could not reliably identify waterborne enterohemorrhagic *E. coli* (EHEC) (Shelton et al., 2006). Others have reported the inadequacies of using the gene (*ehlyA*) encoding for the putative virulence factor enterohemolysin for detecting environmental EHEC (Boczek et al., 2006). They found that although enterohemolysin production among environmental *E. coli* isolates is common, the enterohemolysin positives isolates did not exhibit the necessary virulence factors to be classified as EHEC.

The most useful methods for detecting *E. coli* in water are those that provide information on cell viability. The importance of such methods was illustrated in a study investigating viable but nonculturable cells (VBNCs) in nondisinfected drinking water (municipal tap and private well water) (Bjergbaek and Roslev, 2005). These researchers examined the ability of four different *E. coli* strains to enter a VBNC state using both traditional and molecular-based methods [cultivation, *gfp*-tagged *E. coli*, fluorescent in situ hybridization (FISH) and direct viable counts]. In addition, various resuscitation procedures were used to investigate the recovery of stressed *E. coli* from drinking water samples. Interestingly, they reported that the *E. coli* strains could enter a state where they were undetectable with standard media but able to maintain the potential for metabolic activity and sometimes, cell division. They reported that apparently nonculturable cells were fully resuscitated to a culturable state. The authors conclude that potentially viable *E. coli* may not be detected with standard procedures during the routine analysis of drinking water.

More recently, a highly sensitive method of detecting viable *E. coli* O157:H7 cells in water samples was developed (Liu et al., 2006, 2008). In fact, according to the authors, the procedure resulted in the lowest limit of detection reported to date for viable but not culturable cells in environmental water samples. The technique involved efficient cell capture, RNA extraction and purification, followed by detection via reverse transcription PCR and electronic microarray detection of the *E. coli* O157 lipopolysaccharide gene (*rfbE*) and the H7 flagellin gene (*fliC*) gene of *E. coli* O157:H7. The electron microarray facilitated DNA concentration on the microchip and contributed significantly to assay sensitivity. The assay enabled detection of 3 to 4 CFU/L tap water, 7 CFU/L river water, and 50 VBNCs/L river water. Clearly, this method has great potential for monitoring viable *E. coli* in environmental and drinking water samples.

3.3 METHODS FOR ADDRESSING EMERGING PROBLEMS FOR WATER

3.3.1 Genetic Characterization of Toxic Algae in Water

Harmful algal blooms (HABs) are considered emerging waterborne pathogens because of the toxins produced by these organisms (cyanobacteria or "blue-green algae"). Thus, monitoring the toxin-producing cyanobacteria is critical for the continued protection of our drinking water sources. In fact, cyanotoxins are on the U.S. Environmental Protection Agency's (EPA's) current (version 3) contaminant candidate list. However, because each cyanotoxin can be produced by more than one cyanobacterial species and the same species can produce more than one toxin (Funari and Testai, 2008), this is a

particularly challenging research area. In addition, some genotypes of a single species possess the gene for a given toxin, whereas others will not (Funari and Testai, 2008), and traditional methods, such as microscopy, are unable to distinguish between these organisms. To address this, researchers have focused on molecular monitoring methods based on the detection of the genes responsible for toxin production. A significant number of molecular detection methods have involved the genes responsible (*mcy* operon) for production of the cyanotoxin microcystin (Tillett et al., 2000). These genes have been used in real-time PCR assays to quantify microcystin-producing cyanobacteria from environmental waters (Foulds et al., 2002; Kurmayer and Kutzenberger, 2003; Vaitomaa et al., 2003; Rinta-Kanto et al., 2005; Rinta-Kanto and Wilhelm, 2006). Other molecular detection assays have targeted the genes responsible for production of the cyanotoxins cylindrospermopsin (Fergusson and Saint, 2003; Rasmussen et al., 2008) or nodularin (Koskenniemi et al., 2007), or both microcystin and nodularin (Rantala et al., 2008). Molecular methods clearly have great potential to contribute significantly to our understanding of the mechanisms resulting in cyanotoxin water contamination.

3.3.2 Antibiotic-Resistant Gene Detection

The spread of antibiotic-resistant pathogens is a growing problem. Antibiotic-resistant genes and pathogens have been found in environmental water and waste samples. Various analytical methods have been used for the detection of antibiotic resistance, and these include culture methods and molecular methods. Culture methods identify the resistant pathogens, and molecular methods target specific genes. The DNA extracted for use in molecular methods may be obtained from cultured bacteria or isolated directly by environmental samples.

Molecular ecology tools have been used to study antibiotic resistance. Phylogenetic analysis of tetracycline resistance genes encoding the ribosomal protection proteins revealed the monophyletic origin of these genes (Aminov et al., 2001). Based on the phylogenetic analysis, which showed distinct separate clusters of the genes, a set of PCR primers for the detection, retrieval, and sequence analysis of the corresponding gene fragments for a variety of bacterial and environmental sources was developed and characterized.

Others have used a combination of molecularly and phenotypically based methods to characterize antibiotic resistance genes in nature (Chee-Sanford et al., 2001). Using these methods, the presence of tetracycline resistance genes has been determined in waste lagoons in two swine farms and in the groundwater underlying the two farms. The methods included growing tetracycline-resistant bacteria aerobically on agar amended with antibiotic, DNA extraction, PCR amplification, DGGE analysis, and cloning and sequencing.

Antibiotic-resistant genes have been detected and quantified in various environmental compartments, including river sediments, irrigation ditch water, dairy lagoon water, drinking water treatment plants, and wastewater recycling plants using PCR detection assays for four sulfonamide resistance gene families and five tetracycline resistance gene families (Pei et al., 2006; Pruden et al., 2006). Several gene families were further quantified by real-time quantitative PCR (Q-PCR). Resistance to four classes of antibiotics was also investigated using a culture-based approach. The quantities of resistance genes were normalized to the 16S gene copy number. Combinations of culture techniques, phylogenetic tree analysis, presence/absence molecular techniques, and

quantitative PCR methods can provide new information on the emerging problem of antibiotic resistance spread in the environment.

3.3.3 Microbial Source Tracking

Microbial source tracking (MST) describes a group of methodologies aimed at identifying, and in some cases quantifying, the dominant source(s) of fecal contamination in drinking and environmental waters (Stoeckel and Harwood, 2007). MST approaches can be classified into two categories: library-dependent and library-independent methods. Library-dependent methods typically use databases containing genotypic or phenotypic "fingerprints" of organisms from known sources to which fingerprints from unknown isolates can be compared. Reference libraries can be constructed using isolates collected directly from animals or/and from all over a watershed. Library-dependent MST methods can include ribotyping, pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), repetitive element PCR, antibiotic resistance profiling, and carbon source utilization (Parveen et al., 1999; Dombek et al., 2000; Carson et al., 2001; Gaun et al., 2002; Hartel et al., 2002; Burnes, 2003; Hagedorn et al., 2003; Johnson et al., 2004; Meays et al., 2004).

Library-independent methods, also called host-specific methods, use chemicals, microbes, or their gene markers which are specific to certain hosts. Targets for host-specific methods include *Bacteroides* spp., *Enterococcus* spp., *Bifidobacterium* spp., *Rhodococcus coprophilus*, adenoviruses, enteroviruses, polyomaviruses, and coliphages (Hsu et al., 1995; Bernhard and Field, 2000a,b; Scott et al., 2002; Bernhard et al., 2003; Fong et al., 2005; Jiang, 2006; McQuaig et al., 2006; Brownell et al., 2007; Plummer and Long, 2007). Library-independent methods are now preferred for MST because of their lower time and cost requirements.

The idea of using Bacteroides above other methods as comparisons began for microbial source tracking (Bernhard and Field, 2000a,b; Field et al., 2003), and with the use of length heterogeneity (LH) PCR and terminal restriction fragment length polymorphism which identified fragments from 16S rRNA gene that were specific for humans and bovine. Primers were designed targeting these fragments, which could then differentiate the human feces from the bovine fecal contamination of water samples. Field et al. have estimated that 10% of the Bacteroides cells that comprise 30% of the total fecal bacteria have host-specific markers.

3.4 NOVEL METHODS FOR DETECTION OF PARASITES IN WATER

3.4.1 Waterborne Parasites of Concern

As has been discussed for the bacteria and later in the chapter for the viruses, the new methods for detection and characterization of parasites are moving toward the use of sophisticated molecular tools. Many of the parasites exhibit a biological diversity in the animal kingdom that necessitates this molecular characterization to further understanding of the multiple species and genotypes that cause both human-specific and zoonotic infections. This is particularly true for *Cryptosporidium* and *Giardia*. Yet application of the new methods within a regulatory framework has not moved forward, despite the need for more rapid and specific information for decision making.

There are two main groups of parasites that are of interest in regard to water and public health safety. The first group are the obligate enteric parasites, which are spread by the fecal–oral route and by contaminated water. This includes helminthes such as *Ascaris* and enteric protozoans, including the genera *Balantidium, Cyclospora, Cryptosporidium, Entamoeba, Giardia*, and *Isospora*. (Note that *Toxoplasma*, which is not enteric in humans, is also a parasite of concern and is spread only by feces from infected cats to water to humans.) The other group is the free-living protozoans, which naturally occur in water and include *Ancanthamoeba* and *Naegleria*. Traditionally, the enteric parasites were detected in the stools of infected persons via microscopy, and morphology was used for identification. In fact, *Entamoeba* and *Giardia* were first identified under the early microscope in the 1870s and 1860s in human feces. Now, as then, whether microscopic or molecular methods are used, the presence, the quantification, and finally, the issue of whether these parasites are alive remains of great interest. Table 3.4 lists some of these parasites and describes the relative importance to health and water monitoring.

Ascaris is an intestinal worm that produces ova that the host excretes in its feces and whose prevalence in humans is poorly described. It is transmitted by humans, by soil, by pets, and rarely by water. Outside the United States it is reported that prevalence of *Ascaris lumbricoides* is 0.67% in Spanish populations but as high as 12% and 28.4% in Turkish and Indian populations, respectively (Coskun, 1991; Jarabo et al., 1995; Wani et al., 2007). In water, its importance and detection has been used as an indicator of adequate sedimentation in wastewater lagoons to determine reuse acceptability (WHO/AFESD, 2005) and in biosolids to determine adequate treatment for class A and B material for land application (USEPA, 2003). Neither of these approaches is evidence risk–based; however, more recently, using dose–response methods, a risk assessment for biosolids has been employed to address concentrations of viable *Ascaris* on crops (Navarro et al., 2008).

The enteric protozoans remain an important cause of waterborne disease worldwide. As part of their life cycle, these intestinal parasites produce oo(cysts) which are excreted in the feces of infected hosts and are spread by the fecal–oral route. The contamination of water is one of the important disease transmission pathways. The main waterborne protozoans include *Cryptosporidium, Entamoeba*, and *Giardia. Entamocha histolytica*, a major cause of dysentery, is found in humans and does not have an animal reservoir; thus, only human wastewater and feces are of concern. However, both *Cryptosporidium* and *Giardia* are zoonotic, and pets, agricultural animals, and wildlife can serve as reservoirs of species that are associated with human infections. Thus, animal fecal waste is also a source for these oo(cysts). *Balantidium, Isospora, Cyclospora*, and *Toxoplasma* will not be addressed fully in this review of novel methods for monitoring water. However, the methods described herein, along with the appropriate molecular tools, could be used for any of these parasites.

The treatment of sewage in the developed world has led to a decrease in *Entamoeba*; however, the burden of disease for the remainder of the world is associated significantly with liver abscess and death. (WHO, 1997). *Giardia* is the most significant protozoan infection in total numbers globally, and the burden of disease remains close to that of bacterial foodborne pathogens. Although *Cryptosporidium* infections in the population are generally lower, the disease outcome and the outbreaks are of significant concern. Drinking water and recreational water outbreaks are documented in every continent caused by *Cryptosporidium*. Routine medical treatment is available for giardiasis and

Table 3.4 Key Parasites Associated with Water

Parasite Description	Health Impacts	Water Issues	Monitoring Issues
Ascaris Intestinal worm, produces an ova (20 μm)	Failure to thrive, particularly for infants; worm burden may affect cognitive ability.	Used to address wastewater reclamation for lagoons in the developing world.	Used for biosolids in the United States. Standard procedures to address viability.
Enteric Protozoans			
Cryptosporidium Zoonotic transmission, produces a small oocyst (5 μm)	Diarrhea, high mortality in the immunocompromised.	Oocyst is resistant to chlorine. Recreational transmission becoming an issue.	Mandatory monitoring in UK filtered water and in U.S. water supplies for drinking water. Most highly monitored protozoan in water worldwide.
Entamoeba Human infections only, produces a large cyst (20 μm)	Diarrhea and complications cause liver abcess and colitis.	Cyst is removed more efficiently by sedimentation.	Water monitoring is rare.
Giardia Zoonotic transmission, produces a cyst (12 μm)	Diarrhea; asymptomatic and chronic infections. Can cause failure to thrive.	Cyst concentrations are found at the highest concentrations of all parasites in human sewage.	Mandatory monitoring in the United States for drinking water sources, along with *Cryptosporidium*.
Free-Living Protozoans			
Nagleria Free-living protozoan, produces a cyst (5 μm)	Humans are accidental hosts, whereby the organism moves into the brain, causing primary amoebic meningoencephalitis.	Recreational exposure is the primary concern; exposure is through nasal passages.	Recent cases associated with drinking water in Arizona; now have enhanced the concern regarding the colonization of wells.

amebiasis infections only but not for cryptosporidiosis, and the immunocompromised population is at significant risk of severe outcomes, with mortality rates 10 times higher than in the general population, and cryptosporidiosis continues to cause severe diarrhea throughout the world (Tzipori and Widmer, 2008).

The monitoring of parasites in water has continued to be of great interest because of the robust nature of the oo(cysts) and the difficulty in inactivating the oo(cysts) with chlorine disinfection. *Cryptosporidium* oocysts in particular are very resistant to chlorination. Ultraviolet disinfection is now seen as a better approach for treating water to decrease the level of protozoan contamination (Fayer and Xiao, 2008). It was discovered early on that the parasites had significant genetic heterogeneity and distribution in animals and humans. The zoonotic nature of the parasites and the discovery of new related species that were not disease associated, as well as the numbers of cases of severe illness and outbreaks worldwide, necessitated better genotyping and identification of the parasites in humans who were ill as well as in waters or other

environmental samples. Thus, currently, molecular characterization is very prominent in the published literature.

3.4.2 Collection, Concentration, and Purification Techniques

The original methods for concentrating the parasites included grab samples followed by centrifugation and flotation methods. The *Ascaris* methods for assessing sludge have followed this approach and use a flotation method in a density gradient of magnesium sulfate (USEPA, 2003). However, it was clear that low-level detection required larger-volume processing. Thus, filtration methods began to be used. The new Gelman parasite filter was designed specifically for *Cryptosporidium* and *Giardia* collection and recovery, as part of EPA method 1623 (USEPA, 2001). More recently, ultrafiltration has been used and has improved recovery. The use of hollow-fiber ultrafilters pretreated with sodium polyphosphate (NaPP, 0.01%) and Tween 80 (0.01%) with cross-flow tangential processing decreased clogging, allowing larger volumes of water with higher turbidity to be processed (Hill et al., 2007; Polaczyk et al., 2008).

Further concentration and purification has been undertaken with immunomagnetic separation techniques (USEPA, 2001), which then produces a concentrate that is used for microscopy or molecular testing. This, in particular, has decreased interferences when PCR methods are used (Johnson et al., 1995).

For *Naegleria*, up to 1 L of water is generally filtered through membranes (0.65-μm DVPP Milipore filters), after which the filter is cut up and eluted with 10 mL of PBS via vortexing and sonication, and finally, concentrated by centrifugation (Behets et al., 2007).

As is the case with any molecular techniques, PCR inhibitors are significant problems with parasites. Unlike the bacteria and viruses, however, it is much more difficult to open up and extract DNA from the oo(cysts) and ova. Several procedures have been used after IMS or flotation, including (1) freeze–thaw and or bead-beating and (2) extraction with Ultraclean soil DNA (Behets et al., 2007) or a FastDNA SPIN soil kit (Hill et al., 2007; Xiao and Ryan, 2008). In other studies, both human and water samples have been evaluated after using the QIAamp DNA stool kits (Bertrand and Schwartzbrod, 2007).

3.4.3 Molecular Techniques for Parasite Detection

Molecular tools have been developed over the last 20 years to detect and differentiate the parasites at the species, genotype, and subtype levels. This has led to an improvement in understanding the diversity of the parasites, their epidemiology, and their pathogenicity. A real-time quantitative PCR (qPCR) method has been reported on and used to determine the levels of total and viable *Ascaris* eggs in laboratory solutions (Pecson et al., 2006). This procedure targeted the internally transcribed spacer (ITS-1) region of ribosomal DNA (rDNA) and rRNA. Currently, microscopy remains the method of choice for sludges.

The PCR methods for *Entamoeba histolytica* and *E. dispar* were developed in the 1990s targeting the ribosomal RNA genes (Clark and Diamond, 1991), which resulted from an awareness that one type of infection in humans was seen as benign (Lebbad and Svärd, 2005). In fact, two separate species were suggested as early as 1925, but it was not until 1993 that *E. dispar*, which causes asymptomatic colonization, was finally

described (Clark and Diamond, 1991). These methods have assisted in the clinical laboratory, determining those patients that require treatment (Lebbad and Svärd, 2005) but have yet to be used in water. A multiplex-PCR method has also been utilized for clinical samples (Santos et al., 2007), targeting tandem sequences repeated in the estrachromosomal circular DNA found in these two species, detecting as few as five trophozoites (the replicating form of the parasite, which is also excreted in stools). The PCR methods could have great application as biomonitoring tools in sewage, examining risks at the community level associated with *E. histolytica*.

Cryptosporidium is the most highly studied parasite of all enteric pathogens in the last 20 years. A recent book and several reviews have described the developments in molecular characterization of the genetic variation, in diagnosis, and in detection in the environment, particularly for the water environment (Smith et al., 2006; Fayer and Xiao, 2008; Jex et al., 2008; Xiao and Fayer, 2008). In many studies and publications, both *Cryptosporidium* and *Giardia* are addressed, with identification of species and the genotypes using the 18S subunit of the ribosomal DNA. Unlike *Giardia*, though, *Cryptosporidium* reproduces by both sexual and asexual means, and early on it was recognized that *C. parvum*, one of the key species, was zoonotic and could be transmitted readily between mammals and humans. PCR tools targeted the 18S rDNA, COWP (cell outer wall protein), and in some cases the Hsp70 (heat shock protein), and using RFLP or sequencing, these have been used to describe the human-specific species, *C. hominis*, and other species associated with other animals [*C. andersonii* (in cattle) *C. muris* (in mice), and *C. baileyi* (in birds)] during outbreaks and in other investigations of water (Smith et al., 2006). New approaches to examine *Cryptosporidium* (and *Giardia*) by quantitative PCR have not gained much traction until recently. In 2003, Guy et al. published a qPCR procedure focused on the COWP gene (and the β-giardin gene for *Giardia*). This could be multiplexed and provided numbers that were consistent with oo(cyst) counts under the microscope from sewage samples. These researchers also suggested the need for Chelex 100 and poly(vinylpyrrolidone) (PVP3600 2%) as pretreatment to decrease the impact of inhibitors on the PCR reaction. Finally, Hill et al. 2007 developed a qPCR method that has been used successfully with ultrafiltration to quantify the oocysts from tap water.

Giardia methods are very tied to the methods that have been used for *Cryptosporidium*; this is due partially to the USEPA methods. Early studies had shown that the epidemiology of the two protozoans was similar and that the methods could readily be multiplexed with fluorescently labeled antibodies with microscopy to examine waters for both protozoans. These parasites are also found in similar sources, animals and humans, albeit not at the same prevalence. Molecular studies have focused on development of PCR for both the β-giardin and *gdh* genes, which have been used in water studies and in outbreak investigations (Robertson et al., 2006). *Giardia duodenalis* is, however, described by seven assemblages, A through G. Bertrand and Schwartzbrod (2007) have developed specific real-time PCR assays to examine the *G. duodenalis* assemblages A, B, and E, which they applied in studies of wastewater. The targets were areas in the *tpi* gene and primers amplified at 148 and 81 bp for A and B, respectively. While assemblages A and B were considered to cause most of the infections in humans, these have been found in a number of other animals. In wastewater, *G. duodenalis* assemblage B was found most often at higher concentration and then, secondarily, assemblage A, but E (associated with animals) was not detected.

The free-living amoeba *Naegleria fowleri* cause a rare but fatal disease of the central nervous system, primary amoebic meningoencephalitis (PAM). PAM is acquired through the exposure of the nasal passages to the pathogenic forms of the amoeba from swimming, generally in warm waters. However, two children recently acquired the disease in Arizona, presumably through bath water, with groundwater as the source (Arizona Department of Health, 2003; CDC, 2008). A quantitative PCR method (Behets et al., 2007) has alleviated the long culture times and problems with identification that previously plagued monitoring programs. In the past, programs initiated, for example, in Florida waters after a death had occurred were abandoned because of inadequate methods and inability to determine the risk associated with the pathogenic *N. fowleri* (Behets et al., 2007) targeted a *Naegleria*-specific genetic sequence which had previously been developed showing no cross-reaction with other free-living amoeba or with genes in the data bank [against a 23-kDa protein designated Mp2C15 (Reveiller et al., 2002)]. This qPCR method developed by Behets et al. 2007 provided improved sensitivity over routine PCR and could be used for monitoring swimming areas, cooling towers, and other potential sources of this free-living protozoan. Table 3.5 provides the primer sets and probes for some of the studies discussed previously.

Flow cytometry has also been used as a protocol for the parasites (*Cryptosporidium*, specifically); however, detection limits for water were poor [2×10^3 oocysts/mL (Barbosa et al., 2008)]. Jex et al. 2008) provides a good history of the research to date and approaches that have been evaluated using flow cytometry methods.

3.5 NEW METHODS FOR DETECTION OF WATERBORNE VIRUSES

3.5.1 Introduction

Occurrence of human pathogenic viruses in environmental waters (i.e., surface waters, groundwater, drinking water, recreational water, and wastewater) raises concerns regarding the possibility of human exposure and waterborne infections. Commonly observed waterborne viruses include enteroviruses, adenoviruses, noroviruses, and rotaviruses. Viruses are the smallest of all microorganisms, and their size facilitates transport in environmental media. In addition, viruses have very low die-off rates and low infectivity doses. The ability to detect waterborne viruses effectively is the basis for microbial risk assessment and management of water sources with the ultimate goal to protect public health. However, precise detection, quantification, and infectivity determination of viruses have always been a challenge in water quality laboratories.

Traditionally, cell culture has been recognized as the golden method for virus detection. However, PCR is emerging very rapidly as a method of virus detection in environmental samples. Compared to cell culture, the main advantages of PCR methods for virus detection include fast results, less labor intensive, high specificity and sensitivity, and capability of detecting difficult-to-culture or nonculturable viruses (e.g., human noroviruses and adenovirus 40/41). However, PCR is not free of problems. The main disadvantage of PCR methods is that they are not able to determine infectivity. There are also problems associated with detection limits caused primarily by environmental inhibitors. Sample collection and concentration is a very important aspect of all environmental virology methods, since it directly affects the ability to detect and quantify viruses in water samples.

Table 3.5 PCR Methods Used for Detection and Speciation of Parasites Important in Water

Microbe/Gene	Assay	Forward Primer/Reverse Primer	TaqMan Probe Sequence
Cryptosporidium spp. (Hill et al., 2007)	qPCR	ATGACGGGTAACGGGGAAT/ CCAATTACAAAACCAAAAAGTCC	CGCGCCTGCTGCCTTCCTTAGATG
(Guy et al., 2003) COW P702	qPCR	CAAATTGATACCGTTTGTCCTTCTG/ GGCATGTCGATTCTAATTCAGCT	HEX/TGCCATACATTGTTGTCCTG ACAAATTCAAT/BHQ-1
Entamoeba histolytica (Santos et al., 2007) *E. dispar*	Multiplex	EHP1-5′CGATTTTCCCAGTAGAAATTA3′/ EHP2-5′CAAAATGGTCGTCTAGGC3′ EDP1-5′ATGGTGAGGTTGTAGCAGAGA3′/ EDP2-5′CGATATTGACCTAGTACT3′	
Entamoeba histolytica (Clark and Diamond, 1991); Lebbad and Svärd, 2005) *E. dispar*	PCR	5′GGCCAATTCATTCAATGAATTGAG3′/ 5′CTCAGATCTAGAAACAATGCTTCTC3′	
Giardia duodenalis Assemblage A	qPCR	5′GGAGACCGACGAGCAAAGC3′ / 5′CTTGCCAAGCGCCTCAA3′	5′-FAM CATCTTCTGCGTCGCA NFQ-3′
Assemblage B (Bertrand and Schwartzbrod, 2007)		5′AATAGCAGCACARAACGTGTATCTG3′ / 5′CCCATGTCCAGCAGCATCT3′	5′-FAM CATGGACCGGCGAGACAAGCGT TAMRA-3′
Naegleria fowleri (Behets et al., 2007)	qPCR	NfQF-TGCAGAGAATCAGGAGGCAAA/ NfQR-TCTTGAGTCCAGGTGAAATGATGT	NfQP TCTGGCACTGCACTC

3.5.2 Sample Collection and Virus Concentration

Many methods have been developed to concentrate viruses from large volumes of water, with most of these being based on adsorption of viruses on filter surfaces. Ultrafiltration has also been used for concentrating viruses. The selection of concentration method should follow six criteria: short processing time, high recoverability, ability to capture a wide range of virus, inexpensive, large processing volume, and reproducible data (Block and Schwartzbrod, 1989). However, no single method is likely to fulfill all seven criteria.

The technique most commonly used to concentrate viruses from water samples is the virus-adsorption-elution microporous filter method, also known as VIRADEL. The filters for VIRADEL could be electropositive or electronegative. Negative filters can handle the most turbid water samples and are useful for concentrating viruses from a large volume of tap water and surface water (Maier et al., 2000).

However, adjustment of the cationic salt concentration and pH (to 3.5) is needed before sample processing. Electropositive filters can also handle large volumes of sample and are suitable for sampling tap water, sewage, and seawater. Positive filters are convenient to use since neither cationic salt nor pH preconditioning is needed for most natural waters. Glass wool and glass powder have also been used to concentrate viruses (Joret et al., 1980; Bosch et al., 1988; Vilagines et al., 1993, 1997), but the extent of use is significantly less than the electropositive and electronegative filter. The glass wool method may not require sample conditioning since glass wool will adsorb viruses near neutral pH.

Numerous studies have been undertaken on recoveries by electronegative and electropositive filters, but the results vary widely. Variations in recoveries may be due to sample type, filter material, elution method, and the processing skill of person. In general, both electronegative and electropositive filters could achieve a recovery of 50 to 60% (Wyn-Jones and Sellwood, 2001). Recoveries from 57 to 75% have been reported for glass wool (Vilagines et al., 1993, 1997). For glass powder, recoveries of 20 to 60% have been reported (Joret et al., 1980; Bosch et al., 1988).

After adsorption, viruses need to be eluted from the attachment surface. The elution matrix is usually beef extract at pH 9.5 to 11.5, skimmed milk at pH 9, or glycine/NaOH at 9.5 to 11.5. To increase the virus concentration in the sample, secondary concentration of eluent is usually required. The methods used for secondary concentration can be ultrafiltration, ultracentrifugation, organic floculation, aluminum hydroxide precipitation, and hydroextraction.

Researchers indicated that beef extract, used traditionally for eluting the viruses from the filter, could cause PCR inhibition (Abbaszadegan et al., 1993; Schwab et al., 1993). In a comparison study of a variety of techniques to remove inhibitors during the study of enteroviruses detection in groundwater samples, Sephadex G-100 in combination with Chelex-100 was found to be the most effective technique (Abbaszadegan et al., 1993). Others have found that guanidinium isothiocyanate (GIT) can eliminate RT-PCR inhibitors (Shieh et al., 1995). A number of studies have also shown that the efficiency of PCR may increase with sample dilutions since inhibitor concentrations decrease as samples get diluted.

A virus sampling method that used only NaOH to elute viruses from cation-coated HA filter has recently been developed (Haramoto et al., 2004, 2005; Katayama et al., 2002). Here, adenoviruses, noroviruses, and enteroviruses in different water samples were detected successfully using PCR methods.

An alternative sampling technique is ultrafiltration. Ultrafiltration requires pressurizing of the water samples to pass through 0.01-μm filter. Due to the low flux, a turbid water sample would need prefiltration to remove larger particles. Ultrafiltration can be conducted with soluble filters, membranes, capillaries, or hollow fibers. Capillaries and hollow fibers are more suitable for sampling surface water because soluble fibers and membranes can be clogged even with the low-turbidity water sample (Maier et al., 2000). The major advantage of ultrafiltration is that no sample preconditioning is required. The recovery is generally high. However, the units are expensive and the processing time could be long.

3.5.3 Latest Methods for Virus Detection

Currently, widely studied enteric waterborne viruses include adenoviruses, noroviruses, enteroviruses, hepatitis A and E, rotaviruses, and astroviruses. Various conventional, PCR, and cell culture–PCR combination techniques have been developed for their detection. The methods include cell culture/cell infectivity, PCR and RT-PCR, nested PCR, multiplex PCR, integrated cell culture–PCR (ICC-PCR), and real-time quantitative PCR (qPCR).

The most basic molecular technique is conventional PCR, in which two primers bind to target DNA and amplification takes place during the PCR cycles. To detect RNA viruses, a reverse transcription step is needed to convert RNA to cDNA before amplification. Nested PCR was developed to increase assay sensitivity by having another set of primers to amplify a target DNA sequence within the first amplicon. The multiplex PCR technique incorporates multiple primer sets in one reaction, and the main advantage of using this technique is the ability to detect multiple viruses simultaneously.

Tables 3.6 to 3.8 summarize recent methods for adenovirus, norovirus, and enterovirus detection in various water samples, including qPCR for quantification of viruses and the combination of cell culture and PCR for infectivity determinations.

The principal drawback of using PCR to detect pathogens is that it cannot differentiate between viable/infectious and nonviable targets. Therefore, ICC-PCR, a method combining cell culture with PCR, can be used to detect infectious viruses. Beside the advantage of detecting only infectious viruses, ICC-PCR also has higher sensitivity than conventional PCR, due to the amplification of viruses during the cell culture step. Recently, researchers developed an mRNA RT-PCR method for the detection of infectious adenoviruses in cell culture (Ko et al., 2005a,b). The rationale behind this method is that only infectious adenoviruses can enter cells and transcribe mRNA during replication. Therefore, the positive mRNA RT-PCR result indicates the presence of infectious adenoviruses in the samples.

Real-time quantitative PCR (qPCR) is currently the most advanced technology for virus detection, as it allows visualization of the increase in the amount of DNA as it amplifies. Several types of real-time qPCR methods are available. Commonly used qPCR methods include the TaqMan, molecular beacons, HyProbe, and SYBR green probe methods. The most important advantage of qPCR over other PCR methods is that the result generated by qPCR is both qualitative and quantitative. Quantitative PCR is at least as sensitive or more sensitive than traditional PCR and nested PCR. Most qPCR assays reported in the literature have a detection limit of 10 copies of the target gene or fewer. Another advantage of qPCR is that it does not require post-PCR handling, such as gel elecrophoresis, to view the results. Multiplex assays can be applied to the

Table 3.6 Summary of Quantitative PCR and Integrated Cell-Culture PCR Methods for Human Adenovirus in Environmental Waters

Sample Type	Volume	Sample Pretreatment	Filter Type	Elution	Reconcentration	Method	Reference
Sewage, seawater, river water	100 mL–1 L	$MgCl_2$	HA filter	NaOH	Centriprep YM-50, ultrafiltration	TaqMan	Haramoto et al. (2007)
Wastewater	100 mL–1 L	$MgCl_2$	HA filter	NaOH	Centriprep YM-50	TaqMan	Katayama et al. (2007)
Stormwater	100 L	None	Ultrafiltration	Glycine/NaOH/Tween 80	Filtration	TaqMan	Rajal et al. (2007)
Lake water	250–350 L	Adjusted to pH 7.0–7.5	1MDS	Glycine buffer/BE	$FeCl_3$ flocculation, suspension in $NaPO_3$, centrifugation, neutralization	TaqMan	Xagoraraki et al. (2007)
Sewage river; drinking water	42 mL; 5–50 L	None; none	None; Zeta Plus MK	None; Glycine buffer/BE	Ultracentrifugation, elution by glycine buffer (pH 9.5), centrifugation after addition of PBS, ultracentrifugation, resuspension of PBS	TaqMan	Albinana-Gimenez et al. (2006)
Sewage	40 mL	None	None	None	Ultracentrifugation, elution by glycine buffer (pH 9.5), centrifugation after addition of PBS, ultracentrifugation, resuspension in PBS	TaqMan	Bofill-Mas et al. (2006)
Seawater	1 L	$MgCl_2$	HA filter	NaOH	Centriprep YM-50	TaqMan	Haramoto et al. (2006b)
River water	10 L	None	Tangential flow filtration	None	None	TaqMan	Choi and Jiang (2005)
River water	500 mL	None	HA filter	NaOH	Centriprep YM-50, ultrafiltration	TaqMan	Haramoto et al. (2005)
Sewage	10 mL	None	None	None	Ultracentrifuged, pellet resuspended in supernatant, extraction with chloroform, filtration	TaqMan	He and Jiang (2005)
River, water, sewage	10 L	None	Tangential flow filtration	None	None	TaqMan	Jiang et al. (2005)

River water, drinking water	25 L–200 L	None	Glass wool	Glycine buffer/BE	PEG/NaCl, centrifugation, pellet resuspension in PBS, sonication, centrifugation	TaqMan	Van Heerden et al. (2005)
						Integrated PCR Method (Infectivity)	
Lake water	250–350 L	Adjusted to pH 7.0–7.5	1MDS	glycine buffer/BE	$FeCl_3$ flocculation, suspended $NaPO_3$, centrifugation, neutralization	ICC-nested-PCR (BGMK cell line)	Xagoraraki et al. (2007)
Marine water	114–151 L	None	Zeta Plus micro wound	Glycine buffer/BBL Sparks/BE	Organic flocculation at pH 3.5, centrifugation, resuspension in Na_2HPO_4, centrifugation, neutralization, syringe filtration	ICC-nested-PCR (BGMK cell line)	Ballester et al. (2005)
Surface water, tap water	70–300 L, 1000–3000 L	None	1MDS	Glycine buffer/BE	PEG/NaCl at pH 7.2, centrifugation, resuspension, 0.2-μm filtration	ICC multiplex-nested PCR (BGMK cell line)	Lee et al. (2005)
River water	2 L	Adjusted to pH 5.5, $MgCl_2$	Negatively charged membrane	Glycine buffer/BE	PEG, centrifugation, resuspension in PBS, 0.2-μm filtration	ICC multiplex nested RT-PCR (A549 and BGMK cell lines)	Lee et al. (2004)
Source water, drinking water	200 L, 1500 L	None	1MDS	Glycine buffer/BE	Organic flocculation, suspension in $NaPO_3$, centrifugation, neutralization	ICC-nested PCR (BGMK cell line)	Lee and Jeong (2004)
Raw water, treated water	100–1000 L	None	Glass wool	Glycine buffer/BE	PEG, centrifugation, resuspension in PBS at pH 7.4	ICC-nested PCR (PLC/PRF/5 cell lines)	Van Heerden et al. (2003)
Sewage	1 L	Adjusted to pH 3.5, $AlCl_3$	Filterite cartridge	Glycine buffer/BE	PEG, suspension in NaPO3, centrifugation, neutralization	ICC-PCR (A549 and BGMK cell lines)	Greening et al. (2002)
River water	10 L	None	1MDS	Lysine and arginine	PEG/NaCl, suspension in PBS		
Tap water	1000–3000 L	None	1MDS	Glycine buffer/BE	PEG, centrifugation, resuspension, 0.2-μm filtration	ICC-multiplex-nested PCR (BGMK cell line)	Lee and Kim (2002)
Surface water	None	None	1MDS	BE	Organic flocculation, BE-treated 0.22-μm syringe filtration	ICC-nested PCR (BGMK cell line)	Chapron et al. (2000)

Note: BE = beef extract; PBS = phosphate-buffered solid.

Table 3.7 Summary of Quantitative PCR and Integrated Cell-Culture PCR Methods for Human Enterovirus in Environmental Waters

Sample Type	Volume	Sample Pretreatment	Filter Type	Elution	Reconcentration	Method	Reference
Wastewater	100 mL–1 L	$MgCl_2$	HA filter	NaOH	Centriprep YM-50	Taqman	Katayama et al. (2007)
Surface water	Spike sample	None	None	None	None	Molecular beacon	Hwang et al. (2007)
Stormwater	100 L	None	Ultrafiltration	Glycine/ NaOH/ Tween 80	Filtration	Taqman	Rajal et al. (2007)
Lagoon canals	2 to 12 L	$MgCl_2$	HA filter	NaOH	Centriprep YM-50	Molecular beacon	Rose et al. (2006)
Surface water	1 L	None	HA filter	Lysis buffer	None	Taqman	Fuhrman et al. (2005)
River water	500 mL	None	HA filter	NaOH	Centriprep YM-50, ultrafiltration	Taqman	Haramoto et al. (2005)
Activated sludge	100 mL	None	None	None	Centrifugation, resuspension in 300 μL DEPC buffer	Taqman	Pusch et al. (2005b)
Surface waters	NM	None	None	None	Flocculation by Al_2SO4_3, precipitation by pH adjustment, centrifugation, resuspension in PBS (pH 8.0)	Taqman	Pusch et al. (2005a)
Sewage, river water	10 L, 600 L	$MgCl_2$	HA filter	BE/0.05 M	Ultrafiltration, rinsed with BE (pH 9.0), concentrated by two-phase separation	Nucleic acid sequence-based amplification	Rutjes et al. (2005)
Surface water	20 L	None	Vortex flow filtration	None	None	Taqman	Donaldson et al. (2002)
Seawater	2 L	None	HA filter		Centriprep YM-50	Taqman Integrated PCR Method (Infectivity)	Katayama et al. (2002)
Surface water, finished, tap water	NM	None	1MDS	BE	Organic flocculation, 0.22-μm filtration	ICC-nested PCR (BGMK cell line)	Kim et al. (2006)
Marine water	114–151 L	None	Zeta Plus micro wound	Glycine buffer/BBL, sparks/BE	Organic flocculation at pH 3.5, centrifugation, resuspension in Na_2HPO_4, centrifugation, neutralization, syringe filtration	ICC-nested-PCR (BGMK cell line)	Ballester et al. (2005)

Sewage	1–2 L	Adjusted to pH 7.2	None	None	Centrifugation, resuspension with BE and chloroform containing dithizone, centrifugation, addition of PEG/NaCl, centrifugation, and pellet suspension in PBS.	ICC-PCR (RD and L20B cell lines)	Chowdhary et al. (2005)
Surface water, tap water	70–300 L, 1000–3000 L	None	1MDS	Glycine buffer/BE	PEG/NaCl at pH 7.2, centrifugation, resuspension, 0.2-μm filtration	ICC multiplex-nested PCR (BGMK cell line)	Lee et al. (2005)
River water	2 L	Adjusted to pH 5.5, $MgCl_2$	Negatively charged membrane	Glycine buffer/BE	PEG, centrifugation, resuspension in PBS, 0.2-mm filtration	ICC multiplex nested RT-PCR (A549 and BGMK cell lines)	Lee and Jeong (2004)
Drinking water	100–1000 L	None	Glass wool	Glycine buffer/BE	PEG, centrifugation, resuspension in PBS at pH 7	ICC-nested PCR (PLC/PRF/5 and BGMK cell lines)	Vivier et al. (2004)
Source water, drinking water	200 L, 1500 L	None	1MDS	Glycine buffer/BE	Organic flocculation, suspension in $NaPO_3$, centrifugation, neutralization	ICC-nested-PCR (BGMK cell line)	Lee and Jeong (2004)
River water	1 L	8-μm cellulose prefiltration	Positively charged membrane	Glycine buffer/BE	Centrifugation and adjusted the pH to 7.2	ICC-PCR (BGMK cell line)	Skraber et al. (2004)
Sewage	1 L	Adjusted to pH3.5, $AlCl_3$	Filterite cartridge	Glycine buffer/BE	PEG, suspension in $NaPO_3$, centrifugation, neutralization	ICC-PCR (A549 and BGMK cell lines)	Greening et al. (2002)
River water	10 L	None	1MDS	Glycine buffer/BE	PEG/NaCl, suspension in PBS		
Tap water	1000–3000 L	None	1MDS	Glycine buffer/BE	PEG, centrifugation, resuspension, 0.2-μm filtration	ICC-multiplex-nested RT-PCR	Lee and Kim (2002)
Surface water	NM	None	1MDS	BE	Organic flocculation, BE-treated 0.22-μm syringe filtration	ICC-nested PCR	Chapron et al. (2000)
Marine water, source water, sewage	NM	None	1MDS and filterite	Glycine buffer/BE	Organic flocculation, suspension in $NaPO_3$, centrifugation, neutralization	ICC-PCR	Reynolds et al. (2001)
Drinking water	300–400 L	None	Negatively cartridge filter	Glycine buffer/BE	Organic flocculation, suspension in $NaPO_3$, centrifugation, neutralization	ICC-PCR	Reynolds et al. (1997)

Note: BE = beef extract; PEG = polyethylene glycol.

Table 3.8 Summary of Quantitative PCR Methods for Human Norovirus in Environmental Waters

Sample Type	Volume	Sample Pretreatment	Filter Type	Elution	Reconcentration	Assay	Reference
Wastewater	40 mL	None	None	None	PEG, centrifugation, resuspension in water	Taqman	da Silva et al. (2007)
Wastewater	100 mL–1 L	$MgCl_2$	HA filter	NaOH	Centriprep YM-50	Taqman	Katayama et al. (2007)
Source and tap water	20 L	$NaPO_3$	Hollow-fiber UF	Tween 80/$NaPO_3$	PEG/NaCl, centrifugation, pellet resuspended in PBS	Real time	Hewitt et al. (2007)
Treated raw sewage	NM	None	None	None	PEG/NaCl, centrifugation	Multiplex Taqman	Wolf et al. (2007)
Source and drinking water	10 L	NaPO3	Ultrafiltration	Tween 80/$NaPO_3$	PEG/NaCl, centrifugation		
Wastewater	100 mL–1 L	$MgCl_2$	HA filter	NaOH	Centriprep YM-50	Taqman	Haramoto et al. (2006a)
Surface water	300 L	$MgCl_2$	HA filter	BE/0.05M Tris	Cellulose-acetate filter and two-phase separation	Nucleic acid sequence-based amplification	Rutjes et al. (2006)
River water	500 mL	None	HA filter	NaOH	Centriprep YM-50, ultrafiltration	Taqman	Haramoto et al. (2005)
Surface water	NM	None	None	None	Flocculation by Al_2SO4_3, precipitation by pH adjustment, centrifugation, resuspension in PBS	Taqman	Pusch et al. (2005b)
Tap water	100–532 L	None	HA filter		Centriprep YM-50	Taqman	Haramoto et al. (2004)
Tap water	10 L	Adjusted to pH 3.5	Cellulose nitrate membranes	Glycine buffer/BE	Flocculation by pH adjustment to 3.5, centrifugation and suspension in Na_2HPO_4, pH 7	SYBR green	Laverick et al. (2004)
Sewage	100 mL	None	None	None	BE addition, centrifugation, supernatant, flocculation at pH 3.5, centrifugation, suspension in Na_2HPO_4		
xSeawater	2 L	None	HA filter		Centriprep YM-50	Taqman	Katayama et al. (2002)

Note: PEG = polyethylene glycol; PBS = phosphate buffered saline.

qPCR method. Wolf et al. 2007 used a multiplex qPCR assay to detect norovirus types 1, 2, and 3 simultaneously in environmental samples. Other studies also used multiplex qPCR to detect enteric viruses, but the application is in clinical samples. With the rapid advancement in qPCR technology, it is expected that the limitations of using mulitplex real-time PCR, such as limited available fluorophoric labels and the significant overlap in emission spectra, would soon be overcome.

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CHAPTER 4

Microbiological Transformations of Radionuclides in the Subsurface

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4.1 LEGACY WASTES FROM U.S. NUCLEAR WEAPONS COMPLEX

As a result of the Manhattan Project initiated during World War II to develop the first atomic bomb and the resulting Cold War with the Soviet Union, the United States developed a massive nuclear weapons complex that was initiated in 1942 and grew until the late 1970s, at which time it began to decline (Crowley and Ahearne, 2002). This historical project included some 5000 facilities at 16 major sites across the country whose missions included recovery of uranium from extracted ore, large-scale materials processing and extraction to weapons assembly, maintenance, and testing. Among these facilities, the largest were involved in materials production and processing, including Hanford in southeastern Washington, the Idaho National Engineering Laboratory in southeastern Idaho, and the Oak Ridge and Savannah River sites in eastern Tennessee and South Carolina, respectively.

As a result of nuclear weapons production and associated activities, it has been estimated that over 1 billion cubic meters of groundwater and >75 million cubic meters of soil have been contaminated with organic compounds of various classes and radionuclides, including U, Pu, ^{137}Cs, ^{90}Sr, Ra, Th, and Tc, at approximately 10,000 individual contamination release sites across the United States. In addition to direct disposal to soils, trenches, cribs, and so on, large volumes of waste remain in storage at several locations and present challenges for current and future cleanup operations. At Hanford alone, over 200,000 m^3 of high-level waste is stored in 177 large underground storage tanks. To date, it is estimated that 67 of these tanks have leaked 1.9 million gallons of waste, containing more than 1 million curies of radionuclides to the subsurface vadose zone, with most of the leaks occurring between 1950 and 1975 (Zachara et al., 2007). Some of the anionic waste constituents, including $Tc(VII)O_4^-$, $Cr(VI)O_4^{2-}$, U(VI) carbonate complexes, and NO_3^-, are relatively mobile and have been detected in the groundwater at Hanford. Due to their long half-lives and relatively high environmental mobility, U and Tc are the two primary risk-driving contaminants at Hanford and

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other Department of Energy (DOE) sites. Because of their high environmental concern and susceptibility to biogeochemical redox transformations, in this chapter, we focus largely on those radionuclides with multiple oxidation states U, Tc, and Pu.

The chemistry of radionuclides is key to their environmental behavior but is complex and is not addressed in detail here; the reader is referred to other reviews for more information (Nue et al., 2005; Lloyd and Renshaw, 2005). In general, U and Tc exhibit higher solubility in their most oxidized forms, U(VI) and Tc(VII), respectively, and are considerably less soluble and precipitate as oxides at their lowest oxidation state, +4. Their intermediate oxidation states tend to be unstable and therefore are not believed to persist in the environment. Pu exhibits a complex chemistry because it can coexist in multiple oxidation states, including +3, +4, +5, and +6. Tetravalent Pu species are sparingly soluble in the absence of complexing ligands, while the +3 and +5 oxidation states tend to be much more soluble. The solubility of Pu(VI) is considered to be intermediate between Pu(IV) and Pu(V).

In addition to subsurface contamination resulting from nuclear weapons production, the release of long-lived radionuclides from spent nuclear fuels is also of significant concern. The current preferred storage option is isolation in engineered barriers in deep geologic environments or repositories. In the event of engineered barrier failure, the surrounding geological environment will be the primary impediment to radionuclide transport (Cornaton et al., 2008). A variety of microorganisms can be present in such environments, either introduced with the engineered materials such as bentonite or native to the deep subsurface (Fredrickson and Balkwill, 2006). As a result, microbiological transformation of radionuclides in such repositories have been important considerations for site selection.

4.2 BIOGEOCHEMISTRY OF RADIONUCLIDES

4.2.1 Introduction: Reduction–Reoxidation Reactions

The environmental fate of radionuclides is controlled largely by the chemical form of the contaminant and the properties of the geological medium with which they interact. The chemical forms of the contaminants are primarily a function of their oxidation state, the extent of hydrolysis, and their tendency for complexation by either organic or inorganic ligands. For example, in oxidized environments uranium exists as the highly soluble uranyl ion (UO_2^{2+}) and its complexes, the most common of which in subsurface systems are the uranyl carbonates: largely anions such as $UO_2(CO_3)_3^{4-}$ and $UO_2(CO_3)_2^{2-}$. As *p*e (the negative log of the electron activity) declines, uranyl is reduced to the uranous ion (U^{4+}), which is considerably less soluble and can precipitate as the oxide mineral uraninite (UO_2).

Microorganisms are ubiquitous in subsurface environments, although their population sizes and metabolic activities can vary considerably, depending on energy and nutrient inputs (Fredrickson and Onstott, 2001). As a result of their metabolic activities and the chemical properties of their cell surfaces and the exopolymers they produce, microorganisms can facilitate the biotransformation of radionuclides directly or indirectly, thus altering their solubility and overall fate and transport in the environment. Although biosorption to cell surfaces and exopolymers can be an important factor modifying the solubility of some radionuclides under specific conditions, the oxidation state is often considered the single most important factor controlling their speciation and, therefore, environmental behavior (Lloyd and Renshaw, 2005).

4.2.2 Reduction: General Influences of Bioreduction and Rate-Affecting Factors

Microorganisms catalyze changes in *p*e in subsurface environments as a result of energy-yielding oxidation of organic compounds and inorganic ions coupled to the reduction of various electron acceptors, including O_2, oxidized metal ions such as Fe^{3+} and Mn^{4+}, nitrate, and various oxidized sulfur species and CO_2. The potential energy yield from these oxidation–reduction reactions is related to the ΔG values for the various couples and generally occur in the order of those that are most thermodynamically favorable to those that are the least, although there are a number of factors that can influence this sequence (Fredrickson and Onstott, 2001). The radionuclides of concern in this chapter are similarly subject to microbially catalyzed oxidation–reduction reactions in the subsurface and, in general, are not reduced in the presence of oxidants that exhibit higher midpoint potentials, such as O_2, nitrate, and Mn(III/IV). These include direct, enzymatically catalyzed reactions such as the reduction of the pertechnetate ion (TcO_4^-) by hydrogenases or *c*-type cytochromes (Marshall et al., 2008) and indirect mechanisms such as the reduction of TcO_4^- by biogenic Fe(II) (Fredrickson et al., 2004); these mechanisms are discussed in more detail later. These two types of reactions are not mutually exclusive and may occur simultaneously, or one may dominate over the other, depending on a range of factors including cell population densities, levels and types of metabolic activities, local geochemistry, and sediment mineralogy.

4.2.3 Reoxidation: Stability and Solubility of Radionuclides

While reduced forms of U and Tc such as $UO_{2(s)}$ and $TcO_{2(s)}$ are poorly soluble under anoxic conditions, they are subject to chemical oxidation by O_2 or Mn(III/IV). Because Mn(III/IV) also is poorly soluble at circumneutral pH and can form various oxide minerals, redox disequilibrium can occur at very small spatial scales where Mn(III/IV) oxides can coexist with the reduced radionuclide solids (Fredrickson et al., 2002). Dissolved O_2 is freely diffusible and hence is not subject to the same microscale redox disequilibrium imposed by transport limitation as are solids.

Reduced radionuclides are also subject to oxidation as a result of direct and indirect microbiological processes. Biogenic UO_2 can be oxidized by the intermediates of dissimilatory nitrate reduction, including nitrate, nitrous oxide, and nitric oxide (Senko et al., 2002), by Fe(III) produced by nitrate-dependent Fe(II)-oxidizing bacteria, and by Fe(III) resulting from oxidation of Fe(II) by nitrite accumulating during dissimilatory nitrate reduction (Finneran et al., 2002; Senko et al., 2005). *Thiobacillus denitrificans* has also been shown to oxidize biogenic UO_2 anaerobically at circumneutral pH in a nitrate-dependent manner (Beller, 2005). TcO_2 is also subject to nitrate-dependent microbial oxidation in estuarine sediments, although the responsible mechanisms are unclear (Burke et al., 2006).

4.3 MICROORGANISMS INVOLVED IN RADIONUCLIDE REDUCTION

Significant progress has been made toward understanding the microbiology and reductive transformations, which are largely catalyzed by a diverse group of metal ion–reducing organisms. Dissimilatory metal-reducing bacteria (DMRB) constitute a phylogenetically versatile group that spans from hyperthermophilic archaea to

anaerobic proteobacteria. The hallmark feature of DMRB is extensive respiratory versatility; under anaerobic conditions these organisms reduce a variety of organic and inorganic substrates, as well as various metal ions either as soluble complexes or associated with solid-phase minerals (for a review, see Lovley et al., 2004; Nealson and Saffarini, 1994). Radionuclide reduction has been described extensively within the well-studied genera of *Geobacter* and *Shewanella* (Gorby and Lovley, 1992; Lloyd and Macaskie, 1996; Gorby et al., 1998; Boukhalfa et al., 2007; Sanford et al., 2007) as well as other DMRB (Wu et al., 2006; Sanford et al., 2007; Marshall et al., 2009). Field studies in radionuclide-contaminated sites have found that DMRB are indigenous members of the microbial communities (Anderson et al., 2003; Petrie et al., 2003; Istok et al., 2004; North et al., 2004; Shelobolina et al., 2008). Community analysis of in situ biostimulation experiments within contaminated sediments showed an increase in DMRB within the δ-proteobacteria group, which to a large extent represented an increase in both *Geobacter* spp.–and *Anaeromyxobacter* spp.–related sequences.

Genome annotation of sequenced *Geobacter, Anaeromyxobacter*, and *Shewanella* species reveals that these organisms possess a large number of genes predicted to encode putative redox proteins, including cytochromes, hydrogenases, flavins, ferredoxins, multicopper containing–proteins, and Fe–S proteins (Heidelberg et al., 2002; Methe et al., 2003, 2006; Thomas et al., 2008). One shared characteristic among these organisms is a remarkable abundance of genes that encode multiheme *c*-type cytochromes. Although poor amino acid sequence conservation exists between *c*-type cytochromes produced by different groups of DMRB, the sheer abundance of genes encoding these redox-active multiheme-containing proteins and the innate ability of these proteins to accept and donate electrons suggest that *c*-type cytochromes probably play key roles in electron transfer to metals and radionuclides. Moreover, the abundance of putative *c*-type cytochromes in DMRB suggests that flexibility and redundancy in the respiratory electron transfer networks under a variety of environmental conditions (Methe, 2003).

Mutagenesis and biochemical studies with *Shewanella* and *Geobacter* spp. confirmed the role of *c*-type cytochromes in electron transfer to both Fe(III) and Mn(IV) oxides (Beliaev and Saffarini, 1998; Myers and Myers, 1998, 2000, 2001; Beliaev et al., 2001; Leang et al., 2003; Lloyd et al., 2003; Metha et al., 2005). In these studies it was proposed that *c*-type cytochromes functioned either as an intermediary electron carrier in electron transport or as the terminal reductase transferring electrons directly to the metal oxides. Although these proposed roles are not linked exclusively to their subcellular localization, cytochromes localized to the outer membrane (OM) are generally considered to function as terminal reductases of insoluble electron acceptors while cytochromes anchored to the cell membrane (CM) or localized to the periplasm often function as intermediary electron carriers but may also serve as periplasmic terminal reductases of soluble electron acceptors.

Given the wealth of putative reductases predicted to be localized at the OM or in the periplasm, knowing the subcellular regions where biotransformation products accumulate can provide important information to the locations of the terminal reductases for both metal oxides and radionuclides. Laboratory studies using U(VI)- or Tc(VII)-reducing organisms have demonstrated both extracellular and/or periplasmic accumulation of UO_2 nanoparticles, (Gorby and Lovley 1992; Lovley and Phillips, 1992; Liu et al., 2002; Lloyd et al., 2002; Payne et al., 2004; Marshall et al., 2006;

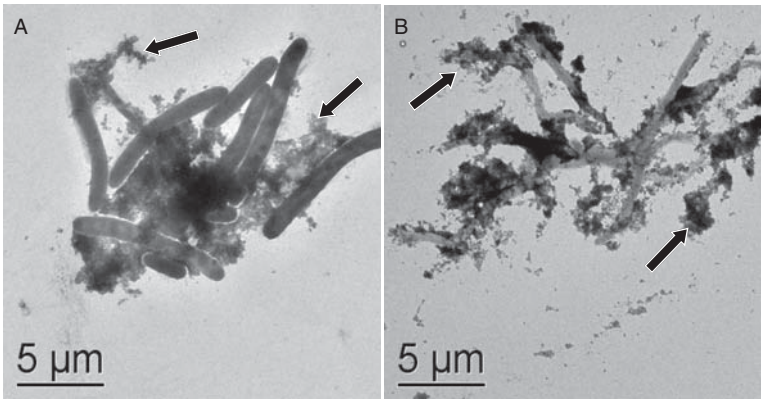

Figure 4.1 Unstained whole-mount transmission electron microscopy (TEM) image of (A) *Shewanella oneidensis* MR-1 and (B) *Anaeromyxobacter dehalogenans* 2CP-C after 24-hour U(VI) reduction. Extracellular UO_2-extracellular polymeric substance (EPS) is highlighted by arrows.

2009) (Figure 4.1) or the localization of reduced TcO_2 particles within the periplasm and at the cell periphery (Lloyd et al., 1999, 2000; Wildung et al., 2000; Liu et al., 2002; Marshall et al., 2009). These consistent subcellular localization patterns among diverse organisms suggest that biological radionuclide reduction has conserved molecular mechanism(s) with possibly wide-reaching implications for radionuclide immobilization strategies.

4.4 MECHANISTIC STUDIES

4.4.1 Direct Mechanisms of Radionuclide Reduction

c-Type Cytochromes Since Fe and Mn are important to the redox cycling of radionuclides, understanding the roles that *c*-type cytochromes play in the reduction of Fe(III) and Mn(IV) is a fundamental aspect of predicting their biogeochemical behavior in the environment (Lloyd et al., 2000; Fredrickson et al., 2002, 2004). The reduction potential for the U(VI)–U(IV) couple is relatively close to that of the γ-Fe(III)OOH–Fe(II) couple (Dale et al., 2007); therefore, *c*-type cytochromes functioning as Fe(III) reductases are strong candidates for being the primary reductases facilitating electron transfer to U(VI). This was confirmed by the isolation of *Shewanella oneidensis* MR-1 mutants lacking components of the *c*-type cytochrome maturation system (Ccm) (Bouhenni et al., 2005). Cultures containing insertional mutations in either *ccmC* or *ccmF1* were found to be deficient in both Fe(III) and Mn(IV) reduction. Cell extracts from anaerobically incubated *ccm* mutants did not exhibit the heme *c* peroxidase activity that is indicative of *c*-type cytochromes, whereas this activity was found in wild-type cell extracts grown similarly. Subsequent studies showed conclusively that a fully functional Ccm is also crucial for reduction of U(VI) in *S. putrefaciens* strain 200 (Dale et al., 2007) and in *S. oneidensis* MR-1 independent of the electron-donor source (H_2 or lactate) (Marshall et al., 2006, 2008).

Given that *c*-type cytochromes may also function in U(VI) reduction, biochemical and mutational studies were performed to identify novel uranyl reductase(s). The

first report of uranyl reductase isolation was from *Desulfovibrio vulgaris* (Lovely et al., 1993). Although this strain was not able to couple U(VI) reduction with growth, a small-molecular-weight cytochrome c_3 was purified and possessed in vitro U(VI) reductase activity in the presence of both H_2 and a H_2-oxidizing hydrogenase as the physiological source of electrons for U(VI) reduction. This was confirmed when a periplasmic cytochrome c_3 (*cycA*) mutant of the closely related *D. desulfuricans* strain G20 was assayed and also found to be impaired in the rate of U(VI) reduction relative to the parental strain (Payne et al., 2002). Interestingly, differences in U(VI) reduction rates were observed by the cytochrome c_3 mutant when either H_2 or an organic acid (lactate or pyruvate) was provided as the electron donor, suggesting that there are electron donor–specific mechanisms for U(VI) reduction. Further evidence for the existence of a periplasmic uranyl reductase was provided when protease-treated *Geobacter sulfurreducens* lost the ability to reduce insoluble Fe(III)-oxides but not soluble U(VI) (Lloyd et al., 2002). Subsequently, the triheme cytochrome c_7 (PpcA) of *G. sulfurreducens* was found to display U(VI) reductase activity in vitro (Lloyd et al., 2003). A deletion of the gene encoding this periplasmic *c*-type cytochrome revealed that PpcA played a role in the acetate-dependent but not H_2-dependent U(VI) reduction (Lloyd et al., 2003). Another unrelated periplasmic *c*-type cytochrome (MacA) has recently been shown to play a significant role in acetate-dependent U(VI) reduction by *G. sulfurreducens* (Shelobolina et al., 2007).

There is little homology between these periplasmic small-molecular-weight cytochrome c_3 or c_7s and the *Shewanella* *c*-type cytochromes (Lloyd et al., 2003; Meyer et al., 2004); however, the periplasmic accumulation of $U(IV)O_2$ nanoparticles suggests that members of the *Shewanella* genus may also possess periplasmic uranyl reductase(s) (Fredrickson et al., 2002; Liu et al., 2002a; 2002b; Marshall et al., 2006), which would directly reduce U(VI) after it enters the periplasm, probably through OM porins. Early mutant analyses of *S. putrefaciens* 200 implicated the periplasmic nitrite reductase in U(VI) reduction because of the simultaneous loss of U(VI) and NO_2^- reduction (Wade and DiChristina, 2000). However, this mutant was later found to contain a point mutation in the *ccmB* gene and subsequently produced an altered *c*-type cytochrome maturation system under anaerobic growth conditions (Dale et al., 2007). More recently, two *c*-type cytochromes located in the cell periplasm and previously linked to Fe(III) and Mn(IV) reduction have been shown to be involved in U(VI) reduction (Bencheikh-Latmani et al., 2005). The protein designated MtrA is a soluble periplasmic decaheme cytochrome, while CymA is a periplasmic tetraheme cytochrome anchored to the CM (Myers and Myers, 1997; Beliaev and Saffarini, 1998). Mutational analysis suggests that during U(VI) reduction, CymA is reduced near the CM by menaquinone and serves as an electron donor to MtrA and possibly other (undetermined) periplasmic *c*-type cytochromes (Bencheikh-Latmani et al., 2005) (Figure 4.2A). It is unknown whether MtrA functions exclusively as an intermediary electron carrier or can also serve as a terminal reductase in U(VI) reduction. These findings suggest that multiple periplasmic *c*-type cytochromes have roles during the reduction of U(VI).

c-Type cytochromes localized to the OM are generally considered to function as terminal reductases of insoluble electron acceptors. In *G. sulfurreducens* the OM-associated *c*-type cytochromes OmcC, OmcE, and OmcF are shown to have roles in the reduction of insoluble Fe(III) oxides (Leang et al., 2003; Kim et al., 2005; Metha et al., 2005). However, only OmcE and OmcF could be implicated in

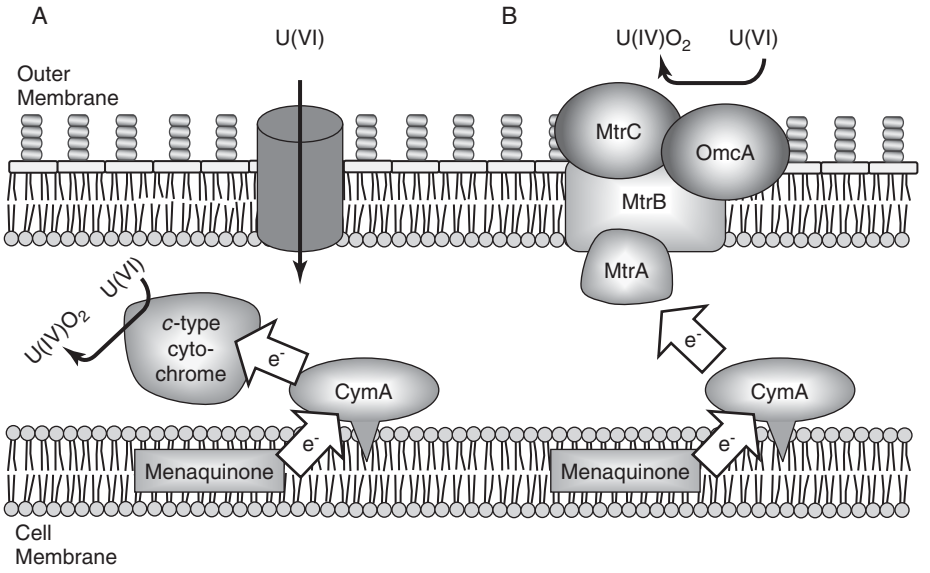

Figure 4.2 Working model for cytochrome *c*–facilitated U(VI) reduction in *Shewanella oneidensis* MR-1: (A) reduction of U(VI) by periplasmic *c*-type cytochromes; (B) reduction of U(VI) by outer membrane *c*-type cytochromes. (See insert for color representation.)

U(VI) reduction (Shelobolina et al., 2007). Two additional previously uncharacterized putative OM cytochromes (OMCs) (GSU0332 and GSU1334) were also shown to decrease significantly the ability of *G. sulfurreducens* to reduce U(VI) (Shelobolina et al., 2007). The involvement of the *S. oneidensis* MR-1 OM metal reductase complex in U(VI) reduction was confirmed, as cultures lacking MtrC, OmcA, or MtrB were found to be impaired in their ability to reduce U(VI) relative to the wild-type (Bencheikh-Latmani et al., 2005; Marshall et al., 2006) (Figure 4.2B). The reductase activity of these OMCs was investigated further, as dithionite-reduced MtrC and OmcA were found to possess in vitro ferric reductase activity, but only reduced MtrC was also oxidized by uranyl citrate in vitro (Marshall et al., 2006). Borloo et al. (2007) later bolstered the hypothesis that a redundant network of uranyl reductases existed when they showed that insertional inactivation of the genes encoding *mtrC* and *omcA* did not inhibit anaerobic growth coupled with dissimilatory U(VI) reduction in a spontaneous rifampicin-resistant *Shewanella* strain (MR-1R). Taken together, these studies show conclusively that *c*-type cytochromes are essential for the reduction of U(VI) and that there is in all likelihood significant functional redundancy among periplasmic and OM *c*-type cytochromes with regard to electron transfer to U(VI) (Figure 4.2).

Compared to U(VI), relatively little is known about the roles of *c*-type cytochromes in the reduction of other radionuclides. Wildung et al. (2000) first speculated that the ferric citrate reducing *c*-type cytochromes of *Shewanella* would theoretically be capable of reducing Tc(VII) by evaluating the similarity in the redox couples of $Tc(VII)O_4^-/Tc(IV)O_{2(s)}$ and Fe(III)–citrate/Fe(II)–citrate. In *D. fructosovorans* a purified periplasmic cytochrome c_3 did not reduce Tc(VII) in vitro. However, when the cytochrome c_3 was added to a hydrogenase in the presence of H_2, the reduction of Tc(VII) was rapid (De Luca et al., 2001).

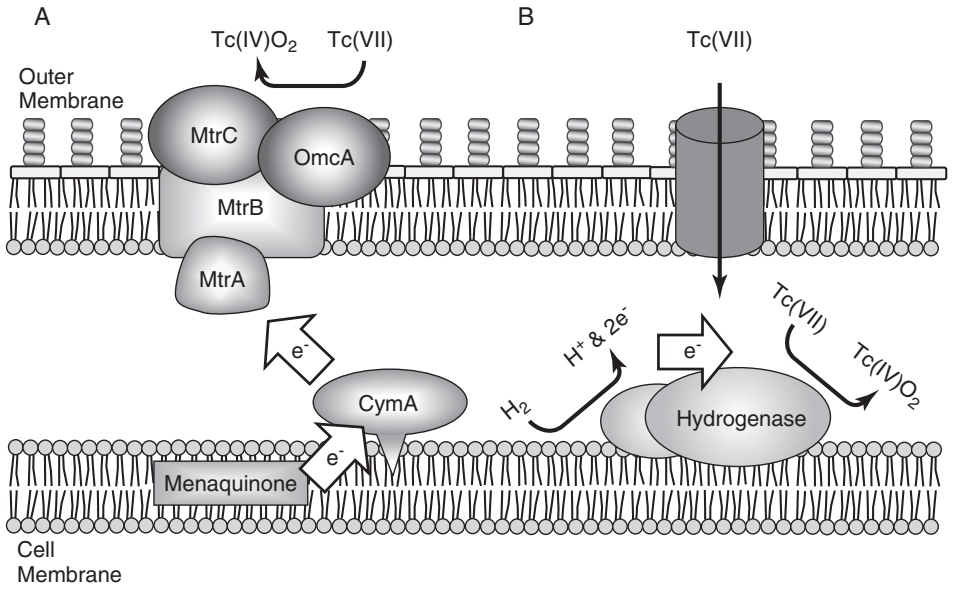

Figure 4.3 Working model for Tc(VII) reduction in *Shewanella oneidensis* MR-1: (A) outer membrane cytochrome *c*–facilitated Tc(VII) reduction; (B) periplasmic hydrogenase-facilitated Tc(VII) reduction. (See insert for color representation.)

The first report of a Tc(VII) reductase activity attributable to the *c*-type cytochromes has recently been described (Marshall et al., 2008). *S. oneidensis* MR-1 cells lacking all functional *c*-type cytochromes ($CcmC^-$) displayed slower Tc(VII) reduction kinetics than did the wild-type cells during H_2-driven Tc(VII) reduction. Moreover, this mutant was unable to reduce Tc(VII) in the presence of lactate, suggesting that a *c*-type cytochrome-mediated Tc(VII) reduction pathway exists in *Shewanella*. Additional research found that the OMCs (MtrC and OmcA) functioned as novel Tc(VII) reductases in the presence of lactate in vivo (Figure 4.3A) and also as Tc(VII) reductases in an in vitro assay independent of an electron donor source. Therefore, *c*-type cytochrome-catalyzed Tc(VII) reduction could be a potentially important mechanism in environments where organic electron donor concentrations are sufficient to allow this reaction to dominate.

Currently, there is no direct evidence to link the reduction of $^{60}Co(III)$, highly insoluble $Pu(IV)O_2$, or complexed Pu(IV) to *c*-type cytochromes. Studies indicate that the reduction of γ-emitting ^{60}Co by metal reducing bacteria is analogous to the reduction of nonradioactive ^{59}Co (Gorby et al., 1998). Similar to soluble U(VI)-carbonate or chelated Fe(III) complexes, in vivo studies with $^{59}Co(III)$ complexed by ethylenediaminetetraacetate (EDTA) found that both *Shewanella* and *Geobacter* rapidly reduced Co(III)-EDTA to Co(II)-EDTA, independent of the electron-donor source provided (H_2 or organic carbons) (Liu et al., 2002). Recent in vitro studies suggest that the *Shewanella* OMCs (MtrC and OmcA) functioned efficiently as ferric reductases when the chelating ligand was EDTA (Wang et al., 2008), suggesting that this metal-chelating ligand may not prevent electron transfer between the reductase and other oxidized metals [either $^{60}Co(III)$ or Pu(IV)].

Given the reduction potential of soluble Pu(IV)–EDTA at circumneutral pH (Boukhalfa et al., 2007), it would be thermodynamically feasible for either MtrC or OmcA (Marshall et al., 2008) to reduce Pu(IV) to Pu(III); however, this remains to be determined experimentally. Boukhalfa et al. (2007) also reported that both *Geobacter* and *Shewanella* can reduce insoluble $Pu(IV)O_2$ in the presence of organic electron donors (acetate or lactate, respectively). Although this work suggested that the reduction of Pu(IV) solids may be driven by the same *c*-type cytochrome-mediated reductase processes as insoluble Fe(III) and Mn(IV) oxides, the presence of exogenous EDTA as well as endogenous complexing agents (bicarbonate produced during either acetate or lactate oxidation) necessitates further mechanistic studies. Taken together, these similarities suggest that *c*-type cytochromes may play roles in the reduction of other radionuclides; however, more focused studies using mutants and biochemical analyses will be required to completely ascertain the processes driving the biotransformation of these radionuclides.

Hydrogenase Hydrogenases are metalloproteins that play a central role in microbial redox metabolism by enzymatically catalyzing the bidirectional reaction of H_2 oxidation with the formation of two protons and two electrons (for a review, see Vignais et al., 2001; Vignais and Colbeau, 2004). An early study with *Escherichia coli* found that although this organism could not couple dissimilatory metal reduction with growth, it produced hydrogenases which also functioned as Tc(VII) reductases (Lloyd et al., 1997). Under anaerobic conditions, *E. coli* was found to accumulate Tc(VII) intracellularly and transform it to Tc oxide (TcO_2). Subsequent analysis found that a cytoplasmic [NiFe] hydrogenase III (Hyc) of the formate hydrogen lyase (FHL) complex was capable of both H_2 oxidation and the reduction of Tc(VII) in *E. coli* (Lloyd et al., 1997). Periplasmic hydrogenase was later implicated indirectly in Tc(VII) reduction in several *Desulfovibrio* spp. based on the periplasmic location of FHL complex and the H_2-dependent periplasmic accumulation of $Tc(IV)O_2$ nanoparticles (Lloyd et al., 1999, 2001). Among these strains, the ability to utilize formate as an efficient electron donor for Tc(VII) reduction was also noted. This was not unexpected since the FHL complex in *Desulfovibrio* consists of a formate dehydrogenase, linked to a hydrogenase via a cytochrome (Peck, 1993) and suggested that formate-dependent Tc(VII) reduction was catalyzed by the hydrogenase in this multienzyme complex (Lloyd et al., 1999). Biochemical studies with *D. fructosovorans* and a *D. fructosovorans* mutant lacking the ability to produce periplasmic [NiFe] hydrogenase confirmed that hydrogenase was essential for Tc(VII) reductase activity in sulfate-reducing bacteria (De Luca et al., 2001). While in vitro assays with purified [NiFe] hydrogenase and H_2 confirmed the Tc(VII) reductase activity for this protein, the addition of both purified [NiFe] hydrogenase and a small periplasmic cytochrome c_3 significantly increased the rate of H_2-dependent Tc(VII) reduction (De Luca et al., 2001).

Early studies with DMRB produced only circumstantial evidence for the involvement of hydrogenase in the reduction of Tc(VII). These predictions were made because of the exclusive requirement for H_2 as the electron donor for direct Tc(VII) reduction (Lloyd et al., 2000) or based on genomic analysis of amino acid homologies with other confirmed hydrogenases (Wildung et al., 2000; Payne and DiChristina, 2006). The genome of *S. oneidensis* MR-1 encodes two heterodimeric hydrogenases (Heidelberg et al., 2002). Biochemical analysis of these proteins suggested that the [NiFe] hydrogenase was the predominant hydrogenase and functioned bidirectionally, both

forming and releasing H_2, while the [Fe–Fe] hydrogenase was involved primarily in H_2 formation (Meshulam-Simon et al., 2007). It was subsequently shown that the [NiFe] hydrogenase and to a lesser degree, the [Fe–Fe] hydrogenase, play a more direct role in the H_2-dependent reduction of Tc(VII) than just the production of H^+ for subsequent electron-transfer reactions (Marshall et al., 2008) (Figure 4.3B). The [NiFe] hydrogenase of MR-1 was shown to be necessary for the rapid and complete reduction of Tc(VII) using H_2 as the electron donor, but was not an absolute requirement for reduction by MR-1 since deletion of both hydrogenases did not completely abolish the ability of cells to transfer electrons to Tc(VII) with lactate. Experiments investigating the [NiFe] hydrogenase mutant also suggested that the [Fe–Fe] hydrogenase can facilitate Tc(VII) reduction, although it is unknown if this hydrogenase transfers electrons directly to Tc(VII) or another reductase, possibly MtrC or OmcA. In additional experiments, the inactivation of the Ccm cytochrome maturation system, which abolished the ability of MR-1 cells to reduce U(VI), did not abolish the ability of cells to reduce Tc(VII) in the presence of H_2 and confirmed that cytochromes were not the solitary electron-transfer proteins involved in Tc(VII) reduction (Marshall et al., 2008).

Relatively little is known about the involvement of hydrogenases in the reduction of other radionuclides. It has been reported that the *S. oneidensis* MR-1 [NiFe] hydrogenase facilitated U(VI) reduction by oxidizing H_2 to provide a source of electrons for cytochrome-facilitated reduction (Marshall et al., 2008). Similar results were observed when chelated metals [Fe(III) or ^{59}Co(III)] or insoluble oxides [Fe(III) or Mn(IV)] were supplied as the terminal electron acceptor. The [Fe–Fe] hydrogenase was also shown to be capable of H_2 oxidation to facilitate U(VI), Fe(III), Mn(IV), or Co(III) via cytochrome-facilitated reduction (Marshall et al., 2008). In contrast, in the presence of lactate, neither hydrogenase was required for the reduction of any of these terminal electron acceptors, suggesting (1) that hydrogenase was not a terminal reductase of these metals, and (2) that lactate-oxidizing enzymes (not hydrogenase) provided electrons to the reduction pathway under these conditions (Marshall et al., 2008).

Extracellular Electron Shuttles Although redox-active *c*-type cytochromes and hydrogenases currently shown to function as terminal reductases of radionuclides are generally considered to be cell associated, additional mechanisms have been proposed by which metal-reducing bacteria may carry out extracellular electron transfer to metal oxides in environments where direct cellular contact may not be possible (for a review, see Gralnick and Newman, 2007; Lovley, 2008). Early studies proposed that *G. sulfurreducens* released a small periplasmic *c*-type cytochrome to serve as an extracellular electron shuttle to insoluble Fe(III) oxides (Seeliger et al., 1998). However, it was subsequently determined that the deliberate release of this periplasmic cytochrome to serve as an extracellular electron shuttle was unlikely (Lloyd et al., 1999a). As a result, studies have continued to focus on identifying putative electron shuttles that either preexist in nature (exogenous) or that can be synthesized and released by some bacteria (endogenous).

Humic substances are organic materials that are common in terrestrial and aquatic environments and can function as exogenous electron shuttles by accepting electrons from reduced bacterial reductases followed by electron transfer to Fe(III) oxides (Lovley et al., 1996) (Figure 4.4A). The innate ability of humics to undergo redox cycling can yield energy to support cell growth and facilitate enhanced rates of Fe(III) oxide reduction (Lovley et al., 1996). Anthraquinone-2,6-disulfonate (AQDS) is a commonly

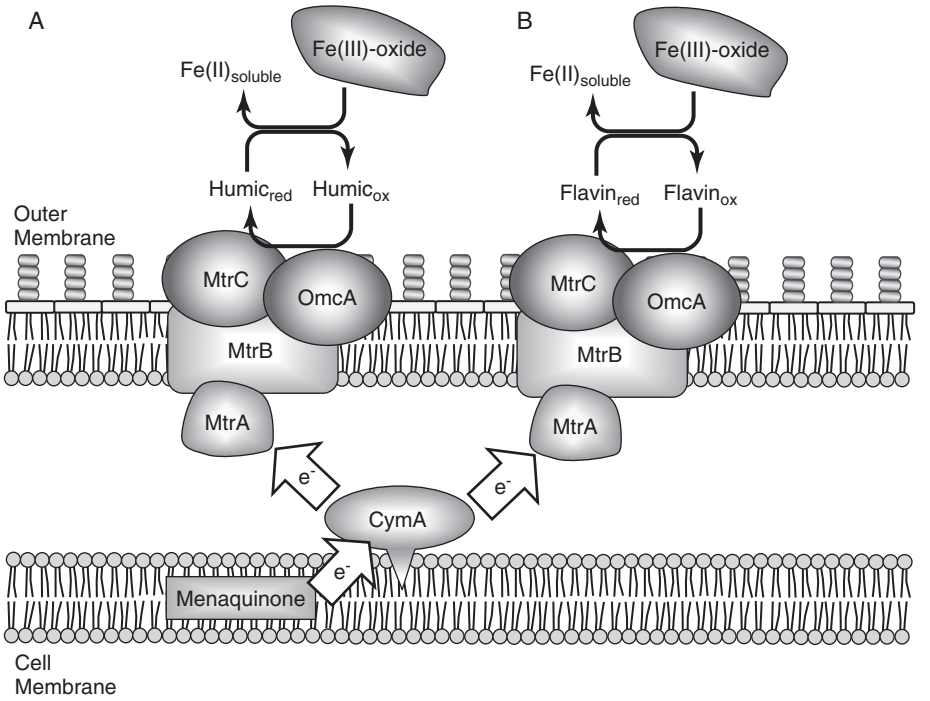

Figure 4.4 Working model for shuttle-facilitated Fe(III)-oxide reduction in *Shewanella oneidensis* MR-1: (A) reduction via an exogenous electron shuttle (humics); (B) reduction via an endogenous electron shuttle (flavins). (See insert for color representation.)

used compound that contains a microbially reducible quinone similar to those associated with some humics (Lovley et al., 1996; Scott et al., 1998). *G. metallireducens* transforms AQDS to 2,6-anthrahydroquinone disulfonate (AH_2DS), which subsequently rapidly reduces Fe(III) to regenerate AQDS. Therefore, electron shuttling through exogenous humics was found to be a mechanism for organisms such as *G. metallireducens* that lack endogenous electron shuttles (described below) to transfer electrons between cellular terminal reductases and Fe(III) oxides (Lovely et al., 1996; Nevin and Lovely, 2000, 2002a). The ability to couple AQDS reduction with Fe(III) oxide reduction was also noted for several *Shewanella* species (Lovley et al., 1996; Zachara et al., 1998; Fredrickson et al., 2000). A cell-free isolation of bacterially reduced AQDS was shown to reduce U(VI), suggesting that it is plausible for naturally occurring quinone-containing humics to transfer electrons to radionuclides (Fredrickson et al., 2000).

Phenazines are redox-active metabolites with antimicrobial properties synthesized by some soil organisms such as *Pseudomonas chlororaphis* PCL1391 (Hernandez et al., 2004). These compounds can reductively dissolve poorly crystalline Fe(III) and Mn(IV) oxides and have also been shown to fortuitously support extracellular Fe(III) reduction by acting as exogenous shuttling compounds for DMRB in laboratory studies (Hernandez et al., 2004). The ability of these compounds to transfer electrons from a cell-associated reductase to extracellular radionuclides remains undetermined.

The production of endogenous compounds for extracellular electron shuttling to Fe(III) oxides was first reported in *Shewanella* and *Geothrix* (Newman and Kolter, 2000; Nevin and Lovley, 2002a, 2002b). This compound was small, diffusible, nonproteinaceous, and exhibited other characteristics similar to menaquinone, but the exact nature of this microbially excreted compound was not identified (Newman and Kolter, 2000). Notably, it was found that *G. metallireducens* did not produce endogenous electron-shuttling compounds suggesting that either an intimate contact between terminal reductases and Fe(III) oxides or exogenous humic shuttle was required (Lovley et al., 1996; Childers et al., 2002; Nevin and Lovley 2000, 2002a).

It has recently been shown that numerous *Shewanella* strains secreted flavin mononucleotide (FMN) and riboflavin as the extracellular electron shuttles to facilitate the reduction of the Fe(III) oxides or soluble compounds such as azo dyes (Marsili et al., 2008; von Canstein et al., 2008) (Figure 4.4B). The redox potentials of FMN and riboflavin reported (von Canstein et al., 2008) suggest that these flavins have the potential to reduce radionuclides at circumneutral pH. However, electron shuttling from a reduced endogenous flavin to radionuclides has not been demonstrated experimentally.

Complexation It is well established that synthetic chelating ligands such as EDTA or citrate can influence the solubility of metals and radionuclides in the environment. Early biotransformation studies demonstrated that the solubility of metals directly influenced the rate of their transformation (Arnold et al., 1988; Lovley and Woodward, 1996). For example, *S. putrefaciens* 200 reduced soluble organic Fe(III) complexes three orders of magnitude faster than did insoluble Fe(III) oxides (Arnold et al., 1988). In the absence of synthetic ligands, bacteria have developed mechanisms to solubilize otherwise insoluble oxides (Nevin and Lovley, 2002a, 2002b; Boukhalfa et al., 2007; Gralnick and Newman, 2007).

Siderophores are biogenic ligands that can promote dissolution of Fe(III) and Pu(IV) oxides via complexation (Neu et al., 2003; Boukhalfa et al., 2007). Several natural siderophores have been shown to form soluble Pu(IV) complexes, suggesting that siderophore-facilitated dissolution can influence interactions of Pu(IV) with microorganisms and potentially the rate of biotransformation (Boukhalfa et al., 2007). The production of an unidentified Fe(III)-chelating compound was noted in the analysis of culture filtrates from *G. fermentans* and *S. alga* BrY (Nevin and Lovley, 2002a, 2002b) but not in culture filtrates from *G. metallireducens* (Nevin and Lovley, 2000). Novel voltammetric techniques have also been used to determine that *S. putrefaciens* 200 produced Fe(III)-solubilizing chelators during anaerobic Fe(III) respiration (Taillefert et al., 2007).

In contrast to chelating ligands, it has recently been reported that some DMRB produce an extended extracellular polymeric substance (EPS) which has the ability to bind (or nucleate) reduced UO_2 nanoparticles (UO_2-EPS) (Marshall et al., 2006, 2009). In *S. oneidensis* MR-1, the complex EPS matrix associated with UO_2 was negatively charged and found to contain OMCs involved in U(VI) reduction (MtrC and OmcA), polysaccharide, and other OM proteins (Marshall et al., 2006). The production of EPS by *S. oneidensis* MR-1 does not appear to be required for U(VI) reduction since OMC mutants that produce little UO_2-EPS remain capable of reducing U(VI). Although the concept of UO_2 nanoparticle association with EPS is quite new, an early

U(VI) bioreduction study demonstrated that fine-grained U(IV) precipitates produced by *G. metallireducens* ultimately resulted in the formation of large uraninite aggregates (i.e., > 0.2 μm in diameter) (Gorby and Lovley, 1992). It is unknown whether the uraninite nanoparticles that comprised such aggregates were associated with EPS or whether these were single- or polydomain aggregates.

Although colloidal transport of UO_2 nanoparticles through a porous medium has yet to be demonstrated, the apparent close, interactive molecular association of the nanoparticulate UO_2 with complex biopolymers such as EPS in the environment could influence (i.e., slow) the oxidation rate of U(VI) and influence the environmental mobility of the small precipitates as dispersed colloids in the subsurface.

4.4.2 Indirect Mechanisms of Radionuclide Reduction

In soils and sediments, the presence of Fe(III) and Mn(III/IV) oxides can impart substantial control on radionuclide contaminant behavior, constituting a substantial redox buffering capacity. As subsurface sediments become progressively reduced, increasing concentrations of Mn(II) and Fe(II) are generated as a result of microbial Mn(III/IV) and Fe(III) reduction. Depending on the form of Fe(II), it can serve as a facile reductant of TcO_4^-. For example, nanocrystalline magnetite, as a product of microbially reduced ferrihydrite, can reduce Tc(VII) to an insoluble form (Lloyd et al., 2000), and sediment-associated Fe(II) was implicated as the reductant of TcO_4^- in anoxic U.S. Atlantic Coastal Plain sediments (Wildung et al., 2004). The reduction of TcO_4^- has also been shown to be facilitated by biogenic Fe(II) associated with subsurface sediments from U.S. DOE sites (Hanford and Oak Ridge) and other locations (Fredrickson et al., 2004). The rates of TcO_4^- reduction generally increased with increasing concentrations of 0.5 N HCl-extractable Fe(II), but major differences in rates between sediments with different mineralogy implied that sorbed Fe(II) associated with residual Fe(III) oxides was more reactive than that associated with layer silicates.

Fe(II) preadsorbed to the surface of goethite, but not aqueous Fe(II), can also reduce U(VI) (Fredrickson et al., 2000), as can Fe(II)-surface complexes of a range of Fe(III) minerals generated abiotically (Liger et al., 1999; Jeon et al., 2005) or as the result of microbial Fe(III) oxide reduction (Behrends and van Cappellen, 2005). Investigations of Pu redox transformations are relatively scarce, although Rai et al. (2002) demonstrated that reduction reactions involving Fe(II) and hydroquinone were relatively rapid and that reductive dissolution of $PuO_{2(am)}$ to aqueous Pu(III) may play an important role in controlling Pu behavior under reducing environmental conditions.

4.5 ELECTRON MICROSCOPIES AND SPECTROSCOPIES FOR STUDYING RADIONUCLIDE TRANSFORMATIONS

Since the first report of radionuclide reduction by DMRB (Lovely et al., 1991), researchers have strived to use rapidly evolving technologies to further an understanding of the processes involved in metal and radionuclide transformation (for a review, see Fredrickson and Zachara, 2008; Geesey et al., 2008; Kemner, 2008). High-resolution transmission or scanning electron microscopy (TEM and SEM, respectively) imaging and analyses have played an integral role in research designed to visualize microbial–mineral–metal interactions and to analyze the newly

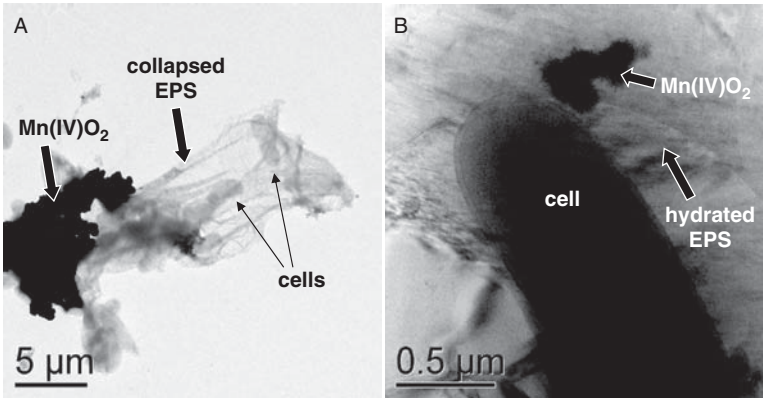

Figure 4.5 TEM images of *Shewanella oneidensis* MR-1 after incubation with $Mn(IV)O_2$: (A) air-dried whole-mount TEM revealing collapsed EPS; (B) cryo TEM image of frozen-hydrated cell, illustrating fully hydrated EPS.

formed biominerals produced by bacterial activity with excellent spatial resolution. Synchrotron-based techniques such as X-ray absorption spectroscopy (XAS) have been used to investigate the local chemical composition of elements, while X-ray fluorescence (XRF) microimaging has been used to produce high-sensitivity, element-specific distributions which correspond to EM images (Kemner, 2008). Microscale X-ray spectroscopy, including X-ray absorption near-edge spectroscopy (XANES) and extended X-ray absorption fine structure (EXAFS), provide information on an oxidation state and local bonding environment that can be used to infer speciation. Collectively, these approaches can provide detailed insights into the speciation and locations of metals and radionuclides within and near bacteria on mineral surfaces (Kemner, 2008) and begin to reveal the molecular mechanisms by which such biotransformations are catalyzed. Technology enhancements are anticipated to continue to improve the resolution of synchrotron X-ray-based microscopies and spectroscopies as the location and chemistry of radionuclides at fine spatial scales (micrometers to nanometers) can be a key to understanding their environmental behavior.

Traditional TEM and/or SEM approaches used for visualizing microbe–mineral–metal interactions are not necessarily straightforward to interpret since chemical fixatives such as glutaraldehyde only cross-link proteinaceous materials and poststaining with osmium tetroxide for enhanced contrast or visualize membranes can also potentially oxidize nascent biominerals. Sample drying either by alcohol dehydration or sublimation for analysis under vacuum can also strongly influence visualization of microbe–mineral interactions in an unperturbed state (Figure 4.5A). Cryo EM has recently been introduced to examine microbial associations with minerals in native, hydrated conditions. Sample preparation for Cryo TEM requires the rapid freezing of samples in liquid ethane (vitrification). Frozen-hydrated samples are visualized using a low-dose, electron beam imaging scheme to provide exceptional cellular detail and unprecedented visualization of minerals associated with bacteria (Figure 4.5B).

4.6 CONCLUSIONS

Microbial processes can exert a dominant influence on the subsurface fate and transport of radionuclide contaminants through alteration of their solubility as a result of complexation reactions with cell surfaces and exopolymers, soluble organic ligands, or via direct and indirect enzymatic redox transformations. For contaminants such as U, Tc, and Pu, microbially facilitated redox transformations can have a pronounced impact on their solubility and therefore mobility in the subsurface. Reduction reactions can be catalyzed directly by electron transfer proteins such as low-potential *c*-type cytochromes or by biogenic and abiogenic electron carriers such as flavins and Fe(II). Although less is known regarding oxidation reactions, there is increasing evidence for the involvement of both direct enzymatic and well as indirect (i.e., via metabolites) mechanisms.

Despite recent advances in understanding some of the mechanisms of radionuclide bioreduction, there are a number of key issues that remain unresolved, such as the role of exopolymers in the reductive precipitation of UO_2 and for formation of uraninite nanoparticle aggregates. More important, future research will required investigating processes under more field-relevant conditions, where system complexities and heterogeneities will require novel approaches and innovative analytical and imaging capabilities to resolve radionuclide distribution and chemistry at small (i.e., submicrometer) scales. Reactions at small spatial scales can be distributed heterogeneously, yet exert a dominant influence on radionuclide reactive transport (McKinley et al., 2006). Ultimately, it will be necessary to scale such reactions to the field in order to predict contaminant transport at sites of concern.

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CHAPTER 5

Eutrophication of Estuarine and Coastal Ecosystems

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5.1 INTRODUCTION

Why would the topic of eutrophication be included in a book on environmental microbiology? First, the organisms involved in the eutrophication process and the subsequent degradation of water quality are microorganisms: picocyanobacteria, nitrogen-fixing cyanobacteria, diatoms, dinoflagellates, sulfide-oxidizing bacteria, and the suite of aerobic and anaerobic bacteria involved in biogeochemical processes, to name a few. Second, the process, the symptoms, and the geographic locations of eutrophication are all increasing worldwide at an accelerated rate.

Eutrophication is the increase in the rate of production of carbon or the accumulation of carbon in an aquatic ecosystem [modified by Rabalais (2004) from Nixon (1995)]. The source of the increased organic carbon may come from within the system (autochthonous) or from outside the system (allochthonous). Knowing the sources and mechanisms of carbon accumulation is critical in developing management strategies to reverse the process. Most lakes follow a path of eutrophication over geologic time in transition from oligotrophic systems to more eutrophic systems. When the addition of organic matter (e.g., from soil erosion or increased inputs of nutrients from natural weathering of soils or from human activities) proceeds at an increasing rate, the process of eutrophication accelerates. For example, a coastal system that responds to a river delivering more organic carbon from terrestrial sources presently than in preceding centuries and subsequent organic matter accumulating in the coastal system would be undergoing eutrophication, but increased organic carbon loading from nutrient-enhanced in situ primary production rapidly surpassed all other causes of eutrophication in the twentieth century.

There is little doubt that human population growth and its associated activities have altered the landscape, hydrologic cycles, and the flux of nutrients essential to plant growth at accelerating rates over the last several centuries (Bennett et al., 2001; Galloway et al., 2008). To support human population growth and the need for fuel, fiber, and food, humans have increased significantly the flux of reactive

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nitrogen and phosphorus to the biosphere and subsequently, to aquatic and terrestrial ecosystems. These excess nutrients are finding their way to the coastal ocean in increasing amounts, especially during the last half of the twentieth century. There are thresholds of nutrient loading above which the nutrient inputs no longer stimulate entirely positive responses from the ecosystem, such as increased fisheries production. Instead land-based sources of nutrients are causing problems: for example, poor water quality, noxious algal blooms, oxygen depletion, and in some cases, loss of fisheries production. Over the last five decades it has become increasingly apparent that the effects of excess nutrients that lead to eutrophication are not minor and localized but have large-scale implications and are spreading rapidly.

5.2 EUTROPHICATION PROCESS

Eutrophication is not a trophic state but a process. A key element of eutrophication is change. Upwelling systems cycle through phases of increased nutrient availability, high primary and secondary productivity, and often, oxygen depletion in the lower water column. The trophic status of upwelling systems would be considered *eutrophic* [an organic carbon supply of 300 to 500 g of carbon per square meter per year, as defined by Nixon (1995)], but upwelling systems are not undergoing eutrophication.

The causes of eutrophication should not be confused with the process itself. The causes may include changes in physical characteristics of the system such as changes in hydrology, changes in biological interactions such as reduced grazing, or an increase in the input of organic and inorganic nutrients. Although the causes may include direct natural or anthropogenic carbon enrichment, eutrophication in the twentieth and twenty-first centuries is more often caused by excess nutrients that would otherwise limit the growth of phytoplankton.

A variety of responses, such as noxious algal blooms, fish kills, oxygen depletion, or seagrass habitat losses, should also not be confused with the process of eutrophication. The responses are multiple and may result in "increases" or "decreases" in components of aquatic ecosystems, to which humans often ascribe beneficial or detrimental values. More subtle responses of aquatic ecosystems to eutrophication include shifts in phytoplankton and zooplankton communities, shifts in the food webs that they support, loss of biodiversity, changes in trophic interactions, and changes in ecosystem functions and biogeochemical processes.

5.2.1 Sources of Nutrients

Reactive nitrogen has increased substantially over the last century and a half through artificial fixation of nitrogen into fertilizers, the emission of nitrous oxides from the consumption of fossil fuels, and the transformation of reduced forms through volatilization processes (Galloway et al., 2008). Phosphorus additions to the landscape enter via phosphorus-containing fertilizers manufactured from mined phosphorus, animal manures, and waste products from animals supplemented with phosphorus-enriched feed, and enter rivers and streams via wastewater effluents, overland runoff, and soil erosion (Bennett et al., 2001). Compared to increased inputs of nitrogen and phosphorus, concentration or loads or both of dissolved silicon have remained the same or decreased due to sequestration in eutrophied systems (Conley et al., 1993) or

accumulation behind dams, so that the relative proportions of silicon to nitrogen and silicon to phosphorus have decreased over time (Turner and Rabalais, 1991; Turner et al., 2003a,b). This is particularly important because the relative proportion of the nutrients and the concentrations control phytoplankton community compositions (see below).

The sources of human-controlled nutrients are numerous, including point or non-point sources. *Point sources* are generally from wastewater treatment or industrial discharges and are usually considered less important as nutrient contributors than are non-point sources on broad regional scales (National Research Council, 2000). In some areas, however, point sources can be a large proportion of the total load. For example, wastewater from New York City contributes 67% of the nitrogen inputs to Long Island Sound on an annual basis. About 25% of the nitrogen and phosphorus inputs to Chesapeake Bay come from wastewater and other point sources (Boynton et al., 1995), and less than 10% from similar sources make up the total annual nitrogen load of the Mississippi River (Goolsby et al., 1999).

Non-point sources are more diffuse, coming off the landscape in stream flow and from the atmosphere in both dry and wet deposition. Fertilizer application, both nitrogen and phosphorus, remains a major contributor to non-point nutrient pollution (Howarth et al., 1996). This source continues to increase on a worldwide basis (Seitzinger and Harrison, 2005). There is a direct relationship between fertilizer applications and river nitrogen and phosphorus fluxes and concentrations (Turner and Rabalais, 1991; Caraco, 1995).

Rivers play a crucial role in the delivery of nutrients to the ocean. These rivers terminate in estuaries or the nearshore coastal ocean, where the effects of nutrient enrichment are most pervasive. In the subbasins to the North Atlantic Ocean, and specifically in the Baltic catchments and the watershed of the Mississippi River, inputs of anthropogenic nitrogen via rivers far exceed other sources of nitrogen input: atmospheric, coastal point sources, atmospheric deposition, and nitrogen fixation (Howarth et al., 1996; Goolsby et al., 1999; Grimvall and Stålnacke, 2001). Phosphorus loads, likewise, come mostly from rivers (Bennett et al., 2001; Grimvall and Stålnacke, 2001).

One of the more rapidly increasing sources of nutrients, behind fertilizer applications, to both freshwaters and the coastal zone is the atmosphere. In estuarine coastal waters and marine waters, up to 40% of the nitrogen inputs are from atmospheric origin, originating from industrial, agricultural, and urban sources (Duce, 1986; Paerl et al., 2001). Along the eastern U.S. coast and eastern Gulf of Mexico, atmospheric nitrogen sources currently account for 10 to over 40% of "new" nitrogen loading to estuaries (Paerl et al., 2001). In other watersheds, such as the Mississippi River basin, the proportion is much lower, less than 10%, and the direct deposition on the area of the northern Gulf of Mexico where seasonal hypoxia occurs is only 1% of the total nitrogen loading to the Gulf (Goolsby et al., 1999). Nitrogen enters the atmosphere in the form of nitrous oxides generated in the burning of fossil fuels. Fertilizer nitrogen can also enter the environment through the atmosphere. Globally, 40% of the inorganic nitrogen fertilizer that is applied to fields is volatilized as ammonia, either directly from fertilizer or from animal wastes after crops grown with the fertilizer have been fed to chickens, pigs, or cattle. Where inputs are high, they have the capacity for stimulating primary production. Algal blooms in the Yellow Sea have increased in frequency over the past several decades and have been related to atmospheric deposition in addition to direct nutrient runoff (Zhang, 1994). It is estimated that a typical rain event over the Yellow Sea may supply sufficient nitrogen, phosphorus, and silicon to

account for over 50% and up to 100% of the primary production of a harmful algal bloom event (Zhang, 1994).

5.2.2 Nutrient-Enhanced Primary Production

Nitrogen and phosphorus limit the growth of autotrophic phytoplankton, and silicon additionally limits the growth of diatoms (e.g., Dortch et al., 2001). Dissolved inorganic nutrients, (nitrite, nitrate, ammonium, orthophosphate, and silicate) are the macronutrients that are most important, but dissolved organic forms of nitrogen, phosphorus, and carbon, while utilized by autotrophs, are critical to the growth of heterotrophic phytoplankton. Phosphorus is usually considered the primary limiting nutrient in most lakes and reservoirs (Schindler et al., 2008), while nitrogen is considered the primary limiting nutrient in coastal and marine waters (Howarth and Marino, 2005; Glibert et al., 2006). However, a strict dichotomy of a single nutrient limitation in either system is an oversimplification. Phosphorus may limit production in some estuarine and coastal systems and during certain seasons, and may also secondarily limit production in combination with nitrogen (Rabalais et al., 2002; Paerl et al., 2004; Sylvan et al., 2006). The likelihood of phosphorus limitation in coastal systems increases as the relative loads of nitrogen to the system far exceed the phosphorus loads, creating an imbalance of high nitrogen/phosphorus ratios. Overenrichment with nitrogen and phosphorus alters the ratios of nitrogen, phosphorus, and silicon to each other such that silicon limitation may occur on a more frequent basis, phytoplankton composition may shift to flagellates, or noxious or toxic forms may predominate (Justić et al., 1995; Turner et al., 1998). Many estuarine and coastal systems display variability in phosphorus, nitrogen, or silicon limitation, or combinations of these, by season and by location along a fresh-to-marine gradient (Mee, 2001; Yin et al., 2001; Rabalais et al., 2002; Paerl et al., 2004).

When a nutrient, multiple nutrients, or micronutrients that otherwise limit the growth of phytoplankton are supplied to an aquatic ecosystem, the initial response is an increase in primary production, usually with some relational increase in phytoplankton biomass. Significant positive relationships exist between nutrient-loading rates and microalgal production and biomass (Cloern, 2001).

There are many examples from aquatic ecosystems that follow similar patterns with an increase in chlorophyll *a* as an indicator of phytoplankton biomass over time as nutrient loads increased, such as the Dutch Wadden Sea (Cadée, 1992), northern Adriatic Sea (Šolić et al., 1997), and Chesapeake Bay (Harding and Perry, 1997). Winter nutrient and summer chlorophyll *a* development for the last 30 years has shown that summer chlorophyll *a* has steadily increased from the 1970s in the northern Baltic Proper, Gulf of Finland, and Bothnian Sea (Fleming-Lehtinen et al., 2008). In other areas, measures of production (annual, summer, or monthly) have increased over time as nutrient loads increased [Belt Sea (Rydberg et al., 1991); Dutch Wadden Sea (de Jonge, 1990); southern Kattegat (Richardson, 1996a)].

5.2.3 Sedimentary Processes

The excess organic material in the form of senescent phytoplankton, fecal pellets, and marine aggregates sinks to the lower water column and the seabed, where the carbon is remineralized by aerobic and anaerobic processes or buried. As aerobic bacteria

decompose the increased organic matter settling onto the seabed and in the lower water column, the dissolved oxygen concentration overlying the sediments becomes hypoxic and approaches anoxia. In this transition, numerous biological and geochemical shifts occur in the benthic community, many with negative feedback into the cycle of eutrophication and declining oxygen levels. With continued eutrophication, concentrations of organic carbon and nitrogen, microbial biomass, microbial decomposition potential of substrates, and community oxygen consumption increase, but not in simple linear relationships (Meyer-Reil and Köster, 2000; Turner et al., 2008).

The redox potential discontinuity layer migrates upward to the sediment–water interface, sulfate respiration replaces oxygen respiration, hydrogen sulfide is generated from the sediments, and oxygen penetrates less deeply into the sediments as the bioturbation potential of the macroinfauna decreases during their demise, due to sulfide toxicity or lack of sufficient oxygen. The sediments become less cohesive, more susceptible to resuspension, and contribute to the turbidity of the overlying water, which in turn reduces the potential for growth of the photosynthetic microphytobenthic community and generation of oxygen into the lower water column.

Settled viable phytoplankton (e.g., the chain-forming diatom *Skeletonema costatum*), phytoplankton resident in the lower water column (e.g. the diatom *Coscinodiscus* spp.), and resident microphytobenthos (e.g., pennate diatoms and cyanobacteria) may help offset the depletion of oxygen in hypoxic bottom waters as long as there is sufficient photosynthetically active radiation reaching the seafloor (Cahoon et al., 1994; Dortch et al., 1994). Some benthic diatoms and cyanobacteria are capable of phytosynthesis in low light levels (Shilo and Fattom, 1984; Paterson, 2001). Typical summertime microphytobenthos in the eutrophic and hypoxic area of the northern Adriatic Sea influenced by the Po River include diatoms (*Pleurosigma* spp., *Gyrosigma* spp., *Navicula* spp.) and filamentous cyanobacteria (Totti, 2003).

Some shifts in the benthic microbial community are visible at the sediment–water interface. Typical black spots from iron sulfide precipitated from intense microbial degradation of organic matter, lacey white colonies and denser, yellowish colonies of sulfide-oxidizing bacteria (*Beggiatoa* and *Thiovulum*), and reddish-to-violet carpets of sulfur-purple bacteria can be observed as oxygen levels decline in the Wadden Sea (North Sea), in shallow-water areas of the Baltic, and on the Louisiana continental shelf, similar to many areas of the world's ocean where the oxygen minimum zone intersects the seabed (reviewed by Rabalais and Turner, 2001).

Biogeochemical cycling of nitrogen and phosphorus in water and sediments is controlled by the availability of oxygen. With the shift in redox potential in the sediments with decreasing oxygen concentration, there is an increase in the flux of inorganic nutrients, ammonium, and particularly phosphate into the overlying water. These inorganic nutrients become available to fuel further phytoplankton production in the overlying water. The degree to which these nutrients diffuse upward through the water column and across strong pycnoclines is not known. High waves or strong currents will probably break down the physical structure of the water column, reaerate the water column, and resuspend sediments and adsorbed and dissolved nutrients into the water column with a subsequent stimulatory effect for primary production. These events, however, are short-lived and may occur during seasons when restratification is unlikely to occur and subsequent accumulation of phytoplankton biomass may be limited: that is, a short-term stimulation of phytoplankton response but not a long-term accumulation of carbon that would lead to further negative feedbacks on the system.

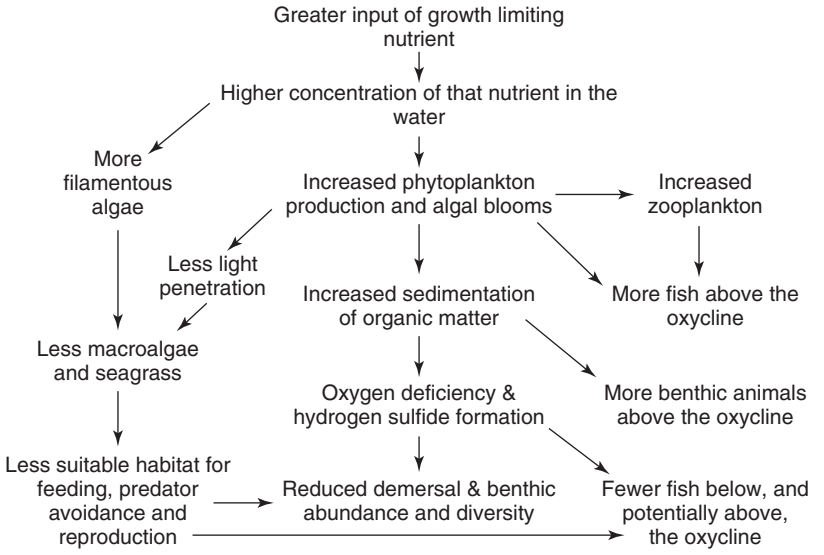

Figure 5.1 Series of responses within a coastal ecosystem to the increased input of a limiting nutrient. Green processes indicate eutrophication. Blue processes or conditions indicate increased secondary production. Red processes or conditions indicate the negative effects of eutrophication. (See insert for color representation.)

5.3 SYMPTOMS

The initial response of an estuarine or coastal system to an increase in limiting nutrients is an increase in phytoplankton growth rate and biomass accumulation, growth of filamentous macroalgae, blooms of noxious or toxic algae, reduction in water clarity, shifts in phytoplankton community structure, or combinations of these (Figure 5.1).

5.3.1 Shifts in Phytoplankton Communities

Phytoplankton are affected not only by the quantity of nutrient loading but also by the relative supply of nutrients. Global patterns of the ratios of dissolved nitrogen, phosphorus, and silica in large rivers indicate that primarily nitrate flux controls the ratios of these nutrients as delivered to the coastal ocean (Turner et al., 2003a). As the N/P ratio rises above the Redfield ratio of 16:1, phosphorus limitation of phytoplankton growth is implied, and in a similar fashion a Si/N ratio below 1:1 implies silica limitation (Turner et al., 2003b). Results from field and laboratory studies have suggested that the lack of silica or phosphorus relative to nitrogen can control phytoplankton community composition (e.g., Rabalais et al., 1996, Dortch et al., 2001).

A series of eutrophication-related ecological changes in the Baltic Sea and Kattegat were caused primarily by increased anthropogenic nutrient inputs (both nitrogen and phosphorus) mainly after World War II (reviewed by Elmgren and Larsson, 2001). One of the system responses was an increase in toxic or noxious algal blooms. Cyanobacterial blooms in the open sea, particularly of the toxic, nitrogen-fixing genus *Nodularia*, are the main problem. However, a number of fish kills by the prymnesiophyte *Prymnesium pravum* have been reported from the Baltic proper

coastal zone. The former is stimulated by low inorganic N/P ratios; the latter is thought to be favored by high N/P ratios.

As the Si/N ratio declines as a response to increased nitrogen, reduced silicon, or both (Turner et al., 2003a), a phytoplankton community of nondiatoms may be competitively enabled (Officer and Ryther, 1980). This alternative community would be more likely to be composed of flagellated algae, especially dinoflagellates, including noxious bloom-forming algal communities. They argued further that the fisheries web would re-form and be composed of less desirable species. Evidence for shifts in the phytoplankton community in the northern Gulf of Mexico indicates a change from heavily silicified diatoms to less silicified diatoms, an increase in the lightly silicified diatom *Pseudo-nitzschia* spp., evidence for more dinoflagellates, and shifts in trophic structure (Rabalais et al., 1996; Turner et al., 1998; Dortch et al., 2001; Parsons et al., 2002).

5.3.2 Secondary Production

There is a variety of evidence that nutrients stimulate secondary production and sometimes fishery yields in marine ecosystems (Caddy, 1993). Colijn and van Beusekom (2002) summarized the responses of the North Sea ecosystem to increased nutrient concentrations and loadings. They found increases in the concentration, production, and changes in species composition for the phytoplankton. There were some indications for an increased biomass of macrozoobenthos. A concomitant increase in higher trophic levels, such as fish and shrimp, was difficult to link directly to the eutrophication process.

Similarly for higher trophic levels, a meta-analysis by Micheli (1999) revealed that the effect of adding nutrients to ecosystems with either two or three trophic levels was to increase the phytoplankton biomass, but not the primary grazers of the phytoplankton. She also reported that the availability of nitrogen and the primary production rate were strongly correlated to the accumulation of phytoplankton, but not of higher trophic levels. Micheli's analyses demonstrated a weak coupling between phytoplankton, mesoplankton, and zooplankton for closed and manipulated systems. She offered three explanations for these results: more complex interactions among zooplankton than our current understanding, differing proportionality of edible and preferred food with higher phytoplankton production, and advection or losses of nutrients or prey in open marine systems.

If the increased primary production that accompanies nutrient enrichment does not result in increased macroconsumer biomass of higher trophic levels then a higher proportion of the total carbon flow must be shunted to smaller consumers/decomposers or it is buried. The carbon burial rates offshore the Mississippi River increased this century as eutrophication occurred (Eadie et al., 1994), but some of the excess carbon may also have entered the microbial food web throughout the water column.

In cases where eutrophication leads to effects such as loss of seabed vegetation and extensive bottom-water oxygen depletion, there are often negative effects of the increased primary production (Caddy, 1993; Boesch et al., 2001; Rabalais and Turner, 2001). In the deepest bottoms of the Baltic Proper, animals have long been scarce or absent because of low oxygen availability. This area was 20,000 km^2 until the 1940s (Jansson and Dahlberg, 1999), but since then, about a third of the Baltic bottom area has intermittent oxygen depletion (Elmgren, 1989). Lowered oxygen concentrations

and increased sedimentation have changed the benthic fauna in the deeper parts of the Baltic, resulting in an impoverished diet for bottom fish. Above the halocline in areas not influenced by local pollution, benthic biomass has increased due mostly to an increase in mollusks (Cederwall and Elmgren, 1990). On the other hand, eutrophication resulting in severely depressed oxygen levels has greatly impoverished or even annihilated the soft-bottom macrofauna, or changed species composition and dominance (Cederwall and Elmgren, 1990).

Eutrophication of surface waters accompanied by oxygen-deficient bottom waters can lead to a shift in dominance of fish stocks from demersals to pelagics. In the Baltic Sea and Kattegatt, changes in fish stocks have been both positive, due to increased food supply (e.g., pike perch in Baltic archipelagos), and negative (e.g., oxygen deficiency reducing Baltic cod recruitment and eventual harvest) (reviewed by Elmgren and Larsson, 2001). Similar shifts are hinted at with limited data on the Mississippi River–influenced shelf with an increase in selected pelagic species in bycatch from shrimp trawls and a decrease in certain demersal species (Chesney and Baltz, 2001), again in an area of increased surface primary production and expanding bottom-water low oxygen. In the case of commercial fisheries in the Black Sea after the mid-1970s, benthic fish populations (e.g., turbot) collapsed, and pelagic fish populations (small pelagic fish, such as anchovy and sprat) started to increase. The commercial fisheries diversity declined from some 25 fished species to about five in 20 years (1960s to 1980s), while anchovy stocks and fisheries increased rapidly (Mee, 2001). The point on the continuum of increasing nutrients versus fishery yields remains vague as to where benefits are subsumed by environmental problems that lead to decreased landings or reduced quality of production and biomass.

5.3.3 Water Clarity

Water clarity decreases with an increase in phytoplankton biomass. These relationships are evident in many coastal areas where long-term data sets derived primarily from Secchi disk depth readings show a decline over time or with increased nitrogen or phosphorus loading. Well-documented examples exist for the northern Adriatic Sea, the Baltic Sea, and the northern Gulf of Mexico (Justić, 1988; Elmgren and Larsson, 2001; Rabalais et al., 2002).

5.3.4 Macrophyte Responses

The reduced availability of light for photosynthesis as a result of increased chlorophyll biomass is a particularly inhibiting feature for the growth of rooted seagrasses in estuaries and macroalgal beds in shallow coastal waters. Increased turbidity from excess phytoplankton growth in the upper water column can affect the amount of light reaching submerged aquatic vegetation, which in turn limits their growth, and ends in the demise of these structurally complex habitats and the functions they serve as refuge, feeding, and nursery areas for fish and invertebrates (Deegan et al., 2002; Hauxwell et al., 2003). Prolonged and persistent brown tides (*Aureoumbra lagunensis* in the Laguna Madre, Texas and *Aureococcus anophagefferens* in estuaries from Narragansett Bay, Rhode Island, to Barnegat Bay, New Jersey) detrimentally affected seagrass beds and suspension-feeding bivalves, including bay scallops (Stockwell et al., 1996; Bricelj and Lonsdale, 1997).

In addition to decreased water clarity, increased nutrient loading favors the growth of fast-growing, short-lived epiphytic macroalgae over slow-growing, long-lived macrophytes (Schramm, 1999). With hypereutrophic conditions, free-floating macroalgae, in particular "green tide"–forming taxa such as *Ulva* and *Enteromorpha*, alternate with dense phytoplankton blooms in dominance and replace the perennial and slow-growing benthic macrophytes until their extinction. A massive bloom of *Enteromorpha prolifera* covered 3800 km^2 (16%) of a 23,000-km^2 area in the East China Sea in June and July 2008 and caused problems for the sailing competition of the 2008 Olympics at Qindao, China (Hu and He, 2008). The suspected mechanisms were the nutrient-rich, sediment-laden plume of the Changjiang River off Shanghai, which probably transported to the northeast some of the algae that proliferated when suspended sediments settled out. Optimal water clarity and high nutrient concentrations supported a proliferation of the *Enteromorpha*. Other factors besides nutrients and water clarity, such as changes in hydrological conditions and grazing communities, influence or determine the varying responses of benthic macroalgae to increased nutrient levels.

Clear water and rocky shores with dense growths of the brown seaweed bladderwrack (*Fucus vesiculosus*) on rocky shores characterized the Baltic Sea in the 1940s (Jansson and Dahlberg, 1999). With increased phytoplankton blooms that reduced light penetration by 3 m compared to the first half of the century (Sandén and HAAkansson, 1996), the lower growth limit moved up by about 3 m since the 1940s, and the bladderwrack does not now grow as densely as before (Kautsky et al., 1986). Bladderwrack habitat functions as a refuge from predators, source of prey, and location of spawning and nursery grounds, but these functions are compromised and no longer support many species previously found there. A similar loss of massive beds of red macroalgae occurred on the northwestern shelf of the Black Sea with concomitant loss of fisheries stocks that were supported by that habitat (Mee, 1992).

5.3.5 Harmful Algal Blooms

Excessive phytoplankton growth in response to nutrient increases or shifts in nutrient ratios or both may result in a bloom of a single species that has some negative impact. These events are typically called *harmful algal blooms* (HABs) and variously encompass red tides, brown tides, and toxic and noxious blooms. Toxic forms may directly affect a variety of life forms, such as macroalgae, invertebrates, and vertebrates, including humans, and indirectly cause impacts through the consumption of toxins accumulated in fish and shellfish. Less obvious impacts are reduced grazing, increased flux of organic matter leading to hypoxia, and changes in trophic dynamics.

There has been some debate as to whether the frequency of harmful algal blooms has increased, but several researchers suggest a clear global expansion (Hallegraeff, 1993; Glibert et al., 2005). At a local scale, there are reports of positive trends in HABs that parallel trends in eutrophication—but this is not necessarily the case with many HABs (Zingone and Wyatt, 2004). Compelling evidence points to a linkage between nutrient loading and the often-cited increased frequency of harmful algal blooms in the Seto Inland Sea, Japan (Okaichi, 1989), Tolo Harbor in Hong Kong (Lam and Ho, 1989), the Baltic Sea (Poutanen and Nikkilä, 2001), and the East China Sea (Zhang, 1994). Other lines of evidence link cultural eutrophication to several HAB species (Glibert et al., 2005). The domoic acid–producing forms of the diatom *Pseudo-nitzschia* occur in the northern Gulf of Mexico (Parsons et al., 1999), and their seasonal abundance correlates

with high dissolved inorganic nitrogen flux from the Mississippi River. In addition, their abundance has been increasing since the 1950s and is coincident with human-related increases in riverine nitrogen flux and decreases in the Si/N ratio (Parsons et al., 2002).

5.3.6 Hypoxia and Anoxia

Dead and senescent algae, zooplankton fecal pellets, and marine aggregates resulting from nutrient-enhanced primary production contribute significant amounts of organic detritus to the lower water column and seabed. Aerobic bacteria consume oxygen during the decay of the carbon and deplete the oxygen in the lower water column at a faster rate than the diffusion of oxygen from surface waters to bottom waters, particularly when stratification of the water column occurs. The result is hypoxia (low oxygen) and sometimes anoxia (no oxygen). In a recent compilation of anthropogenic hypoxic zones, Díaz and Rosenberg (2008) noted that there was a consistent trend of increasing severity (either in duration, intensity, or size) where hypoxia occurred historically, or hypoxia existed presently when it did not occur before. Although hypoxic environments have existed through geologic time and are common features of the deep ocean, oxygen-minimum zones, or adjacent to areas of upwelling, their occurrence in estuarine and coastal areas is increasing, and the trend is consistent with the increase in human activities that result in nutrient overenrichment.

The coastal areas of the Baltic Sea and northern Gulf of Mexico are the largest such coastal hypoxic zones in the world, reaching 84,000 km^2 and 22,000 km^2, respectively (Rosenberg, 1985; Conley et al., 2002; Rabalais and Turner, 2006). An equally large area of hypoxia became more frequent and widespread on the northwestern shelf of the Black Sea in the 1970s and 1980s (Zaitsev, 1992; Mee, 2001), reaching over areas of the seafloor up to 40,000 km^2 in depths of 8 to 40 m, as nitrogen and phosphorus loads from the Danube River increased. Similar declines in bottom-water dissolved oxygen have occurred elsewhere as a result of increasing nutrient loads and cultural eutrophication: for example, the northern Adriatic Sea (Justić et al., 1987), the Kattegat and Skaggerak (Rosenberg, 1985; Andersson and Rydberg, 1988), Chesapeake Bay (Officer et al., 1984; Hagy et al., 2004), the German Bight and the North Sea (Dethlefsen and von Westernhagen, 1983), and Long Island Sound (O'Shea and Brosnan, 2000).

The obvious effects of hypoxia/anoxia are displacement of pelagic organisms and selective loss of demersal and benthic organisms (Rabalais and Turner, 2001). These impacts may be aperiodic so that recovery occurs, may occur on a seasonal basis with differing rates of recovery, or may be permanent so that long-term ecosystem structure and function shifts. A variety of behavioral and physiological impairments affect the animals that reside in the water column or in the sediments or attached to hard substrates as oxygen concentrations reach critical thresholds (Gray et al., 2002; Vaquer-Sunyer and Duarte, 2008). Mobile animals such as shrimp, fish, and some crabs flee waters where the oxygen concentration falls below 25 to 30% oxygen saturation. As oxygen levels decline to anoxia, there is a fairly linear decrease in benthic infaunal diversity, abundance, and biomass (Rabalais et al., 2001), leading to an impoverished diet for bottom-feeding fish and crustaceans. In waters of Scandinavia and the Baltic, Karlson et al., (2002) estimated a reduction of 3 million tons in benthic macrofaunal biomass during the worst years of hypoxia occurrence. Estimates of missing biomass for about one-third of the world's human-caused hypoxic areas indicate that as much as 343,000 to 734,000 metric tons of carbon is displaced over

an area of 245,000 km^2 (Díaz and Rosenberg, 2008). These losses may, however, be partly compensated by the biomass increase that occurred in well-flushed organically enriched coastal areas not subject to hypoxia. In most instances, longer-lived, deep-burrowing organisms are replaced by opportunistic, surface deposit feeders, and therefore changes in bioturbation activity and biogeochemical processes (Rosenberg, 1990; Gutiérrez et al., 2000; Rabalais et al., 2001).

5.4 HISTORICAL COURSE OF EUTROPHICATION

Changes in nutrients as well as the symptoms of cultural eutrophication follow similar time courses on a global scale among developed countries (Cloern, 2001; Boesch, 2002). This same sequence of events is becoming more evident in developing countries (Figure 5.2). As scientists began documenting the sequence of symptoms progressing toward eutrophication and correlating them with changes in water quality, particularly nutrient loads, the patterns and similar trajectories became evident globally in developed countries in Europe, North America, and Asia. This pandemic cultural eutrophication was well established before scientists started to recognize the patterns of coastal eutrophication and implications for those ecosystems (Rosenberg, 1985). The data continued to emerge in the same or additional ecosystems (Nixon, 1995; Cloern, 2001). Boesch (2002) summarized the "explosive and synchronous intensification of eutrophication" in susceptible coastal ecosystems over the narrow period 1960–1980. This period somewhat lagged the beginning increases in nutrient loads, but clearly occurred during the period of accelerated nutrient additions.

Given the trajectories of future nutrient loads in both developed and developing countries, principally from terrestrial drainage, atmospheric deposition, and urban discharges, it is likely that coastal eutrophication will continue to expand globally. Populations will continue to expand with higher consumptive requirements for food and fuel, and the current trend is for fertilizer use to escalate as the industrialization of agriculture intensifies in the developed world and spreads even more rapidly in

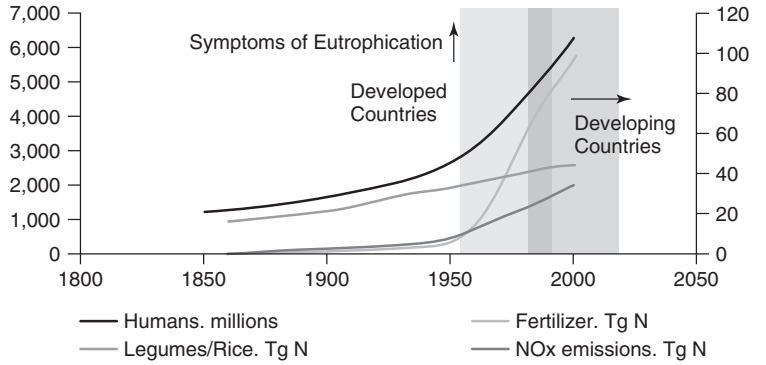

Figure 5.2 Period of the explosive increase in coastal eutrophication in relation to global additions of anthropogenically fixed nitrogen. Most of the symptoms for developed nations were manifested in the 1960s to 1980s (aqua) but are becoming more evident in developing countries with increases in fossil-fuel consumption and use of artificial fertilizers (lavender). (Modified from Boesch, 2002; Galloway and Cowling, 2002.) (See insert for color representation.)

developing countries. Coastal ecosystems in many parts of the world, where conditions are conducive to stratification and retention of water, are at high risk for developing eutrophication and the associated direct and indirect responses, bringing to bear Rosenberg's (1985) prediction for coastal eutrophication to become the future coastal nuisance and supporting the National Research Council's (2000) statement that "given the growing magnitude of the problem and the significance of the resources at risk, nutrient over-enrichment represents the greatest pollution threat faced by the coastal marine environment."

5.5 MANAGEMENT CHALLENGES

The path to human-caused eutrophication of estuarine and coastal waters follows centuries of human population growth and changes in landscapes and land use, so that lands lose more nutrients than historically when watersheds are less functional at removing them naturally. Beginning in the middle of the twentieth century, an increasing human population, emissions of oxidized forms of nitrogen (NO_x), production and use of artificial fertilizers, and volatilization of reduced forms of nitrogen (NH_y) accelerated the rate of reactive nitrogen and phosphorus in global nutrient budgets. Management of both terrestrial and coastal ecosystems is therefore integrally linked.

5.5.1 Nutrient Mitigation

While Schindler (2008) clearly demonstrated that phosphorus inputs must be decreased to mitigate eutrophication of lakes and reservoirs, reactive nitrogen *and* phosphorus are the key controlling factors of eutrophication along a gradient from the head of estuaries through the coastal ocean (see above) with sufficient evidence to support this course, despite Carpenter (2008) indicating that arguments about the relative roles of nitrogen and phosphorus in coastal ecosystems remain unresolved because of the inability to conduct ecosystem-level experiments. It is clear that both nitrogen and phosphorus controls, in concert, are necessary for the slowing or reversal of eutrophication symptoms in estuarine and coastal systems (Paerl et al., 2004; Howarth and Marino, 2006; SAB, 2008). It is also naive to expect that nutrient management practices for nitrogen and phosphorus each will be suitable for reducing the opposite nutrient, because the sources and relative inputs differ among landscape uses and watersheds (Figure 5.3). Nitrate contamination is more likely to come from seepage through soils, drainage from tiled croplands, volatilization of nitrogen from fertilizers and manure, and atmospheric deposition of reduced nitrogen from burning of fossil fuels. Phosphorus is more likely to come with erosion of soils and movement of artificial phosphorus fertilizers off the land via manure and wastewater treatment plants.

From the late 1960s to the mid-1980s, the nutrient inputs to the Baltic increased fourfold for nitrogen and eightfold for phosphorus (Elmgren, 2001). Initially, the focus there was on phosphorus as the key element in the eutrophication process, but this was soon questioned. Algal growth-potential experiments and water chemistry both implicated nitrogen as the main limiting nutrient in the open Baltic Proper as well as in open Danish waters. The spring phytoplankton bloom in the Baltic Proper is clearly nitrogen limited, but summer production may sometimes be phosphorus limited. The seasonal variation of nutrient limitation and time course of nutrient changes in the Baltic

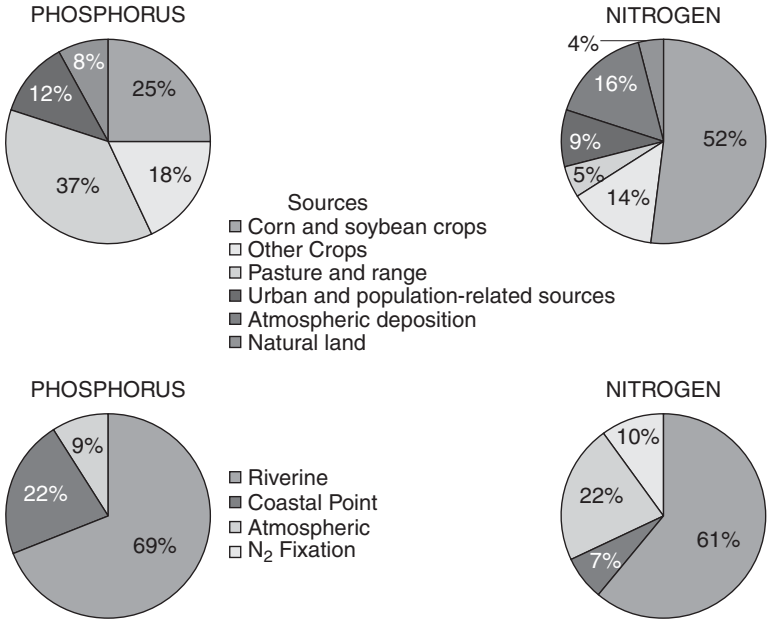

Figure 5.3 Relative proportion of sources or transport mechanisms of nitrogen and phosphorus to the northern Gulf of Mexico from the Mississippi River watershed (upper panels) (from Alexander et al., 2008, http://water.usgs.gov/nawqa/sparrow/gulf_findings/) and to the Baltic Proper (from Grimvall and Stålnacke, 2001.) (See insert for color representation.)

raised practical issues related to their management. Fourteen nations agreed through the Helsinki Commission to restore the Baltic by reducing loads of both nitrogen and phosphorus. In addition to the complexities of nutrient limitation, there is evidence that loads from the land have been rather constant since 1970, but nutrient concentrations in the Baltic gradually increased up to the late 1980s. This disconnect in loads and concentrations is probably related to landscape alterations that allow quicker transport of nitrogen from field to sea and lower denitrification losses en route (e.g., lowering of lakes, hydrologic modifications including dams that trap silicate, draining of wetlands, and straightening of waterways). Thus, nutrient management involves both repair to the landscape and reductions in inputs (Mitsch et al., 2001).

A similar situation exists in the Mississippi River watershed that fuels the nutrient-enhanced primary production and subsequent seasonally severe oxygen depletion in the Gulf of Mexico (Rabalais et al., 2002; SAB, 2008). The nitrogen load increased threefold from the 1960s to 1980s, due primarily to an increase in nitrogen concentration, but began to stabilize in the 1990s. There is a slight indication of increase in total phosphorus over this time, but not in orthophosphate. Since then the loads of nitrogen continued to rise because land drainage facilitated flux from artificially drained agriculture fields (Raymond et al., 2008) and a further increase in the concentration of nitrate-nitrogen in the Mississippi River related to biofuel production (Turner et al., 2008). In addition, an equal load of nitrate-nitrogen delivered by the Mississippi River results in a larger area of hypoxic water than it did historically, indicating that a regime shift has occurred in the coastal ecosystem. This makes

the mitigation of nitrogen with time more critical. "Each year without a significant reduction in nutrient loading rates means that it will take longer to realize the Action Plan goal [reduction in the size of hypoxia], because the legacy of accumulated organic matter and its respiratory demand increases with time" (Turner et al., 2008). Although there is clear evidence of phosphorus limitation of phytoplankton growth near the Mississippi River during high flow and in lower salinity waters, it remains true that nitrogen is the overall limiting nutrient for phytoplankton growth over the broad area where hypoxia occurs. In fact, the P limitation is mostly driven by the dramatic rise of N, so that the N/P ratio is well above the Redfield ratio. A conclusion on Gulf of Mexico hypoxia by the Science Advisory Board (2008) was that nitrogen and phosphorus controls need to be addressed simultaneously and that targeting areas where the yields of nitrogen and phosphorus are highest is recommended.

5.5.2 System Response and Inertia

Any nutrient management strategy applied to the landscape or the chemicals applied to the landscape to reduce excess nutrients will probably result in changes in the reverse of those seen over the last decades and centuries, but the change will probably be gradual because of the strong buffering capacity of the soil ecosystem. It is likely to take the many decades it took for the present system to develop to succeed in water quality rehabilitation. The strong buffering capacity of the soils is becoming evident (Stålnacke et al., 1999; Bennett et al., 2001). There is inertia in terrestrial systems and rivers and streams with regard to losses from land to sea following nutrient reductions actually achieved or planned (Grimvall et al., 2000). Nitrate leaching from a grain field in Sweden continued almost unabated 13 years after fertilizers were no longer added (Löfgren et al., 1999). Data on river water quality following the collapse (circa 1990) of agriculture in the former Soviet republics of Estonia, Latvia, and Lithuania demonstrated that although fertilizer application fell to the level of the 1950s, the concentration of inorganic phosphorus and nitrogen was the same in 1994 as in 1987 (Löfgren et al., 1999).

Public and private funds were expended within the Chesapeake Bay watershed to reduce the controllable sources of nitrogen and phosphorus entering the bay by 40% by the year 2000. Efforts targeted both point sources, such as treated sewage discharges, and non-point sources, especially those from agriculture, and the trapping of nutrients in the watershed by wetland and riparian zone restoration. Assessing whether the reduction targets were reached was difficult, but it appears that the goal was nearly met for phosphorus, but nitrogen loadings, although reduced, did not achieve the goal. Similarly, Grimvall et al. (2000) reported that there was a remarkable lack of response in eastern European river nutrient loads in response to the dramatic decrease in the use of commercial fertilizers that started in the late 1980s. In western Europe, while studies of decreased phosphorus emissions have shown that riverine phosphorus loads can rapidly be reduced from high to moderate levels, a further reduction, if achieved at all, may take decades.

The accumulated loads of organic matter and the internal load of inorganic and organic nutrients in the sediments underlying eutrophic waters perpetuate conditions of eutrophication as they continue to be processed by normal geochemical processes in the sediments (Rabalais et al., 2002). Within estuaries and coastal systems, a decrease in external nutrient loads does not produce an immediate shift in the eutrophic state of the

system, in part because of the continued remineralization of labile carbon and releases of regenerated nutrients. Boynton and Kemp (2000) suggested a "nutrient memory" over time scales of a year rather than seasonal periods as suggested by Chesapeake Bay water residence times. Turner et al. (2008) suggested that at least a year of continued carbon respiration contributed to oxygen demand on the Louisiana continental shelf in the subsequent summer. Besides an inherent lag in system responses, climatic variability may mask any attempts to reduce nutrients within a watershed. For the Kattegat, given the variability present in the system, a statistically significant detectable improvement in oxygen conditions will take 18 years (Richardson, 1996b). These predictions are relevant to management strategies to mitigate nutrient loads to estuaries and coastal waters and the perceived projection for "recovery."

As a result of the economic collapse of the former Soviet Union and declines in subsidies for fertilizers, the decade of the 1990s witnessed a substantially decreased input of nutrients to the Black Sea, with resulting signs of recovery in some aspects of the pelagic and benthic ecosystems (Mee, 2001; Lancelot et al., 2002). There is a recovery in zoobenthos species diversity; phytoplankton biomass has declined. Some trophic levels in the coastal ecosystem have recovered, but others have not. Oxygen deficiency decreased and was nonexistent or minimal within six years. There should be little doubt of the strong relationships among human activities, northwestern shelf Black Sea eutrophication, and demise of pelagic and benthic coastal ecosystems (Lancelot et al., 2002), as well as similar linkages in the partial recovery of those systems following reduced nutrients. Although the mediator of the nutrient reductions from the watershed of the Black Sea was economic hardship and decline (i.e., not a preferable means of reducing nutrient loads worldwide), the resilience of the coastal ecosystem within periods of a few years to a decade is heartening.

5.5.3 Progress

Reducing excess nutrient loading to estuarine and coastal waters requires individual, societal, and political will. Proposed solutions are often controversial and may extract societal and economic costs. Yet, multiple cost-effective methods of reducing nutrient use and delivery can be integrated into a management plan that results in improved habitat and water quality, within both the watershed and the receiving waters (NRC, 2000). These efforts are usually more successful in reducing point sources of nitrogen and phosphorus than with the multiple non-point sources of high solubility and growing atmospheric inputs of nitrogen. The growing decline in coastal water quality and expansion of symptoms of eutrophication, and the proven successes of reducing nutrients, are reasons enough for continuation and expansion of efforts to reduce nutrient overenrichment.

Countermeasures may take decades for effective restoration to be realized, and as noted above, it may take decades for management intervention to be realized in the landscape and the coastal ecosystem. The landscapes and the ecosystems that depend on them are held within social structures that can be modified for the betterment of the ecosystems and the humans that depend on them, or for the worse. The social infrastructure supporting the human-made landscape is nontrivial and important to many. It involves food, fiber, and fuel supplies, national political agendas, international trade, and global climate change. It will take political and societal will for scientists, citizens, land and water managers, agriculturalists, industrialists, and environmentalists

to work toward a mutually satisfying equilibrium of interests that is fair, sustainable, ethical, and socially responsive (Turner and Rabalais, 2003).

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CHAPTER 6

Microbial Deterioration of Cultural Heritage Materials

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6.1 HERITAGE MATERIALS AND ENVIRONMENTAL SCIENCE

A chapter on microbial deterioration of historic materials may strike many as slightly out of place in a book devoted to environmental microbiology. For most microbiologists and environmental scientists, the word *conservation* undoubtedly calls to mind natural resources. Our goal in this chapter is to introduce a field of study that we believe is viewed correctly as a branch of environmental science: one that deals with the study and conservation of historic and cultural resources.

During the past 35 to 40 years, we have become acutely aware of threats to the natural environment and the need to conserve the world's resources. Research into the effects of human activity on the biology and chemistry of the biosphere has resulted in increased understanding of threats to the environment and in efforts to protect natural resources. We now understand that our historic manuscripts, buildings, monuments, and archaeological sites, all of which are the cultural analogs of the biosphere, are also at risk from human activities and in need of conservation.

Some of our most important cultural and historic symbols show evidence of deterioration. The Taj Mahal is threatened by air pollution, Da Vinci's *Mona Lisa* is cracking, and Michaelangelo's *David* was recently cleaned to remove sulfates. The consequences of human activities, such as acid rain and urban air pollution, are among the main causes of deterioration (Figure 6.1). In addition to physical and chemical processes of deterioration, many historic and culturally important objects are also at risk of biodeterioration. Microbial processes act in conjunction with chemical deterioration caused by air pollution to damage stone. Improper storage of objects under conditions of high temperature and humidity can stimulate bacterial or fungal growth and deterioration. No material is immune to microbial attack; microorganisms have been shown to play a role in the deterioration of historic paintings, wood,

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Figure 6.1 Effects of pollution caused deterioration on the statue of an angel at the cathedral of Cologne, Germany: (a) picture taken in 1880; (b) the same statue in 1993. (From Warscheid and Braams, 2000.)

paper, glass, textiles, metals, waxes, polymers and coatings, and stone (Koestler et al., 2003).

6.1.1 Biofilms

Microbial deterioration of cultural heritage materials occurs primarily through the formation and growth of biofilms. With respect to the deterioration of heritage materials, there are several characteristics of biofilms that are of importance. Biofilms are extremely difficult to eradicate and can serve as a source of microorganisms that disperse to other habitats (Costerton et al., 1999). Harsh physical and chemical treatments that are needed to disinfect surfaces may damage fragile heritage materials. Less stringent treatments may allow biofims to regrow and contaminate other objects in the collection. The biofilm exopolymer matrix may also play a role in the deterioration of some materials. Acidic functional groups on the polymer may damage stone structures. Finally, the close association of different types of organisms within the biofilm allows for cooperative growth that can enhance deterioration. For example, large communities of heterotrophic bacteria have been found growing on stone surfaces. Presumably, a major source of organic carbon for these organisms is the associated cyanobacteria and algae also found on the stone surface.

6.2 DETERIORATION OF SPECIFIC MATERIALS

6.2.1 Paper

There are two separate processes of concern in the study of microbial deterioration of paper, both of which are primarily the result of fungal growth. The first is degradation of the paper itself through fungal hydrolysis of the cellulose fibers (Szczepanowska and Cavaliere, 2000). The second and more interesting phenomenon is foxing, the appearance of reddish-brown colored stains (Figure 6.2). The origin of fox spots on paper (so-called because the reddish-brown to yellow stains may resemble the color of fox fur) has historically been attributed to both metal (iron or manganese) contamination and fungal growth. Questions about the role of fungi in foxing arose from two problems. First was the inability to determine if fungal growth was the actual cause of the staining and not simply a secondary problem. Second, if one were to assume that fungi were responsible, how would one determine if observed or cultured organisms were the fungi responsible for discoloration? Solutions to each of these problems were hampered by difficulties reproducing the foxing effect with cultured fungi in the laboratory.

Evidence for fungal growth as the cause of foxing comes from several sources. Fungi are invariably found associated with fox spots (Meynell and Newsam, 1978; Florian, 1996). Fox spots also appear to occur in areas of low iron concentration on paper (Arai, 2000; Press, 2001). Finally, the reddish-brown color of fox spots appears to contain a variety of organic acids, oligosaccharides, and amino acids (combinations of some of these components can induce similar stains on paper, particularly, γ-aminobutyric acid, β-alanine, glycine, ornithine, and serine) (Arai, 2000).

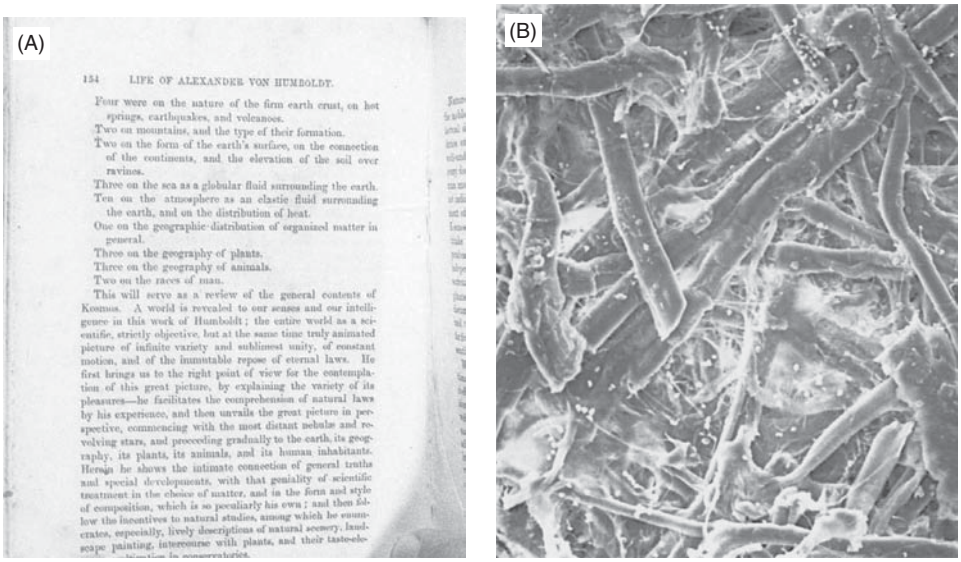

Figure 6.2 (A) Reddish-brown fox spots in the book *Lives of the Brothers Humboldt*, published in 1854. (B) Scanning electron micrograph of fungal growth occurring in a fox spot on paper. (Adapted from Florian, 1996.) (See insert for color representation.)

A wide variety of fungi have been isolated from foxed paper, including *Aspergillus, Chaotomium, Cladosporium, Penicillium*, and yeasts (Corte et al., 2003). Bacteria have also been found but rarely identified, and it is not clear that they play a role in the discoloration (De Paolis and Lippi, 2008). Growth of fungi associated with foxing is dependent on the environmental conditions in which the paper was stored. Arai (2000) found that a water activity of 0.84 and temperatures above 25°C were necessary to culture fungi from fox spots. Due to the hygroscopic nature of paper, recommended storage conditions are 16 to 20°C and 40 to 60% relative humidity (Gallo, 1992).

6.2.2 Glass

Although there are instances of bacterial and lichen-caused deterioration, fungi appear to be the most common organisms to cause the deterioration of glass. Organic matter to support microbial growth is deposited on the glass from various sources, such as dust, dirt, and dead microorganisms. Other significant environmental factors include water availability and temperature. In urban areas, pollutants such as carbon dioxide and sulfur dioxide can stimulate biodeterioration (Garcia-Vallès et al., 2003). In addition to the availability of organic matter, the extent of biofilm growth is also related to glass composition (the presence of essential trace elements) and durability (Müller et al., 2001).

Damage to glass caused by microbial growth is the result of both physical and chemical processes. Acidic microbial metabolites can cause pitting and etching, which can enhance physical processes such as crack formation. Microbial metabolites may mobilize components of the glass, such as iron or manganese, which are then deposited on the surface, causing discoloration. Analysis of 18S rDNA from fungi found on glass from historic churches in Germany revealed a complex fungal assemblage, including *Aspergillus, Aureobasidium*, and *Geomyces* (Table 6.1) (Schabereiter-Gurtner et al., 2001a).

6.2.3 Paintings

Painted objects and surfaces constitute a wide range of different substrata, pigments, and binders. Additionally, they are found in conditions ranging from constant, protective museum environments to highly exposed outdoor locations. Easel paintings, in one sense, present the simplest case. Due to their size and indoor location, microbial growth can be restricted through properly controlled environmental conditions of temperature and humidity. On the other hand, materials used in easel paintings are varied and can provide a wide range of substrates for microbial growth. These include cellulose or protein in the support materials (such as canvas, paper, wood, or silk), organic material in the sizing, and a wide range of organic binders (lipids, sugars, and in some cases substances such as egg yolk or bile).

Wall paintings present a somewhat different set of conditions for microbial growth. Their size and location may make environmental control of microbial growth difficult or impossible. On the other hand, they may initially present a much narrower range of substrates to support microbial growth—primarily organic binders. However, colonization by photosynthetic microorganisms can provide organic material for subsequent growth of heterotrophic microorganisms.

Table 6.1 Fungal Taxa[a] on Nineteenth-Century Window Glass in a Church in Stockkämpen, Germany and the Church of St. Michael and Johann Baptist in Brakel, Germany

Fungal Taxa with Most Similar Sequence in EMBL Database	% Similarity[b]
Ascomycetes	
Aspergillus fumigatus	95.3
Aureobasidium pullulans	93.7–98.5
Capnobotryella renispora	95.3
Coniosporum perforans	97.8
Engyodontium album	97.1
Geomyces asperulatus	99.8
G. pannorum	95.5–96.5
Kirschsteiniothelia elaterascus	95.1
Leptosphaeria maculans	97.5
Stanjemonium ochroroseum	95.4
Verticillium lecanii	98.8–99.7
V. psalliotae	98.8
Basidiomycetes	
Rhodotorula minuta	98.1
Ustilago spp.	99.0

Source: Adapted from Schabereiter-Gurtner et al. (2001a).
[a]Identified from 18S rDNA sequences.
[b]Ranges are given for multiple clones related to the same taxa.

Ciferri (1999) provides an excellent review of the microorganisms usually found on easel and wall paintings. These commonly consist of soil fungi (*Penicillium, Aspergillus, Chaotomium, Cladosporium, Alternaria*) and soil bacteria (*Pseudomonas, Arthrobacter, Streptomyces*). Gurtner et al. (2000) presented a detailed analysis of bacteria on two wall paintings in Austria and Germany using denaturing gradient gel electrophoresis and 16S rDNA sequence analysis. There was little overlap in sequences between the two paintings, and most were related to the actinobacteria and proteobacteria. Furthermore, there was little similarity between these sequences and isolates cultured from the paintings identified by fatty acid methyl ester analysis. Subsequent investigation of frescos at the same location in Austria (Catherine Chapel of the Castle Herberstein) also revealed the presence of halophilic and alkaliphilic archaea (Piñar et al., 2001). The presence of halophiles and alkaliphiles is probably indicative of the high-pH high-salt environment on the surface of the frescos, which are painted on a lime plaster base.

A third group of paintings that are susceptible to microbial attack are prehistoric cave paintings. The caves and the paintings are host to a wide range of microorganisms (Groth et al., 2001). In these caves, paintings that were stable for thousands of years can suddenly initiate dense microbial growth that endangers the paintings. The growth is caused primarily by human activities. Tourist traffic and lighting may alter the temperature and humidity of the caves, as may misguided attempts to alter or control the cave environment. Additionally, it has been suggested that climate change may play a factor, increasing the temperature and humidity, especially in small and shallow caves. The best known example is the explosive fungal growth that has occurred in

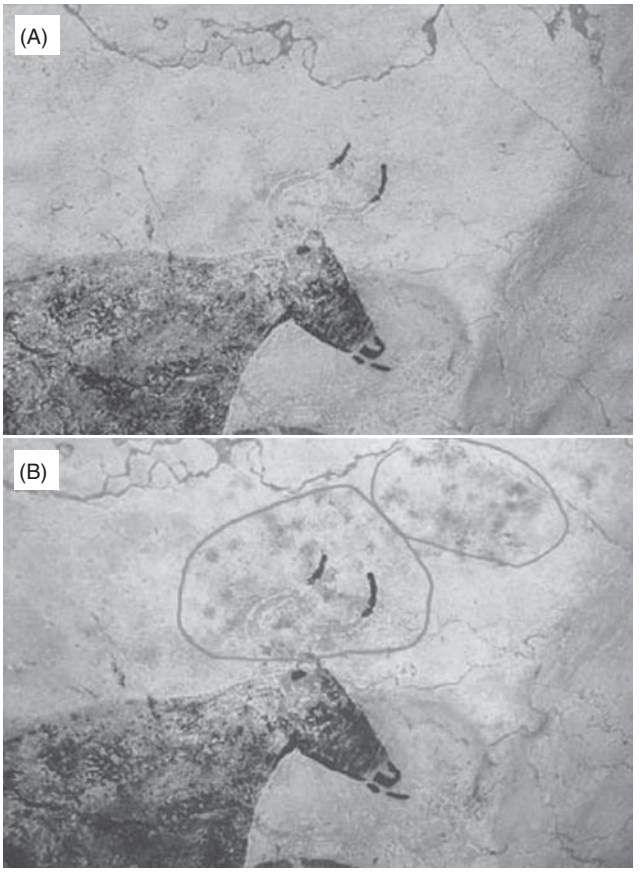

Figure 6.3 Painting of a bull on a wall in Lascaux before (A) and after (B) growth of grayish-black fungi. (From Bahn, 2008.) (See insert for color representation.)

Lascaux, France (Figure 6.3). A bloom of *Fusarium solani* that began in 2001 has been followed by an outbreak of gray and black fungal growth. The great age and fragile nature of the paintings make treatment of the fungal outbreaks difficult. For example, during the *F. solani* outbreak, conservators resorted to removing visible fungal growth by hand.

6.2.4 Polymers

Polymeric materials are found in a variety of culturally, historically, and artistically significant materials, ranging from modern art and photographs to protective coatings for bronze and stone. Additionally, many modern archival materials, such as video, audiotape, and compact disks contain polymeric materials. Although bacteria and algae can also cause deterioration, most cases of polymeric heritage materials degradation have been attributed to fungal growth [see Cappitelli and Sorlini (2008) and references therein].

In addition to aesthetic damage caused by microbial growth, acid and enzyme production by microorganisms can result in deterioration of the polymers and selective leaching of material components (Mitton et al., 1993). Breuker et al. (2003) isolated

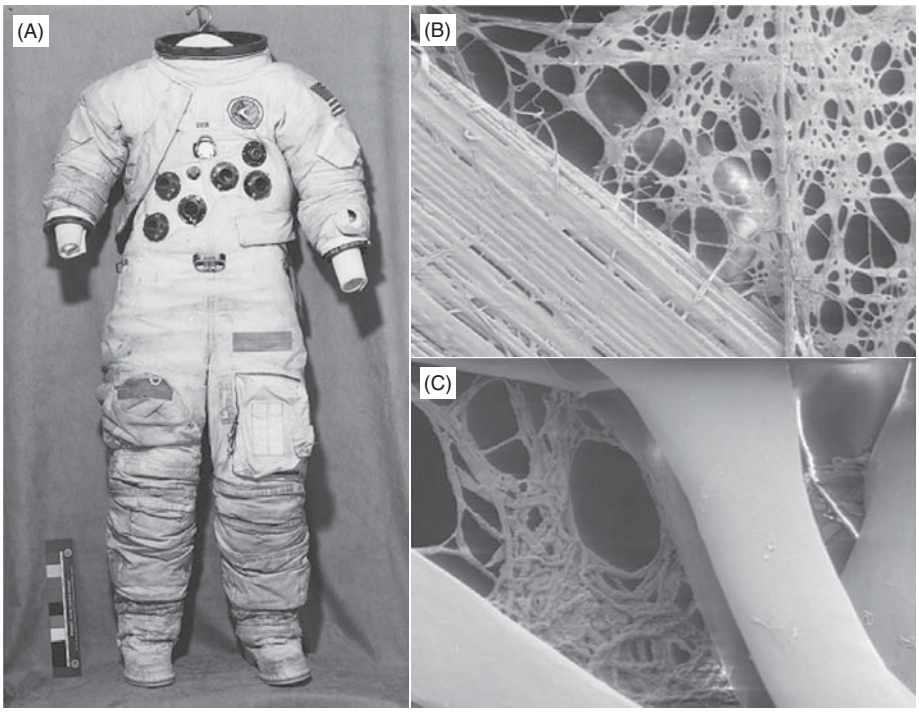

Figure 6.4 Fungal growth on materials from an *Apollo* space suit. (A) The suit worn by David R. Scott, commander of the *Apollo 15* mission. (B). Growth of *Paecilomyces* sp. on beta marquisette. (C). Growth of *Cladosporium cladosporioides* on nonwoven Dacron. (Adapted from Breuker et al., 2003.)

two species of fungi (*Penicllium* and *Aureobasidium*) from an *Apollo* space suit in the National Air and Space Museum and demonstrated that the fungi could degrade synthetic cloths found in the suits (Figure 6.4). McNamara et al. (2004) isolated an unidentified yeast from the coating on a bronze monument and showed that the yeast could cause deterioration of the polymer coating. Tarnowski et al. (2007) isolated a mixed bacterial community from soil that could degrade antigraffiti coatings used to protect stone. Like other materials, growth of fungi on these and other polymeric materials is influenced by environmental conditions. Without sufficient temperature and humidity, microbial growth will be inhibited.

6.2.5 Wood

Blanchette (2000) described the microbial decay processes of historic wood in detail. Terrestrial decay occurs primarily through the action of fungal growth and there are three mechanisms of deterioration. White rot fungi, a heterogeneous group within the *Basidiomycota*, degrade cell wall components and cause the characteristic bleaching of wood. Brown rot fungi cause rapid depolymerization of cellulose and degradation of cell wall carbohydrates, leaving behind a lignin-rich brown-colored wood. The most destructive brown rot fungus is *Serpula lacrymans*. A third type of terrestrial degradation, soft rot, is caused by fungi within the *Ascomycota* and *Deuteromycota* and is

characterized by degradation of the secondary cell wall of the wood cells. In contrast, wood in aquatic environments is attacked primarily by bacteria. Three different patterns of bacterial attack have been characterized: erosion of the secondary wall, formation of pits (cavitation) in the secondary wall, and tunneling through the secondary wall and middle lamellae.

Microbial growth interacts with environmental factors in the degradation of wood. In terrestrial environments, temperature and relative humidity regulate microbial growth and deterioration. Sulfur dioxide in air pollution reacts on wood to form gypsum, which can accumulate on the wood surface as well as inside the wood. During formation, the gypsum traps hydrocarbons generated in the urban environment from burning fossil fuels, which then serve as a substrate for microbial growth (Genestar and Pons, 2008).

6.2.6 Stone

In some instances, the presence of microbial growth on stone is a purely aesthetic issue, because visible growth of pigmented microorganisms can alter the appearance of buildings and statues. However, in many cases microorganisms are one of the main causes of stone deterioration (Warscheid and Braams, 2000) or may exacerbate physical weathering of stone (Papida et al., 2000). Microbial biodeterioration of stone is widely thought to occur through the action of organic and inorganic acids produced as metabolic by-products (Sand and Bock, 1991). However, in addition to metabolic acids, biofilm exopolymers can increase the dissolution rate of calcium carbonate, suggesting that they may also cause deterioration of stone cultural heritage materials (Perry et al., 2004).

Not all organic acids and polysaccharides produced by microorganisms cause immediate dissolution of stone. Depending on environmental conditions (e.g., pH), bacterial polysaccharides may precipitate calcium carbonate. Some acids may react with calcium from stone to form other minerals. Sulfuric acid produced by sulfur-oxidizing bacteria can react with limestone to form a gypsum (calcium sulfate) crust. Exfoliation of this crust results in deterioration of the stone. Similarly, oxalic acid, which is produced by many fungi and lichens as well as some bacteria, can form the minerals wewelite and weddellite (calcium oxalate) on limestone. However, calcium oxalate is less soluble in water than is calcium carbonate, and it has been suggested that formation of a calcium oxalate layer could be protective.

In addition to growth on the surface, microorganisms have been found in the interior of porous rock, such as limestone (McNamara et al., 2006). These organisms colonize the interior of the stone through pores and cracks formed due to weathering. The endolithic microflora may cause deterioration of culturally important stone objects through the same mechanisms as those used by microorganisms on the surface. In addition, water absorption by the biofilm matrix results in shrinking and swelling of the extracellular polymeric substance, causing mechanical stress that opens cracks and fissures in the stone (Warscheid and Braams, 2000). In the case of endolithic cyanobacteria, this process has been shown to cause exfoliation of surface layers and crusts (Krumbein, 1988).

Culture-based studies have identified a few groups of easily cultured bacteria (e.g., *Pseudomonas, Actinobacteria*, and *Bacillus*) as well as organisms related to the local fauna (e.g., *Staphylococcus* and *Salmonella*), as the most common

microorganisms on stone heritage materials (Videla et al., 2000; Gaylarde et al., 2001; McNamara et al., 2003). Studies using molecular techniques to examine microbial diversity on stone cultural heritage items have detected a wide range of bacteria. Phyla most commonly reported include the *Proteobacteria, Actinobacteria*, the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group, *Acidobacteria*, and the low GC *Firmicutes* group (Gurtner et al., 2000; Schabereiter-Gurtner et al., 2001b, 2002). One of the most striking aspects of these studies is the similarity of the organisms from site to site; these five phyla formed the majority of the community at all sites studied. Most of the sites investigated using these methods are located in western Europe. However, one study from southern Mexico yielded similar results (McNamara et al., 2006). There are insufficient data from molecular studies of this type to draw conclusions about geographic or climatic variation in the microbial populations. Furthermore, much of the similarity between communities may be a result of analysis of the 16S rDNA. This technique will identify both active and inactive microorganisms, and much of the observed similarity among sites may be due to the detection of microorganisms that are not active on the stone.

Tayler and May (2000) found that roughly 20 to 50% of bacteria on sandstone monuments were active. Unfortunately, the methodology did not provide information about the activity of specific taxonomic groups. In attempts to determine which taxa are active on stone, recent studies have compared both polymerase chain reaction (PCR)-amplified 16S rDNA and RNA reverse transcribed and amplified with 16S primers. A study of Altamira Cave, Spain found that many microorganisms, such as *Escherichia* and *Pseudomonas*, found in the DNA-based analysis were absent from the RNA analysis (Portillo et al., 2008).

Algae and *Cyanobacteria* are common on stone cultural heritage materials. In addition to causing deterioration of the stone, growth of these microorganisms is visibly obvious and results in green-to-black staining of the stone. A large number of different algal and cyanobacterial species have been found on historic stone buildings and monuments (described extensively in Ortega-Calvo et al., 1993). Light intensity seems to be an important factor in determining the composition of these photosynthetic communities. Interior areas with low light levels (e.g., catacombs and caves) tend to be dominated by cyanobacteria (Albertano and Urzi, 1999). In areas with high light levels, such as the exterior of Mayan ruins in southern Mexico, a more diverse community, including green algae, has been found (Videla et al., 2000). Environmental factors such as exposure to salt spray may also affect the types of algae and cyanobacteria growing on stone cultural heritage materials (Videla et al., 2000). Finally, whereas there appear to be differences in the photosynthetic microorganisms that colonize various types of stone and building materials (Tomaselli et al., 2000), the organisms found in tropical and temperate climates appear quite similar (Crispim et al., 2003). The role that algae and cyanobacteria play in supporting the growth of other microorganisms on stone cultural heritage materials remains largely unexplored. Large communities of heterotrophic bacteria and fungi have been reported on historic stone. Airborne deposition and throughfall from surrounding vegetation undoubtedly provide substantial quantities of organic matter. Algal and cyanobacterial photosynthesis are also likely to provide a large source.

Fungi and lichens are also frequently observed on historic structures. They have been implicated in damage to some stone cultural heritage materials, primarily through production of organic acids (Jones and Wilson, 1985; Sterflinger and Krumbein, 1997).

In some cases, a protective role for lichens has been proposed, based on the formation of a calcium oxalate patina (Di Bonaventura et al., 1999). The diversity of these organisms on stone is considerable (see Jones and Wilson, 1985; de la Torre et al., 1993; Gomez-Alarcon et al., 1994; Sterflinger and Krumbein, 1997 and the references therein). Fungal hyphae are capable of penetrating beneath the stone surface and can cause severe deterioration, through the production of organic acids, through shrinking and swelling of the hyphae and polysaccharides, and by providing an entry for water that can freeze and thaw. Penetration of stone by fungal hyphae results in transport of water, organic compounds, and nutrients through the stone, and this may facilitate colonization of the interior by bacteria. Although the influence of hyphal transport of materials has not been explored in historic stone, it is well documented in other cases, such as the transport of iron by the dry rot fungus *Serpula lacrymans* (Low et al., 2000).

6.3 EFFECTS OF AIR POLLUTION

Monuments and archeological sites throughout Europe and the United States are being discolored and degraded because of air pollution (Figure 6.1). For example, pollution has been linked to the decay of stone monuments in Italy (del Monte and Vittori, 1985). The eighteenth-century Royal palace in Stockholm is situated close to heavy traffic where particulates and harmful gases are emitted. The result has been signs of serious decay of the sandstone facades (Nord and Tronner, 1995).

The burning of fossil fuels for energy production and for transportation is an important source of destructive chemicals. Sulfur dioxide, hydrocarbons, and particulates function together in the formation of gypsum crusts. Sulfur dioxide emitted from fossil fuels into the atmosphere is oxidized to sulfuric acid, which attacks limestone to form calcium sulfate or gypsum. There are two mechanisms of deterioration resulting from gypsum formation. In a process called *exfoliation*, deterioration of the stone behind the gypsum causes the crusts to fall off, exposing the fresh stone to deposition of additional pollutants. Gypsum crusts can be substantial, and exfoliation poses a severe threat to historic stone. The sculptures on the Acropolis have accumulated gypsum to a depth of about 1 cm (Gauri, 1978).

Additionally, during formation, hydrocarbons and particulates are incorporated into the gypsum, giving the crust its characteristic black color. The incorporation of these sources of organic matter influence the microbial community found on the stone (Saiz-Jimenez, 1997). Mitchell and Gu (2000) compared headstones in polluted and unpolluted locations and found that the sulfur-oxidizing and hydrocarbon-utilizing microflora were enriched in the polluted location (Figure 6.5).

Air pollution also contains significant quantities of nitrogen oxides. Nitrogen oxides on stone are converted by nitrifiers to nitric acid, which is extremely damaging to stone (Bock and Sand, 1993). Deterioration of the Cologne Cathedral in Germany is linked to the complex interaction of air pollutants with nitric acid–producing bacteria.

6.4 CONCLUSIONS

Scientists studying heritage materials face the same issues as those confronting environmental microbiologists in other areas. Environmental pollutants, particularly sulfur

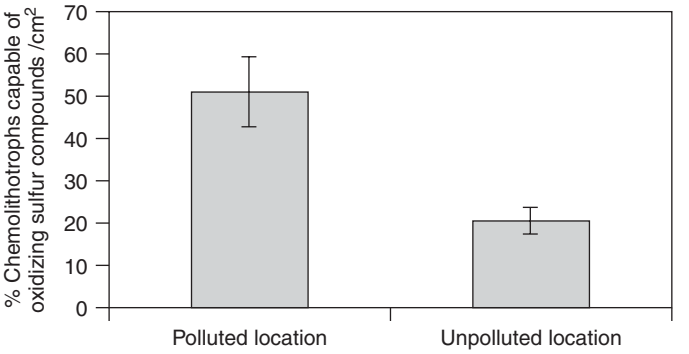

Figure 6.5 Enrichment of sulfur-oxidizing bacteria on limestone in polluted locations. (From Mitchell and Gu, 2000.)

dioxide and hydrocarbons in urban environments, act synergistically with microorganisms to accelerate deterioration of many materials. Problems accurately describing the diversity of microbial communities plague heritage studies just as they do other systems. Similarly, determining function (in this case, the ability to cause deterioration) of uncultured microorganisms is also a major area of study. However, there are significant challenges present in working with cultural heritage materials that do not arise in other fields of environmental science. These challenges arise from the unique, historic, and/or culturally significant nature of the objects being studied.

The biggest obstacle to working with heritage materials is sample collection. Because each painting, object, manuscript, monument, or building is regarded as unique and irreplaceable, it is frequently impossible to collect samples of the actual material. Often, sample collection is limited to nondestructive techniques such as swabbing or tape lifts. These sampling techniques bias subsequent analyses and decrease the odds of detecting those organisms responsible for deterioration.

A second obstacle to the study of biodeterioration of heritage materials is the small amount of microbial biomass that is available for study. Typically, in the marine environment, for example, low densities of microorganisms can be overcome by collecting large volumes of water. Collection of larger samples is not always possible, particularly with small objects and with structures or objects to which access is limited.

A third challenge arises from the prevailing view that all heritage objects are unique. Although this may be true from a historic standpoint or with respect to provenance, from a materials science or microbiological point of view, this is hardly the case. The situation is somewhat analogous to the issue of eutrophication in freshwater lakes. As most environmental scientists are probably aware, the role of phosphates in eutrophcation was contentious until the classic experiments by Schindler. The following statement by a representative from the chemical industry, although clearly an exaggeration, illustrates the argument (Edmondson, 1991):

> The problem of man-caused eutrophication is the most complex subject in our world. It truly encompasses the ‘mystery of life on earth.’ Thus we are attempting to understand and answer the questions: Why do plants grow? How can we retard or stop their growth?

Although lakes vary in many aspects, such as depth, area, surrounding land use, soil type, water chemistry, and trophic structure, the cause of eutrophication in most cases is phosphate. A detailed knowledge of algal physiology was not needed to understand the cause of eutrophication. Similarly, the types of materials used in the construction of any heritage object has a large influence on the microbial community. Environmental conditions and the history of the structure also exert some influence, but as studies of stone have demonstrated, there are underlying similarities in the microbial communities based on material composition. One of the major challenges facing the study of microbial deterioration is the need to integrate the large volume of information that we have collected on the taxonomic composition of communities of heritage objects. As we hope is clear from this chapter, we now have an excellent idea of the types of microorganisms found on many types of heritage materials. The challenge is to move past this identification stage, to determine which microorganisms cause deterioration and to prevent colonization, growth, and deterioration.

Despite these challenges, we have seen significant advances in studies of microbial deterioration. Many of these challenges have provided the impetus for new methods and techniques, or for adaptation of methods from other areas, including the application of techniques from molecular biology and genomics and the development of nondestructive techniques to analyze microbial growth. In addition, major research initiatives are exploring ways to use microorganisms to protect heritage materials (Webster and May, 2006). The study of microbial interactions with heritage materials is an exciting and rapidly developing area within the larger field of environmental science.

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CHAPTER 7

Sorption and Transformation of Toxic Metals by Microorganisms

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7.1 INTRODUCTION

Metals are essential physical components of the ecosystem, whose biologically available concentrations depend primarily on geological and biological processes (Ehrlich, 2002). To carry out their normal physiological and biochemical functions requires that organisms have various specific requirements met. Elevated levels of metals at specific sites can create a significant environmental and health problem when the release of metals through geological processes of decomposition and anthropogenic processes far exceeds that of natural processes of metal cycling. Metal contamination of both aqueous and terrestrial environments is of great concern, due to the toxicity and persistence of metals in the ecosystem and their threat to animal and human health. Unlike organic pollutants, metal contaminants cannot be degraded or eliminated completely, and they are normally remediated through immobilization or valence transformation to reduce their biological accessibility, through bioavailability intervention, or through phytoremediation. Bacteria play an important role in the biogeochemical cycle of metals in the environment, and their capabilities and mechanisms in transforming toxic metals are of significant interest in the environmental remediation of contaminated sites. Microorganisms colonize and shape the Earth in many different ways, and their ability to adsorb and transform metals can shed light on solving pollution problems and proposing solutions in the cleanup of contaminated sites.

7.2 PHYSICOCHEMICAL INTERACTIONS

Biosorption of metal ions takes place primarily on the outer surface of microorganisms and is the first step in the mechanism involved when interactions between metals and cell wall components take place. This process provides a means to immobilize

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toxic metals and prevents further entry into living cells to cause significant damage to the functional enzymes. Physical adsorption via electrostatic or van der Waals forces can retain metal ions on the outer surfaces of bacterial cells and thereafter make the adsorbed metal ions bind with chemical functional groups of biomolecules on the cell surface and then inside cellular structure through chemical interactions.

Both carboxyl and phosphoryl sites on a bacterial cell wall are the dominant functional groups for metal immobilization and contribute to the adsorption of various metal ions, such as Cd(II), Cu(II), Pb(II), Zn(II), and Al(III). Besides these two chemical functional groups, amine and hydroxyl groups also contribute to the uptake of metal cations. Fourier transform infrared spectroscopy (FTIR) is a powerful tool in the qualitative determination of the functional groups involved in metal biosorption, and the dissociation constants (pK_a) and concentrations of the functional groups on the cell wall can be determined quantitatively by potentiometric titration. Different pK_a values of functional groups present on the biomass have also been summarized: 1.3 for sulfonate, 1.7 to 4.7 for carboxyl, 6.1 to 6.8 for phosphoryl, 8 to 11 for amine, 8.3 to 10.8 for sulfhydryl, and 9.5 to 13 for hydroxyl (Volesky, 2003). Differences between these pK_a values of the same functional group may be due to the different conformation or the steric effect on cell surfaces.

In addition to physical adsorption, ion exchange and complexation are always believed to be the dominant mechanisms involved in metal biosorption. Target metal ions can exchange reversibly with the protons, alkali, or alkali earth ions present on the bacterial cell surface during the ion-exchange process. This mechanism explains many of the observations made on adsorption of metal ions by bacteria. Complexation is a much broader term; metal ions can form unidentate or multidentate with the functional groups on the cell surface, depending on the bacterial species and growth conditions. When most of the metal ions in the complex formed on the cell wall could be replaced by protons, alkali, or alkali earth ions, ion exchange is the predominant mechanism. Stable complexes formed between Cu(II), Cd(II), and Pb(II) with functional groups on the cell wall of *Calothrix* spp. and the thermodynamic stabilities of the complexes have been calculated further using the surface complex model by assuming that only the carboxyl group complexes with metal ions (Yee et al., 2004).

Although surface complexation models can account for competitive proton and metal adsorption on bacterial surfaces and explain changes in pH and the ratio of bacteria to metals on metal adsorption behavior, a lack of direct proof of the specific formation mechanism of complexes is still a major obstacle in understanding the physicochemical interactions between metal ions and bacteria. With the use of such x-ray absorption spectroscopy as x-ray absorption near-edge structure spectroscopy analysis (XANES) and extended x-ray absorption fine structure (EXAFS) in recent years, information about the binding atoms, coordination numbers, and the distance of binding atoms from the metal ions that occur in the surface complexation reactions can be identified further. Previous x-ray absorption analysis has confirmed that phosphoryl and carboxyl groups play a major role in metal complexation reactions (Kelly et al., 2002; Boyanov et al., 2003), which is consistent with the information derived from FTIR and potentiometric titration. Kelly et al. (2002) found that at the lowest pH (pH 1.67), UO_2^{2+} bound exclusively to phosphoryl functional groups on the cell surface, with an average U–P distance of 3.6 Å, indicating an inner-sphere complex with an oxygen atom shared between the UO_2^{2+} and the phosphoryl ligand, whereas at slightly higher pH (3.22 and 4.80), UO_2^{2+} bound increasingly to bacterial surface carboxyl functional groups,

with an average U–C distance of 2.9 Å, indicating an inner-sphere complex with two oxygen atoms shared between the UO_2^{2+} and the carboxyl ligand. Apart from the mechanisms mentioned above, the occurrence of redox reactions also contributes to the removal of metal ions. Under acidic conditions, Cr(VI) can be reduced to Cr(III) by a reductant such as reducing sugar on the cell wall, and most Cr adsorbed on the *Aspergillus niger* is in the form of Cr(III), as demonstrated by x-ray photoelectron spectroscopy (XPS) (Park et al., 2005). X-ray diffraction (XRD) analysis suggests that precious metals such as Pd(II), Ag(I), and Au(III) can be reduced to nanocrystals of zero-valence form on the bacterial surface.

Biosorption is strongly affected by environmental conditions, particularly pH, by changing the bacterial surface charges, because chemical functional groups on the cellular surface are susceptible to protonation or deprotonation. Using x-ray absorption fine structure (XAFS) spectroscopy, Boyanov et al. (2003) indicates that Cd(II) binds predominantly to the phosphoryl ligands of *Bacillus subtilis* at a pH value below 4.4, whereas at higher pH, adsorption to carboxyl groups becomes increasingly important. Considering the availability of the functional groups involved in metal adsorption and their dependence on pH range, the biosorption mechanism may also vary greatly. On the other hand, metal speciation can also have a significant impact on biosorption performance. Many metal ions can undergo hydrolysis and make the biosorption process more complicated. In addition to the hydrolysis reaction, the presence of accompanying anions in a solution can affect metal speciation. Concentrations of free ions such as Pb(II), Cu(II), Hg(II), Al(III), and Fe(III) remain very low, in the pH range 7.5 to 9.0 in fresh water, due to the complexation with inorganic ligands available (Sigg and Xue, 1994), and the adsorption behavior of these complexed ions may be different from that of the free ions. Ahuja et al. (1999) suggest that Zn^{2+} biosorption by *Oscillatoria anguistissima* is inhibited by the presence of anions and the inhibitory order is $SO_4^{2-} > Cl^- > NO_3^-$. By means of time-resolved laser-induced fluorescence spectroscopy, Texier et al. (2000) demonstrated the involvement of NO_3^-, Cl^-, and SO_4^{2-} in the chemical structure surrounding the Eu–ligand complex on the bacterial cell surface.

7.3 BIOCHEMICAL INTERACTIONS

Mechanisms involved in biochemical interactions between bacteria and metal ions always involve specific enzymes for oxidation, reduction, methylation, dealkylation, and precipitation. Microorganisms transform a number of metals and metalloids by reducing or oxidizing them directly to a lower or higher redox state. A range of multivalent metals, such as Fe(III), Mn(IV), Cr(VI), Hg(II), As(V), Co(III), Pd(II), Se(VI), U(VI), Np(V), Tc(VII), Au(III), Ag(I), Mo(VI), and V(V), have been reported to be reduced to a lower valence by metal-reducing bacteria. The oxidation of metals such as Fe(II), As(III), Se(0), Cr(III), and U(IV) from low to high valence can also take place, which may change their stability and bioavailability in the environment. In addition, indirect oxidation or reduction is an alternative for the immobilization of toxic metals in the environment. Sulfide, produced during the enzymatic reduction of sulfate by the ubiquitous sulfate-reducing bacteria, can form insoluble metal sulfide by a chemical reaction between H_2S and metal ions, including Ag, Cd, Hg, Ni, Pb, Se, and Zn ions (Bolton and Gorby, 1995). In anaerobic sediments, As(V) reduction to

As(III) can be facilitated by a sulfate reduction process before sulfide and As(III) are combined to form insoluble As_2S_3 (Rittle et al., 1995).

Methylation is important for geochemical cycling of metals and the removal of metal pollutants from contaminated sites. Methylation processes derive the methyl group from methylocabolamine (CH_3B_{12}), a compound from many microbial species and implicated in the methylation of a number of metals and metalloids, such as Pb, Sn, Pd, Pt, Au, Ti, As, Se, and Te (Ridley et al., 1977). The methylation and oxidation of arsenic compounds (III to V) reduces their toxicity and is hypothesized to be a protective mechanism in some organisms. On the contrary, demethylation is a process used to break the metal–methyl bond and generally results in a charged metal ion.

Recently, microbial transformation of multivalent toxic metals has attracted more attention, for the mechanisms in these processes are very complicated and some of them are still unclear. Table 7.1 summarizes possible reactions involved in the biotranformation processes. Recent developments in the biotransformation of such toxic metals as Cr(VI), As(V), Se(VI), and U(VI) are introduced briefly.

7.3.1 Biotransformation of Chromium

Cr(VI) forms mainly $HCrO_4^-$, $Cr_2O_7^{2-}$, CrO_4^{2-}, $HCr_2O_7^-$, and H_2CrO_4 in the environment and is more toxic than Cr(III) because of its carcinogenic and mutagenic effects. It is generally believed that Cr(III) is 1000 times less toxic than Cr(VI). Moreover, Cr(VI) is highly soluble and thus mobile and biologically available in ecosystems, while Cr(III) displays a high affinity for organics, resulting in the formation of complexes that precipitate as amorphous hydroxides. Therefore, the reduction of Cr(VI) to Cr(III) is a common detoxification mechanism involved by most organisms.

A number of aerobic bacteria are capable of reducing Cr(VI), and in the presence of oxygen, microbial reduction of Cr(VI) is commonly catalyzed by soluble enzymes in the cytosol, except in *Pseudomonas maltophilia* O-2 and *Bacillus megaterium* TKW3, which utilize membrane-associated reductases (Blake et al., 1993; Cheung et al., 2006). Park et al. (2000) purified 600-fold a soluble Cr(VI) reductase, ChrR, from *Pseudomonas putida* MK1, and the ChrR-coding gene, *chrR*, was identified from the genomic sequence of *P. putida* MK1, based on the known amino acid sequences of the N-terminal and internal amino acid segments of the pure enzyme. An open reading frame (ORF), *yieF*, on the *Escherichia coli* chromosome with no assigned function is found to have a high homology to *chrR*. This gene has been cloned and the encoded protein, YieF, shows maximum reduction of Cr(VI) at 35°C (Park et al., 2002).

In the presence of oxygen, bacterial Cr(IV) reduction commonly occurs as a two- or three-step process with Cr(VI) initially reduced to the short-lived intermediates Cr(V) and/or Cr(IV) before further reduction to the thermodynamically stable end product Cr(III) (Figure 7.1). Nevertheless, it is unclear whether the reduction of Cr(V) to Cr(IV) and then Cr(IV) to Cr(III) is spontaneous or enzyme mediated. NADH, NADPH, and electrons from the endogenous reserve are implicated as electron donors in the Cr(IV) reduction process (Appenroth et al., 2000). The Cr(VI) reductase ChrR transiently reduces Cr(VI) with a one-electron shuttle to form Cr(V), followed by a two-electron transfer to generate Cr(III). Although a proportion of the Cr(V) intermediate is spontaneously reoxidized to generate reactive oxygen species (ROSs), its reduction through two-electron transfer catalyzed by ChrR reduces the chance to produce harmful radicals (Ackerley et al., 2004). Enzyme YieF is unique in that it catalyzes the direct reduction of Cr(VI) to Cr(III) through a four-electron transfer in which three electrons are

Table 7.1 Microbial Transformation of Multivalence Metals

Metals	Possible Reactions	Microorganisms Involved	References
As	Reduction of As(V) to As(III)	*Escherichia coli, Shewanella* sp. strain ANA-3, etc.	Stolz et al. (2006)
	Oxidation of As(III) to As(V)	*Hydrogenophaga* sp. NT-14, *Rhizobium* sp. NT-26, etc.	Stolz et al. (2006)
	Methylation of As(V) or As(III) to methylated As compounds	*Desulfovibrio gigas, Methanobacterium formicicum*, etc.	Stolz et al. (2006)
Cr	Reduction of Cr(VI) to Cr(III)	*Pseudomonas maltophilia* O-2, *Shewanella putrefaciens* MR-1, etc.	Cheung and Gu (2003)
Fe	Reduction of Fe(III) to Fe(II)	*Geobacter merallireducens, Desulfuromonas acetoxidans, Shewanella putrefaciens*, etc.	Nealson and Saffarini (1994)
	Oxidation of Fe(II) to Fe(III)	*Leptothrix ochracea, Gallionella ferruginea*, etc.	Emerson (2000)
Hg	Methylation of Hg(II) to methylated Hg compounds	*Desulfovibrio desulfuricans* LS	Barkay et al. (2003)
	Reductive or oxidative demethylation of $CH_3Hg(I)$ to Hg(0) or unidentified Hg compounds	*Desulfovibrio gigas, Escherichia coli*, etc.	Barkay et al. (2003)
	Reduction of Hg(II) to Hg(0)	*Pseudomonas aeruginosa* PU21	Barkay et al. (2003)
	Oxidation of Hg(0) to Hg(II)	*Escherichia coli*	Barkay et al. (2003)
Mn	Reduction of Mn(IV) to Mn(II)	*Geobacter merallireducens, Desulfuromonas acetoxidans, Shewanella putrefaciens*, etc.	Nealson and Saffarini (1994)
	Oxidation of Mn(II) to Mn(IV), or oxidation of Mn(II) to Mn(III) and then Mn(IV)	*Leptothrix discophora* strain SS-1, *Bacillus* sp. SG-1, etc.	Tebo et al. (2005)
Se	Reduction of Se(VI) to Se(IV), Se(0), or even Se(-II)	*Thauera selenatis, Enterobacter cloacae* SLD1a-1, *Desulfomicrobium* sp., etc.	Schröder et al. (1997); Hockin and Gadd (2006)
	Reduction of Se(IV) to Se(0)	*Thauera selenatis*	Schröder et al. (1997)
	Oxidation of Se(0) to Se(VI) or Se(VI)	*Bacillus megaterium*	Sarathchandra and Watkinson (1981)
	Methylation of Se(VI) or Se(VI) to methylated Se(-II) compounds	*Enterobacter cloacae, Pseudomonas* strain Hsa.28, etc.	Ranjard et al. (2003)
U	Reduction of U(VI) to U(IV)	*Geobacter, Shewanella, Desulfovibrio*, etc.	Wall and Krumholz (2006)
	Oxidation of U(IV) to U(VI)	*Acidithiobacillus ferrooxidans, Thiobacillus denitrificans*	DiSpirito and Tuovinen (1982); Beller (2005)

consumed in reducing Cr(VI) and the other is transferred to oxygen. Since the quantity of ROSs generated by YieF in Cr(VI) reduction is minimal, it is regarded as a more effective reductase than ChrR for Cr(VI) reduction (Park et al., 2002). The membrane-associated Cr(VI) reductase recently isolated from *B. megaterium* TKW3 utilizes NADH as an electron donor, but the kinetics of Cr(VI) reduction is not characterized in more detail (Cheung et al., 2006).

Besides aerobes, a number of facultative anaerobes have also been able to reduce Cr(VI) (Cheung and Gu, 2007). Moreover, *Desulfotomaculum reducens* MI-1 is capable of utilizing Cr(VI) as a sole electron acceptor (Tebo and Obraztsova, 1998), and this capability has only been reported in another sulfate-reducing bacteria (SRB) consortium (Cheung and Gu, 2003).

Both soluble and membrane-associated enzymes are found to mediate the process of Cr(VI) reduction under anaerobic conditions. Unlike the Cr(VI) reductases isolated from aerobes, Cr(VI)-reducing activities of anaerobes are associated with their electron transfer systems ubiquitously catalyzing the electron shuttle along the respiratory chains. The cytochrome families (e.g., cytochromes *b* and *c*) are frequently shown to be involved in anaerobic Cr(VI) reduction.

In the past, the anaerobic reduction of Cr(VI) was considered a fortuitous process that provides no energy for microbial growth. More recently, however, an SRB isolate was found to grow from the energy generated during anaerobic Cr(VI) reduction (Tebo and Obraztsova, 1998). In the absence of oxygen, Cr(VI) can serve as a terminal electron acceptor in the respiratory chain for a large array of electron donors, including carbohydrates, proteins, fats, hydrogen, nicotinamide adenine dinucleotide [NAD(P)H], and endogenous electron reserves (Wang, 2000). A typical anaerobic Cr(VI) reduction is shown in Figure 7.1.

Although the microorganisms involved in direct oxidation of Cr(III) to Cr(VI) have not been found, indirect oxidation by Mn(II)-oxidizing bacteria such as *Ps. putida* strain GB-1 and *Bacillus* sp. strain SG-1 have been studied (Murray and Tebo, 2005, 2007). Murray and Tebo (2007) suggest that *Bacillus* sp. strain SG-1 can oxidize Mn(II) to Mn(III) or Mn(IV) oxides via a multicopper oxidase-like enzyme located on its spore coat, which in turn catalyzes Cr(III) oxidation, and the initial rates of Cr(III) oxidation by such biogenic oxides were approximately seven times faster than Cr(III) oxidation rates by equivalent amounts of synthetic δ-MnO_2. Moreover, the requirement of the Mn(II) in the process further excluded the possibility that Cr(III) can be oxidized directly by Mn(II)-oxidizing bacteria, although it was nonspecific (Murray and Tebo, 2007).

7.3.2 Biotransformation of Arsenic

Naturally, arsenic occurs primarily in four oxidation states: As(V), As(III), As(0), and As(-III). As(V) and As(III) are the most common inorganic species in nature. As(V) predominates in oxygen-rich aerobic environments, whereas As(III) is more stable in moderately reducing anaerobic environments such as groundwater. Elemental arsenic occurs rarely, and arsines have been identified from fungal cultures and strongly reducing environments. At neutral pH, As(V) exists as anionic species, $H_2AsO_4^-$ and $HAsO_4^{2-}$ ($pK_{a1} = 2.24$, $pK_{a2} = 6.94$, and $pK_{a3} = 12.19$), whereas As(III) is electronically neutral ($pK_{a1} = 9.29$). Therefore, As is more mobile than arsenate in soil and sediment environments. Moreover, As(III) is at least 100 times more toxic than As(V).

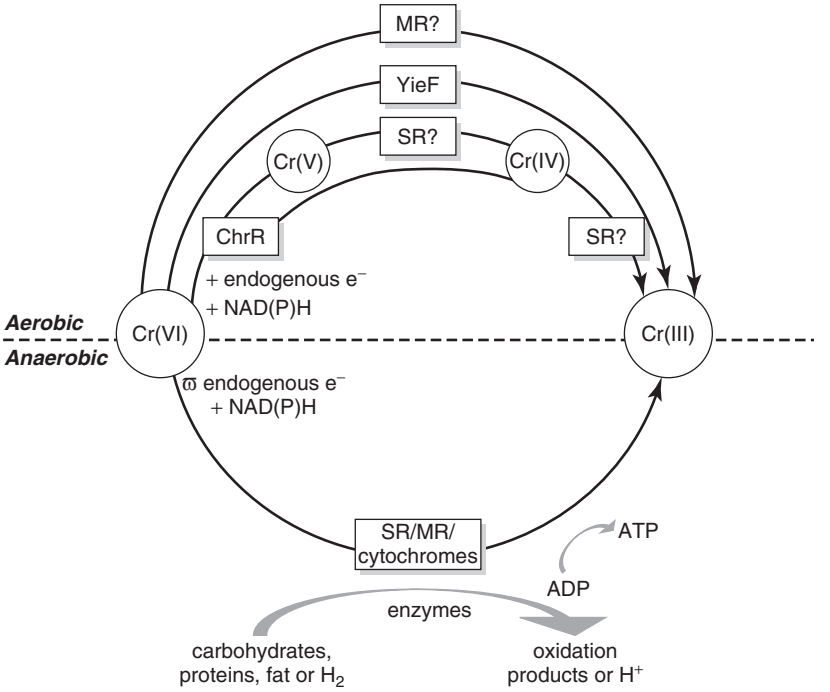

Figure 7.1 Plausible mechanisms of enzymatic Cr(VI) reduction under aerobic (upper) and anaerobic (lower) conditions. Under aerobic conditions, ChrR of *P. putida* MK1 catalyzes a combination of one- and two-electron transfers to Cr(VI) with the transient formation of Cr(V); YieF of *E. coli* mediates a four-electron shuttle for the direct reduction of Cr(VI) to Cr(III) with the remaining electron transferred to oxygen. An uncharacterized membrane-associated Cr(VI) reductase has been isolated from *B. megaterium* TKW3. Under anaerobic conditions, both soluble and membrane-associated Cr(VI)-reducing enzymes, including cytochromes, associated with the electron transfer system have been reported. Enzymes involved in the reduction of Cr(VI) are shown in boxes. SR and MR represent soluble and membrane-associated reductase, respectively.

As-resistant microbes (ARMs) are widely distributed in the environment, and aerobic reduction of As(V) to As(III) as a resistant mechanism has been studied extensively. Once entering into cytoplasm, As(V) can be reduced by an As(V) reductase, ArsC, which is a small-molecular-weight protein related to a class of tyrosine phosphatases that mediate the reduction of As(V) to As(III) in the cytoplasm, followed by As(III) removal from the cell with an efflux pump, ArsB, a multisubunit As(III) efflux pump, playing an important role in the extrusion of As(III) out of the cytoplasm. The *ars* operon of plasmid R773 from *E. coli* has been well studied and is composed of five genes, *arsR, arsD, arsA, arsB*, and *arsC*, whereas the chromosomal locus has only *arsB, arsC*, and *arsR* (Gladysheva et al., 1994; Martin et al., 2001). Both *arsR* and *arsD* encode two regulatory elements, and *arsA* encodes a membrane-associated ATPase subunit that interacts with the *arsB* gene product. In *E. coli*, a cysteine residue near the N-terminal of ArsC binds As(V), which is then reduced with electrons donated by the GSH and glutaredoxin (Shi et al., 1999). The As(III) is then expelled from the cytoplasm through an ATP-dependent arsenite transporter formed by ArsAB (Rosen, 2002). In the *Staphylococcus aureus* system, the reduced thioredoxin provides the

electrons to reduce As(V), and As(III) is expelled from the cells via an ATP-independent ArsB (Oremland and Stolz, 2003).

In addition to arsenate-resistant mechanisms, some microorganisms may also actively respire As(V) dissimilatorily under anaerobic conditions, and such microorganisms are always called *dissimilatory arsenate-reducing prokaryotes* (DARPs). They can be strict anaerobes, facultative anaerobes, or microaerophiles capable of utilizing arsenate as a terminal electron acceptor. Figure 7.2 shows the DARPs involved in the reduction of As(V) to As(III). Different from ARMs, which can only reduce aqueous As(V), DARPs are able to reduce both aqueous and solid-phase As(V). A new As(V) respiring species, *Shewanella* strain ANA-3, contained two systems for reducing As(V): One is similar to the well-conserved *ars* detoxification system from *E. coli* plasmid R773, but not required; the other is a separate system required for As(V) anaerobic respiration (Saltikov et al., 2003).

The respiration process is energetically favorable when coupled with the oxidation of organic matter. These microorganisms can use hydrogen or a variety of simple organics as electron donors, including acetate, formate, pyruvate, butyrate, citrate, succinate, fumarate, malate, and glucose, or even more complex aromatic molecules such as benzoate and toluene. Many species are also capable of using other terminal electron acceptors, such as nitrate, nitrite, Fe(III), fumarate, sulfate, thiosulfate, sulfur, dimethyl sulfoxide, and trimethyl amine oxide. Oremland et al. (2005) suggest a SLAS-1 under the conditions of a salt-saturated, alkaline brine, capable of using either an organic (lactate) or inorganic (sulfide) electron donor to sustain its growth while respiring As(V). However, Hoeft et al. (2004) found that an obligate chemolithoautotrophic arsenate respirer, a δ-proteobacterium strain, MLMS-1, could use only arsenate and sulfide as the respective electron acceptor and donor to sustain its growth.

A heterodimer of respiratory arsenate reductase (ArrA and ArrB) has been identified in DARPs, and Saltikov and Newman (2003) suggest that mutants with in-frame deletions of either *arrA* or *arrB* are incapable of growing on As(V). ArrA, with sequence motifs similar to the molybdenum-containing enzymes of the dimethyl sulfoxide reductase family, is predicted to bind a molybdenum cofactor, and ArrB is predicted to contain iron–sulfur clusters (Saltikov and Newman, 2003). The location of Arr from *Chrysiogenes arsenatis* and *Shewanella* sp. strain ANA-3 has been identified in the periplasm (Macy and Santini, 2002; Saltikov and Newman, 2003), whereas the enzyme purified from *Bacillus selenitireducens* is located on the membrane (Afkar et al., 2003). Although the detoxifying and respiratory As(V) reductases share a common reaction substrate and product, they are completely different in structure and function. In the case of the ArrAB respiratory reductase from *Shewanella* sp. strain ANA-3, a periplasmic protein complex containing a molybdopterin subunit and iron–sulfur clusters accepts electrons delivered by *c*-type cytochromes in the cytoplasmic membrane (Saltikov and Newman, 2003). The mechanism of respiratory reductases in the reduction of As(V) remains to be fully elucidated.

DARPs can also immobilize the reduced As(III) in the anaerobic respiration via the precipitation pathway. A gram-positive sulfate-reducing bacterium *Desulfitomaculum auripigmentum* has been described for reduction of As(V), resulting in the formation of orpiment (As_2S_3) (Newman et al., 1997). Lee et al. (2007) further suggest the production of a filamentous network of arsenic–sulfide (As–S) nanotubes (20 to 100 nm in diameter and 30μm in length) on the extracellular of a DARP, *Shewanella* sp. HN-41.

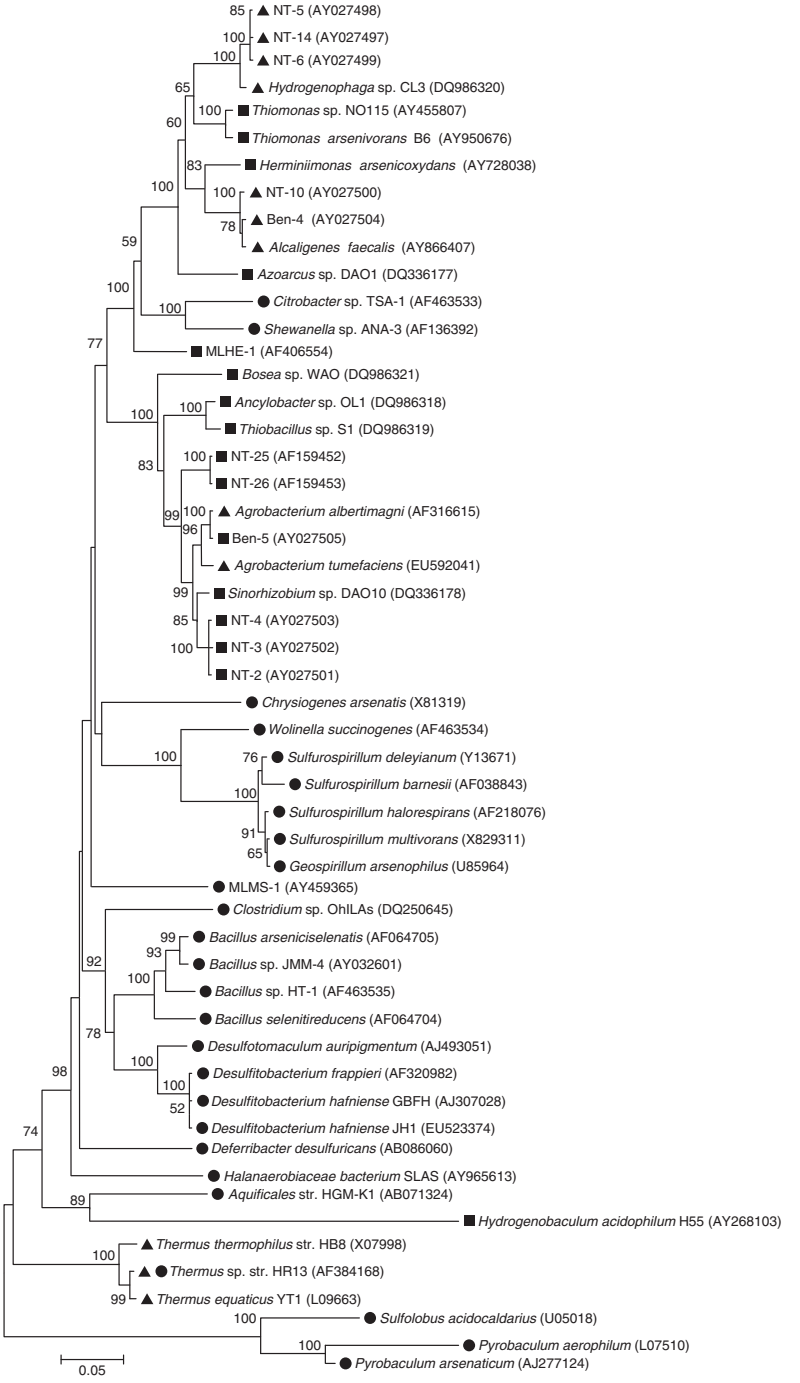

Figure 7.2 Phylogenetic diversity of known species that have been reported to reduce or oxidize arsenic compounds on the basis of 16S rRNA gene sequences. Dissimilatory arsenate-respiring prokarotes (DARPs) are indicated by the circles, chemoautotrophic arsenite oxiders (CAOs) are indicated by the squares, and heterotrophic arsenite oxidizers (HAOs) are indicated by the triangles. Arsenate-resistant microbes (ARMs) are not shown in the figure.

Since the first identification of an arsenite-oxidizing bacterium *Bacillus arsenoxydans*, isolated from an arsenical cattle dip in South Africa, microbiological oxidation of As(III) to As(V) in the environment has been investigated closely, and the commonly found arsenite oxidizers are shown in Figure 7.2. Interestingly, Gihring and Banfield (2001) detected a facultative bacterial isolate, *Thermus* HR13, capable of rapidly oxidizing inorganic As(III) to As(V) under aerobic conditions, whereas growth with As(V) respiration can be coupled with lactate oxidation in the anaerobic conditions. Two types of reactions are always involved in the process. Heterotrophic oxidation of As(III) by heterotrophic arsenite oxidizers (HAOs) is a reaction that transforms As(III) on the outer membrane of the cells into less toxic As(V). The coupling oxidation of As(III) and the reduction of oxygen or nitrate (electron acceptor) by chemolithoautotrophic arsenite oxidizers (CAOs) can use the derived energy to fix CO_2 or CO_3^{2-} into organic cellular material and sustain their growth.

The only information for the crystal structure of the arsenite oxidase (Aox) isolated from *Alcaligenes faecalis* strain NCIB 8687 has recently been elucidated (Anderson et al., 1992; Ellis et al., 2001). Aox is a heterodimer, a member of the DMSO reductase family in molybdenum enzymes. This enzyme is located on the outer surface of the inner membrane and exhibits arsenite oxidation activity in the presence of azurin or cytochrome *c* as an electron acceptor. The large subunit, which is associated with the catalytic site of the enzyme, contains a molybdenum binding site and a [3Fe–4S] cluster, whereas the small subunit contains a Rieske type [2Fe–2S] site. *aoxB* and *aoxA*, required for encoding arsenite oxidase in As(III) oxidation, are detected in a heterotrophic arsenite oxidizer ULPAs1, a member of the β-proteobacteria (Muller et al., 2003). The proteins encoded by *aoxA* and *aoxB* share 64% and 72% identity with the small Rieske subunit and the large subunit of the purified and crystallized arsenite oxidase of *Alcaligenes faecalis*, respectively. Another pair of As(III) oxidoreductase genes (*aroA* and *aroB*) is also detected in a chemolithoautotrophic As(III)-oxidizing bacterium strain NT-26 (Santini and vanden Hoven, 2004). *aroA* is 48% identical at the amino acid level to *aoxB*, and *aroB* is 52% identical to *aoxA*, and translated sequences of these genes share high identity with proteins from *A. faecalis*. However, the mechanism of arsenite oxidase in the oxidation of As(III) remains unclear.

Methylation is a complicated process involved in arsenic detoxification, and so far, little is known to the methylation reactions occurring in the bacterial system. Several intermediates, including monomethylarsonic acid [MMA(V), $CH_3AsO(OH)_2$], monomethylarsonous acid [MMA(III), $CH_3As(OH)_2$], dimethylarsinic acid [DMA(V), $(CH_3)_2AsO(OH)$], dimethylarsinous acid [DMA(III), $(CH_3)_2As(OH)$], trimethylarsine oxide [TMA(V), $(CH_3)_3AsO$], trimethylarsine [TMA(III), $(CH_3)_3As$], and other unidentified compounds, may be produced. Arsenic methylating bacteria are widely distributed in the environment, and Fe(III)-reducing, Mn(IV)-reducing, sulfate-reducing, and broad-spectrum anaerobic heterotrophic mixed cultures all produced methylarsenicals (Bright et al., 1994). The facultative marine *Serratia marinorubra* can also convert arsenate to arsenite and methylarsonate when growing aerobically (Vidal and Vidal, 1980). The Challenger mechanism is generally believed to be involved in the methylation process, and the initial reaction is the reduction of inorganic arsenate to arsenite via the loss of the oxygen double bond (Figure 7.3). Thereafter, the methylation–oxidation of arsenite to MMA(V) occurs, with re-formation of the As=O bond and the replacement of OH with CH_3. The sequence repeats with the reduction of MMA(V) to MMA(III), followed by the methylation–oxidation

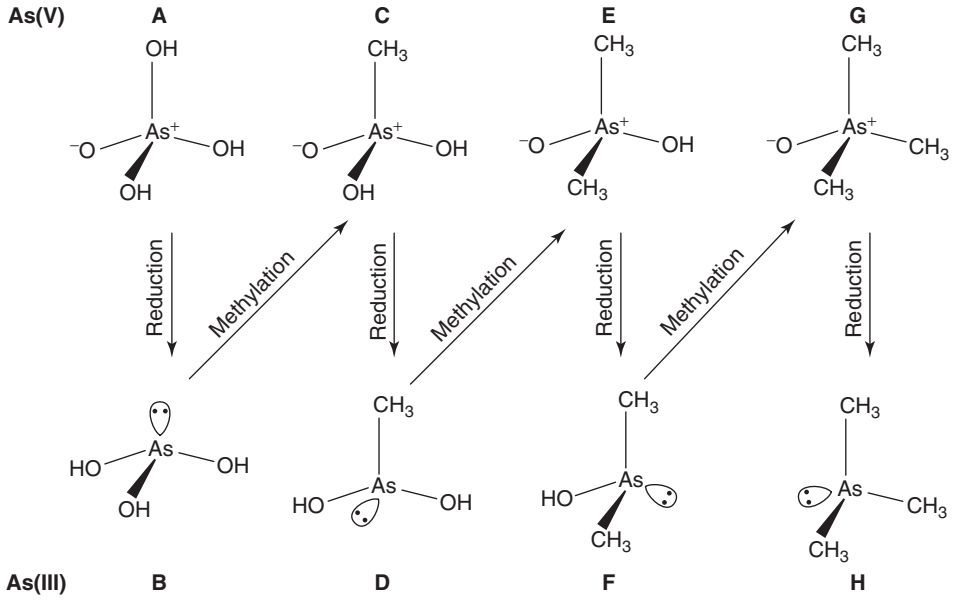

Figure 7.3 Challenger mechanism for the methylation of arsenic compounds: (A) arsenate; (B) arsenite; (C) monomethylarsonic acid, MMA(V); (D) monomethylarsonous acid, MMA(III); (E) dimethylarsinic acid, DMA(V); (F) dimethylarsinous acid, DMA(III); (G) trimethylarsine oxide, TMA(V); (H) trimethylarsine, TMA(III).

of MMA(III) to form DMA(V). The reaction continues to form DMA(III), then TMA(V), and finally, TMA(III). Different from using *S*-adenosylmethionine (SAM) as the methyl donor in fungi, anaerobic bacteria may use methylcobalamin to provide a methyl group (Kräutler, 1990). ArsM [As(III) *S*-adenosylmethyltransferase] plays an important role in the arsenic methylation process, and so far, 125 bacterial and 16 archaeal ArsM homologs have been identified, most likely AdoMet methyltransferases with unknown physiological functions. Qin et al. (2006) suggest that ArsM characterized in *Rhodopseudomonas palustris* is able to catalyze the formation of a number of methylated intermediates from As(III), with trimethylarsine as the end product.

7.3.3 Biotransformation of Selenium

Selenium is an essential trace element and is incorporated into an amino acid (selenocysteine); however, higher concentrations may be toxic to humans. In the natural environment, selenium exists in several oxidation states in both inorganic and organic forms. Among all of the inorganic forms, selenate [Se(VI)] and selenite [Se(IV)] are the most commonly found in oxic environment, whereas elemental selenium [Se(0)] and selenide [Se(-II)] are predominant in anoxic conditions.

The transformation among different Se species is complicated, and in a natural ecosystem it occurs primarily via biotic processes. Reduction from selenate or selenite to elemental selenium is widespread in microorganisms, and selenium reduction from selenate to selenide has also been described. Microorganisms capable of reducing selenium oxyanions are not restricted to particular groups or subgroups of prokaryotes and are widely distributed throughout the bacterial and archaeal domains. Most

bacteria prefer to use selenium oxyanions in their anaerobic metabolism as an electron acceptor, whereas bacteria such as *Enterobacter cloacae* SLD1a-1 (Watts et al., 2003), *Azospira oryzae* (Hunter, 2007), and *Rhodobacter sphaeroides* (Bebien et al., 2001) are able to reduce selenium oxyanions to elemental selenium under both anaerobic and aerobic/microaerophilic conditions. Preference in utilizing selenium chemical species is also different among different bacteria. Several bacteria [e.g., *E. coli* (Turner et al., 1998), *Wolinella succinogenes* (Tomei et al., 1992), *Pseudomonas stutzeri* (Lortie et al., 1992), and *B. subtilis* (Garbisu et al., 1995)], are able to reduce both selenate and selenite into elemental selenium, whereas species such as *Rhodobacter sphaeroides* (Bebien et al., 2001) and *Ralstonia metallidurans* CH34 (Roux et al., 2001) can only reduce selenite to elemental selenium. The distribution of the reduced Se(0) differs among different bacteria after the reduction phase. *R. sphaeroides* accumulates metallic selenium in the cytoplasmic compartment, whereas *Rhodospirillum rubrum* expels the elemental selenium across the plasma membrane and the cell wall (Bebien et al., 2001).

Reduction of selenate to selenite has been studied extensively in different bacterial systems. Although membrane-bound nitrate reductase (NAR) and periplasmic nitrate reductase (NAP) can catalyze the reduction of selenate in some cases, their contribution is not as significant as that of selenate reductases (Sabaty et al., 2001). The dissimilatory selenate reductase (Ser), a periplasmic enzyme in *Thauera selenatis*, was first purified by Schröder et al. (1997). The enzyme is a heterotrimer, consisting of three heterologous subunits, SerA (96 kDa), SerB (40 kDa), and SerC (23 kDa), with a native molecular weight of 180 kDa. It contains molybdenum, iron, acid-labile sulfide, and heme *b* as cofactor constituents. Ser has been demonstrated to be specific for selenate reduction to selenite, but cannot use nitrate, nitrite, chlorate, or sulfate as an electron acceptor. Different from that in *T. selenatis*, selenate reductase in *Sulfurospirillum barnesii* is localized in the membrane fraction, and the reductase is a heterotetramer with subunits of 82, 53, 34, and 21 kDa and contains molybdenum in the active site (Stolz and Oremland, 1999). In addition to selenate, *S. barnesii* showed an appreciable activity with nitrate, thiosulfate, and fumarate, indicating a broader substrate specificity than that of the enzyme from *T. selenatis* (Stolz and Oremland, 1999). The selenate reductase in *E. cloacae* SLD1a-1 is located in the cytoplasmic membrane and its active site faces the periplasmic compartment (Watts et al., 2003). The enzyme is a heterotrimeric complex with individual subunit sizes of 100, 55, and 36 kDa and an apparent molecular mass of 600 kDa with molybdenum, heme, and nonheme iron as prosthetic constituents (Ridley et al., 2006). The selenate reductase of *E. cloacae* also displays activity toward chlorate and bromate but not toward nitrate, dimethyl sulfoxide (DMSO), or trimethylamine N-oxide (TMAO) (Watts et al., 2003; Ridley et al., 2006).

Compared with selenate reduction, mechanisms for selenite reduction remain unclear. DeMoll-Decker and Macy (1993) suggested that the nitrate reductase might catalyze the reduction of selenite to elemental selenium. Schröder et al. (1997) also found that selenite was the predominant product in anaerobic respiration when *T. selenatis* was grown with selenate as the sole electron acceptor, whereas complete reduction of selenite to elemental selenium took place when both nitrate and selenate were available as electron acceptors. Although previous studies have suggested that selenite reduction could be mediated by the nitrite reductase (DeMoll-Decker and Macy, 1993), sulfite reductase (Harrison et al., 1984), or DMSO reductase (Afkar

et al., 2003), more work needs to be carried out to characterize the selenite reductases and their functions in the reduction process.

Reoxidation of elemental Se(0) to Se(VI) or Se(VI) by bacteria is another important step toward a better understanding of the biogeochemical cycle of selenium in the environment, however, very limited attention has been paid to this topic. A strain of *Bacillus megaterium* has been found to oxidize elemental selenium to selenite and a trace of selenate (Sarathchandra and Watkinson, 1981). Dowdle and Oremland (1998) suggest that the oxidation of ^{75}Se(0) to ^{75}Se-selenite and ^{75}Se-selenate in soils is a slow process at physiological pH and dark conditions. The reactions are carried out by at least two physiologically different types of microorganisms. The first class includes heterotrophic microorganisms such as *Leptothrix* strain MNB-1, *B. megaterium*, and possibly fungi; the second class includes bacteria of the aerobic sulfur cycle (e.g., *Thiobacilli*), which carry out the oxidation of Se(0) with enzymes that are used for generating energy from reducing sulfur compounds.

Inorganic selenium compounds can also be converted to volatile methylated species (i.e., dimethyl selenide, dimethyl diselenide, dimethyl selenone, and methyl methylselenite) by microorganisms, and dimethyl selenide and dimethyl diselenide are the most common forms of methylated selenium. Bacteria have been identified as the predominant Se-methylating organisms, and Se-methylating bacteria belong mainly to the proteobacteria and the cytophagales (Ranjard et al., 2003). The *tpm*-encoded bTPMT (bacterial thiopurine methyltransferase) characterized previously in *Pseudomonas syringae* (Ranjard et al., 2002) and *Pseudomonas* strain Hsa.28 (Ranjard et al., 2003) is involved in the methylation of selenite and (methyl) selenocysteine into dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe), but cannot methylate Se from selenate. Recently, a novel genetic determinant, named *mmtA* was found to encode a group of methyltransferases, and the expressed proteins MmtA and MmtQ in *E. coli* cells are found to produce DMSe from selenite and selenate (Ranjard et al., 2004).

7.3.4 Biotransformation of Uranium

Uranium occurs in oxidation states ranging from U(III) to U(VI), with the most stable species, U(VI) and U(IV), existing in the environment. U(VI) is predominant in the oxic surface waters, and UO_2^{2+} (uranyl) always forms stable, soluble complexes with ligands such as carbonate, phosphate, and humic substances. In natural waters, the solubility of U(VI) usually increases by several orders of magnitude at higher pH values, due to complexation with carbonate or bicarbonate. By contrast, U(IV) is commonly found in the anoxic conditions and is present primarily as an insoluble uraninite (UO_2). Therefore, reduction of the soluble uranyl to the insoluble uraninite seems to be an effective means to immobilize uranium in the anoxic environment to decrease the potential release of the mobile species.

More research interests in the bioreduction of U(VI) are demonstrated in the dissimilatory metal-reducing bacteria (DMRB) under anaerobic conditions. During the process, the soluble U(VI) species acts as an electron acceptor for microorganisms and is reduced to highly insoluble uraninite UO_2, with the consumption of hydrogen, acetate, lactate, ethanol, and so on, as the electron donors. Lovley et al. (1991) first demonstrated the occurrence of dissimilatory U(VI) reduction by the Fe(III)-reducing bacteria *Geobacter metallireducens* and *Alteromonas putrefaciens* (later, *Shewanella putrefaciens*), which could conserve energy for anaerobic growth via the reduction of

U(VI). Thereafter, other Fe(III)-reducing bacteria, sulfate-reducing bacteria, and so on, were also confirmed to reduce uranium subsequently. However, besides *Shewanella putrefaciens* and *Geobacter metallireducens*, only *Desulfovibrio vulgaris* UFZ B 490 (Pietzsch et al., 1999), *Desulfotomaculum reducens* (Tebo and Obraztsova, 1998), and *Thermoterrabacterium ferrireducens* (Kennedy et al., 2004) have been reported to gain sufficient energy from U(VI) respiration to support the growth.

Mechanisms involved in U(VI) reduction have been studied with bacteria belonging to the genera of *Geobacter, Shewanella*, and *Desulfovibrio*. *c*-type cytochromes are always believed to play an important role in U(VI) reduction, although the specific mechanism involved in microbial U(VI) reduction remains unclear. TEM results from previous studies indicate that uraninite deposit outside the cells or accumulate in the periplasm of DMRB, suggesting that the reductases responsible for reduction are mainly electron-carrier proteins or enzymes exposed to the outside of the cytoplasmic membrane, within the periplasm, and/or in the outer membrane (Wall and Krumholz, 2006). However, uraninite accumulating in the cytoplasm was also reported in a pseudomonad isolate, and McLean and Beveridge (2001) assumed that uranium might form strong complexes with the internal phosphate and other components within the cytoplasm. Clearly, further work should be focused on U(VI) reductases and the associated mechanism of the biochemical processes in the microbial cytoplasm.

Both periplasmic and extracellular UO_2 nanoparticles are detected in the cell suspensions of *Shewanella oneidensis* MR-1, and multiple pathways for electron delivery to U(VI) have been confirmed in *S. oneidensis* MR-1 (Marshall et al., 2006). A complex network of *c*-type cytochromes with some functional redundancy, including MtrC, other OMCs, as well as periplasmic cytochromes, may function as uranyl reductases and influence the localization of both periplasmic and extracellular UO_2 nanoparticles (Marshall et al., 2006). MtrC, an outer membrane (OM) decaheme *c*-type cytochrome in *S. oneidensis* MR-1, previously reported to be involved in Fe(III) and Mn(IV) reduction, can function as a terminal electron acceptor and transfer electrons directly to U(VI), whereas another OM decaheme cytochrome, OmcA, cannot enhance U(VI) the reduction rate in vitro without the aid of the unidentified protein(s), although it also contributes to U(VI) reduction (Marshall et al., 2006).

Although *Geobacter sulfurreducens* can reduce both Fe(III) and U(VI), the different effects resulting from the deletion of cytochromes in both Fe(III) and U(VI) reduction suggest that different electron-transfer pathways may be involved in the reduction of U(VI) and Fe(III) (Shelobolina et al., 2007). U(VI) reduction in *G. sulfurreducens* is mediated primarily by specific outer membrane cytochromes such as OmcE, OmcF, GSU3332, and GSU1334, and periplasmic amorphous uranium accumulation in *G. sulfurreducens* reflects the ability of uranium ions to penetrate the outer membrane rather than the active occurrence of enzymatic U(VI) reduction (Shelobolina et al., 2007). However, periplasmic cytochromes also contributes to U(VI) reduction, and the deletion of a periplasmic electron shuttle may affect both periplasmic and cell surface electron-transfer processes. MacA is believed to transfer electrons from the inner membrane to the periplasm, and the deletion of *macA* decreases the rate of U(VI) reduction by 98% (Shelobolina et al., 2007). Another periplasmic *c*-type cytochrome, PpcA, purified from *G. sulfurreducens*, a member of the c_7 cytochrome family, has also been proved to function as an intermediary electron carrier in the electron transport from electron donors to the outer membrane reductases in acetate-dependent U(VI) reduction (Lloyd et al., 2003).

By means of EXAFS spectroscopy analysis, Renshaw et al. (2005) have demonstrated that dissimilatory reduction of U(VI) is via a one-electron reduction in *G. sulfurreducens* with an intermediate UO_2^+ species formed from the process, and the unstable pentavalent UO_2^+ ion is subsequently transformed disproportionately to U(IV) and U(VI).

Although the biogenically produced uraninite (UO_2) is stable under anaerobic conditions, it can be oxidized abiotically to the more mobile U(VI) via chemical oxidation by O_2, Fe(III), and Mn(IV) when appropriate conditions are provided. Moreover, the occurrence of reoxidation of U(VI) is also possible in the presence of U(VI)-reducing bacteria. Sani et al. (2004) indicate that in the presence of crystalline Fe(III) (hydr)oxides, *Desulfovibrio desulfuricans* G20 reduces soluble U(VI) to an insoluble U(IV) oxide as long as a suitable electron donor is available, whereas depletion of the electron donor may result in partial reoxidation of the U(IV) to soluble U(VI) species by the crystalline Fe(III) (hydr)oxides. U(IV) can also be oxidized by bacteria. Abiotic oxidation of U(IV) with nitrite in heat-killed sediments was slow compared to U(IV) oxidation when nitrate was added to the biologically active sediments, suggesting the involvement of bacteria in U transformation (Finneran et al., 2002). Some of the U(IV) oxidation may be the result of direct oxidation of U(IV) by microorganisms that can couple the oxidation of U(IV) to the reduction of nitrate. Another likely mechanism for U(IV) oxidation is the abiotic oxidation of U(IV) by freshly precipitated Fe(III) oxides that are produced as a result of microbial oxidation of Fe(II) coupled to nitrate reduction (Finneran et al., 2002). More interestingly, Senko et al. (2007) observed that the relatively slow rates of U(VI) bioreduction would yield larger and less reactive U(IV) phases that would be more resistant to oxidation than relatively rapidly formed biogenic U(IV) phases. In addition to heterotrophic bacteria, autotrophic bacteria can also oxidize U(IV). An aerobic autotrophic bacterium, *Thiobacillus ferrooxidans* (later *Acidithiobacillus ferrooxidans*), was reported to oxidize U(IV) under acidic conditions (DiSpirito and Tuovinen, 1982). Recently, Beller et al. (2005) isolated an obligate chemolithoautotrophic bacterium, *Thiobacillus denitrificans*, capable of catalyzing the oxidation of both synthetic and biogenic U(IV) oxides under nitrate-dependent conditions.

In addition to microbial redox transformation in a natural environment, U(VI) can form precipitates with phosphate facilitated by the phosphatase enzymatic activities of microorganisms, which offers an alternative for the remediation of the U(VI) under oxic conditions (Beazley et al., 2007; Martinez et al., 2007). Two aerobic heterotrophic bacteria, *Bacillus* sp. Y9-2 and *Rahnella* sp. Y9602, are able to constitutively hydrolyze sufficient organophosphate to precipitate up to 95% of the total U(VI) available, and the precipitates are composed of calcium autunite, $Ca(UO_2)_2(PO_4)_2$, as confirmed by EXAFS (Beazley et al., 2007).

7.3.5 Electron Shuttles

Humic substances are a group of high-molecular-weight organic molecules from decaying residues and polymerization process. These complex macromolecules are very diverse structurally and are involved extensively in both oxidation and reduction reactions in the environment. Lovley et al. (1996) first proposed that the biochemically inert humic substances could shuttle electrons between humic-reducing microorganisms and Fe(III) oxide under anoxic conditions, thus significantly increasing the reducing rate

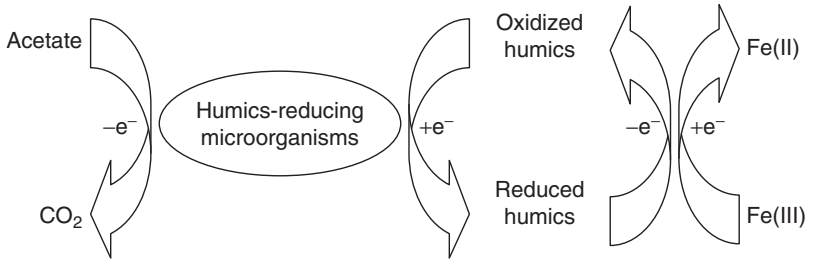

Figure 7.4 Proposed mechanism as to the role of humic substances (electron shuttle) in the reduction of Fe(III).

of the Fe(III) oxide. In their study, although *Geobacter metallireducens*, a dissimilatory Fe(III)-reducing bacterium, could reduce Fe(III) oxide and utilize acetate as an electron donor, the rate was very slow. When a small amount of anthraquinone 2,6-disulfonate (AQDS) was added to the experimental system, the Fe(III)-reducing rate increased remarkably. The mechanism can be shown schematically (Figure 7.4). As acetate is oxidized to CO_2 by *G. metallireducens*, AQDS serves as an electron acceptor and is reduced to AHDS or AH_2DS, which in turn abiotically reduces Fe(III) to Fe(II) to balance the electron production and utilization. By means of electron spin resonance (ESR) analysis, Scott et al. (1998) confirm that the quinones are the important electron-accepting groups in humic substances since they can form organic radicals such as semiquinones and hydroquinones after accepting electrons from anaerobic bacterial respiration, which are subsequently used for the reduction of Fe(III). Electron-shuttling mechanisms are widely distributed in the environment, and in addition to humic substances, other electron shuttlers have also been reported. Some *Shewanella* species could excrete flavins and utilize them as electron-shuttling molecules to reduce poorly soluble Fe(III) compounds in the anoxic environment (von Canstein et al., 2008). Natural products such as phenazines may also act as electron shuttles to promote microbial mineral reduction in the environment (Hernandez et al., 2004). These findings have important environmental significances for the biotransformation of multivalent toxic metals and can be dealt with by a different approach. However, in some cases, the presence of electron-shuttling molecules may negatively affect the bioremediation of toxic metal ions. Soluble U(VI) is more readily reduced to U(IV) by *G. metallireducens* and other Fe(III)-reducing microorganisms than are insoluble Fe(III) oxides, and once produced, U(IV) can be reoxidized to U(VI) with the reduction of Fe(III) to Fe(II) (Nevin and Lovley, 2000). In the presence of nitrate, Fe(II) also accelerates the oxidation of U(IV), presumably due to the oxidation of Fe(II) to Fe(III) by nitrate and the Fe(III) produced further oxidized U(IV) (Finneran et al., 2002). Sani et al. (2005) have demonstrated the reoxidation of U(IV) by Fe(III) hydroxide in the presence of *D. desulfuricans* G20, and they point out that the reoxidation of U(IV) is enhanced more readily by hematite than by goethite or ferrihydrite, and reoxidation of U(IV) by hematite can occur only when sulfate-reducing activity is active, suggesting that a sulfate-reducing process may make Fe(III) available from the mineral surface to react with microbially reduced U(IV).

7.4 INTERACTIONS BETWEEN NANOMETAL (OXIDES) AND BACTERIA

With the rapid development of nanotechnology and the extensive applications of nanometal (oxides) in nanotechnology-based industries, more concerns have been paid to their toxic effects to ecosystem health. Bacteria are the important mediators in the global biogeochemical cycling of elements and may also play an indispensable role in the transport and fate of nanoparticles in the environment; however, to date, the information available focuses mainly on the environmental risk assessment of nanomaterials and their toxicology to human cells. A comprehensive understanding of the interactions between nanometal (oxides) and bacteria is still in the very early stage.

Nanoparticles refer to materials with at least one dimension below 100 nm. Various nanometals (oxides), such as nano zero-valence ion, nano Fe_3O_4, nano TiO_2, nano ZnO_2, and nano CuO, have been produced commercially to date. In contrast to bulk metals, nanoparticles are much smaller and display more reactive atoms or molecules that should be in the interior of bulk materials on the surface, which may, therefore, generate adverse biological effects on living cells that will not be possible with the same material in bulk forms. Griffitt et al. (2007) suggest that nanocopper is acutely toxic to zebrafish, with a 48-h LC_{50} concentration of 1.5 mg/L. Other studies also found that iron oxide nanoparticles could cause cell death (Berry et al., 2004; Gupta and Gupta, 2005). Nel et al. (2006) suggest that nanoparticles can readily migrate throughout the body, deposit in target organs, penetrate cell membranes, lodge in mitochondria, and may trigger injurious responses. Thill et al. (2006) studied the impact of a water dispersion of CeO_2 nanoparticles (7 nm) on the gram-negative bacterium *E. coli*, and they found that positively charged CeO_2 nanoparticles at neutral pH displayed a strong electrostatic attraction toward bacterial outer membranes, and then Ce(IV) was reduced to Ce(III) at the surface of the bacteria. However, the specific enzymes involved in the process have not been identified.

Some problems should be addressed before a better understanding on the interactions between nanometals and bacteria can be made. The first problem is the existence and speciation of nanometals in an aqueous environment. Since a large number of activate sites are exposed in an aqueous environment, interactions between activate sites and water or other ions are prerequisites for a better understanding of the interactions between nanoparticles and bacteria. However, the solution chemistry of nanometals is quite limited, and thermodynamic data such as solubility and reaction constants of nanoparticles are virtually nonavailable. Additionally, the aggregation of nanoparticles always occurs due to the highly reactive characteristics of the surfaces associated with individual particles, and this may be why the true size in suspension differs so significantly from that of the physical nanosized particles in their dry form, which strongly influences the availability of nanoparticles to bacteria.

The second problem involves the physicochemical interactions between the nanoparticles and bacterial cell surfaces. In general, nanoparticles are much larger than cations and anions in solution, and they do not carry charges but expose lots of activate sites on the surface. Apart from the biosorption mechanisms mentioned above, more complicated reactions may take place. Furthermore, in some cases, coatings are always found on the nanoparticles, which in turn may alter the surface characteristics and reactivity of the nanoparticles, and this will make the physicochemical interactions even more complicated in the solution phase. Although coatings may make the nanoparticles less toxic to bacteria, coated particles may be able to access the cell surface more readily

than uncoated particles, due to the similar solubility of the surfactant with the cell membrane (Lubick, 2008). Auffan et al. (2006) suggest that the chemical nature of the coating on the nano γ-Fe_2O_3 determines different cell responses in terms of cytotoxicity. The morphological characteristics and sizes of the nanoparticles are another set of important factors that should be taken into consideration, and it is logical to postulate that the physiochemical and biochemical interactions of the spherical nanoparticles are different from those of the others with the bacteria. In addition, chemical and catalytic properties of nanomaterials should also be considered carefully in future research (Limbach et al., 2007).

The last but most interesting problem is the speciation of the nanoparticles in intracellular sites, the pathways of nanoparticle transport into bacterial cells, and the specific enzymes that assimilate or detoxify the metals or expel them from the cells. Although the generation of ROS under the lighted conditions in the cells of an organism is generally believed to be a toxic mechanism to the host (Nel et al., 2006), Adams et al. (2006) point out that the inhibitory effects of nano TiO_2, nano ZnO_2, and nano SiO_2 to *E. coli* and *B. subtilis* observed under dark conditions suggest that the new mechanisms may contribute additionally to toxicity. We believe that other mechanisms will be uncovered soon because new methodologies and novel analytical methods are being developed and made available. A comprehensive physicochemical and biochemical understanding of the interactions between nanometals and bacteria should soon be realized.

7.5 CONCLUSIONS

Both physicochemical and biochemical reactions are involved in interactions between metals and microorganisms. The physicochemical interaction is always referred to biosorption, which is the first step in the interaction process between the metals of concern and the microbial cells, including physical adsorption, ion exchange, and complexation. Functional groups such as carboxyl, phosphoryl, and amino on the surface of cellular components play a significant role in metal biosorption and immobilization. Although surface complexation models may partly explain some of the experimental observations in metal biosorption systems, a better comprehensive understanding of the physicochemical mechanism of interactions between metal ions and microorganisms should be based on direct proof from the metal speciation, existing format, and structure of the complexes formed on bacterial surfaces, which can be explored by x-ray analysis, such as XPS, XRD, EXAFS, and XANES. Biochemical interactions between microorganisms and toxic metals are more complicated processes, containing primarily oxidation and reduction, methylation and dealkylation, and precipitation. In the biotransformation of multivalent toxic metals such as As(V), Se(VI), Cr(VI), and U(VI), more interest has focused on identifying the enzymes involved in these processes. The biochemical diversity of the enzymes indicated in previous studies suggests the complication of the metal biotransformation processes. Additionally, differences in environmental factors such as substrates and humic acids may also lead to different enzymes and biochemical pathways involved in the metal biotransformation. With the rapid development of the nanotechnology, the transport and fate of nanometal has attracted more interest in recent years. Compared with aqueous metal ions, nanometal is less soluble, with larger particle sizes. However, due to the lack of thermodynamic data about nanoparticles in aqueousions, investigation of the interactions between bacteria and nanoparticles is still scarce. Although significant progress has been made in

the past 20 years in understanding the interactions between microorganisms and metal ions, further investigation into the physicochemical and biochemical mechanisms is still required to provide new insights into the fundamental biochemical mechanisms involved and potential application of metal bioremediation in cleaning up contaminated sites.

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CHAPTER 8

Bioremediation of Hazardous Organics

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8.1 INTRODUCTION

Many microorganisms possess the inherent ability to transform hazardous compounds. However, the long-term persistence of many of these contaminants in the environment is a testament to the fact that these naturally occurring processes often do not occur at rates that are fast enough to protect ecosystem and human health. Frequently, the microorganisms are limited by the availability of the pollutant or another key substrate or are not present in sufficient numbers. In many cases, bioremediation can overcome these limitations through careful engineering of the contaminated environment, thereby enhancing the rates of key microbial processes. Thus, successful bioremediation involves the integration of environmental microbiology and engineering techniques with other disciplines, such as geochemistry and hydrology.

The popularity of bioremediation is increasing because it often consumes less energy and fewer resources and thus is less expensive and more sustainable than physicochemical treatment approaches, such as land filling or incineration. Further, many alternative remediation techniques simply transfer organic contaminants to another medium without detoxifying the compounds. Nevertheless, the implementation of bioremediation is not without its challenges. Difficulties caused by the inaccessibility and heterogeneity of many contaminated environments are encountered in all remediation endeavors. However, additional complexities are faced in bioremediation because of the need to understand how (1) microorganisms alter contaminant structures, (2) microbial activities are affected by environmental conditions and interactions with other populations (microbial ecology), and (3) beneficial microbial processes can be enhanced through engineering approaches.

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In this chapter we address several common themes that have emerged from the growing body of bioremediation research and its application in practice. First we review the basic biological principles of bioremediation, including the relationships between contaminant biotransformation and microbial growth. Next, the most common strategies used by microorganisms to attack key structural features of organic contaminants are described, following the approach of Schwarzenbach et al. (2003), so that when unfamiliar contaminant structures are encountered, the reader can anticipate their potential for biotransformation under various conditions. These initial microbial attack mechanisms are then described in the context of the overall biodegradability of several major classes of contaminants. A key concept is that the biological transformations observed in the laboratory may not be realized in bioremediation endeavors under field conditions. Thus, in the remainder of the chapter we focus primarily on describing (1) factors that may limit biotransformation in the environment, including the genetic capability at a site; (2) various bioremediation strategies that have been developed to overcome these limitations; and (3) tools that aid in the design of these technologies, including reaction stoichiometry and microbial kinetics.

8.2 BIODEGRADATION MECHANISMS

Many environmental contaminants are subject to chemical or photochemical reactions. However, biological organisms—particularly microorganisms—play a more important role in the removal of many hazardous organics from the environment. Thermodynamically feasible contaminant transformations often do not occur in the absence of a biological catalyst, due to kinetic limitations, but are facilitated by microorganisms via enzymes, which lower the activation energy that must be overcome for a reaction to proceed, and the investment of biochemical energy to convert oxygen and other key coreactants to more reactive forms.

8.2.1 Extent of Biodegradation

Biodegradation is the general term used to describe the biological conversion of organic contaminants to products that are generally lower in free energy (ASCE, 2004). This term is often used loosely and interpreted in various ways. However, it does not imply anything about the extent of contaminant transformation or detoxification. Thus, biodegradation refers to *biotransformation* reactions that result in only minor changes in contaminant structures, as well as *mineralization*, which is the conversion of organic compounds into their inorganic constituents (e.g., H_2O, CO_2, NO_3^-, SO_4^{2-}, PO_4^{3-}, and Cl^-). In some cases, biotransformation reactions generate products that have similar or greater levels of toxicity than those of the parent contaminant. Examples include the conversion of nitroaromatic compounds to more reactive and toxic nitroso and hydroxylamino derivatives (Spain, 1995), and the anaerobic conversion of the suspected carcinogen trichloroethene (TCE) to the known carcinogen vinyl chloride (VC) (Freedman and Gossett, 1989). In contrast, the inorganic products of mineralization usually pose no health risks at the concentrations produced by contaminant biodegradation in the environment. Thus, care should be taken in interpreting simple observations of

parent compound removal in terms of hazard reduction, and biodegradation products should be identified to ensure that bioremediation goals are being met.

8.2.2 Relationship of Biodegradation to Energy Conservation and Growth

The extent of contaminant transformation and detoxification is often related to the ability of an organism to conserve energy and grow via a biodegradation reaction. Complete mineralization of a contaminant is frequently the result of a metabolic process and is linked to energy conservation and biomass synthesis (Alexander, 1981). In contrast, biotransformations that cause only minor changes in contaminant structure are often the result of co-metabolic processes that do not yield free energy or carbon that can be used by the cells carrying out the reactions. Although alternative definitions exist, the term *co-metabolism* is generally applied to both reactions that occur only in the presence of a growth substrate, as well as reactions that occur without concurrent growth of the organisms carrying out the reactions (Alexander, 1999). Compounds that support microbial growth are known as *primary substrates*. Co-metabolic substrates are called *secondary substrates* because they do not support growth. Some compounds can serve as primary substrates at relatively high concentrations but may act as secondary substrates when they are present at levels below S_{min}, the threshold concentration needed to supply the organism with sufficient energy for net growth (Rittmann and McCarty, 2001), as discussed below.

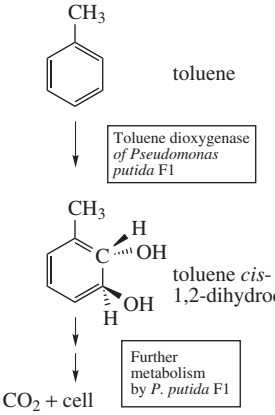
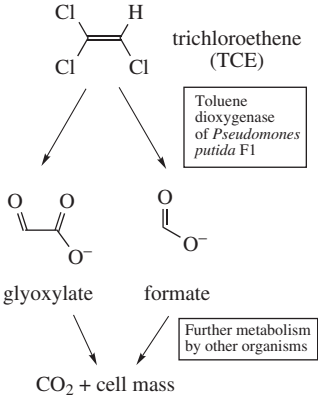
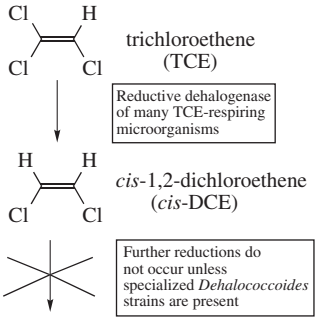
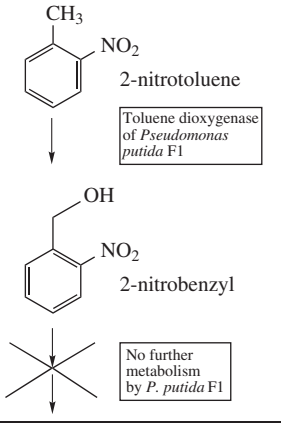
Co-metabolic reactions are frequently catalyzed fortuitously by oxygenases or other broad-specificity enzymes. The toluene dioxygenase from *Pseudomonas putida* F1 is an example of a broad-specificity enzyme and has been shown to act on over 100 substrates with varying structural characteristics (Ellis et al., 2006). The toluene dioxygenase initiates growth of *P. putida* F1 on toluene, a common groundwater contaminant, by introducing hydroxyl groups into the aromatic ring (Wackett et al., 1988), which leads to the formation of compounds that can be funneled into central metabolic pathways (Seagren and Becker, 2002). The toluene dioxygenase can also catalyze mono- and dioxygenase attack on the mononitrotoluenes in toluene-grown cells of *P. putida* F1; however, the products of these co-metabolic reactions are not degraded further, and the mononitrotoluenes do not serve as growth substrates for *P. putida* F1 (Robertson et al., 1992). In this example, toluene serves as a primary substrate for *P. putida* F1, and the mononitrotoluenes act as secondary substrates.

There are numerous exceptions to these general relationships between the ability of a contaminant to serve as a growth substrate and the extent to which it is transformed. For example, co-metabolic reactions sometimes generate products that are not subject to further enzymatic transformations and accumulate in pure cultures but may be acted upon by other species in the environment. The toluene dioxygenase from *P. putida* F1 co-metabolically converts another common groundwater contaminant, TCE, to glyoxylate and formate (Li and Wackett, 1992). TCE is not used as a growth substrate by *P. putida* F1; however, glyoxylate and formate may ultimately be mineralized by other populations within mixed microbial cultures. CO_2 is also a product of aerobic TCE co-metabolism by other species. Thus, co-metabolism sometimes results in partial or complete mineralization of contaminants, particularly in mixed cultures.

There are also examples of metabolic biotransformations that do not achieve mineralization or detoxification. Most notably, TCE and other chlorinated organic compounds are subject to reductive dechlorination reactions under anaerobic conditions. In several bacterial strains, reductive dechlorinations occur as a form of anaerobic respiration (known as *dehalorespiration*) in which the chlorinated organic compound serves as the terminal electron acceptor and the free energy that is released through the reaction is conserved. Most of the strains that grow via dehalorespiration of TCE cannot remove more than one chlorine from TCE, which results in the accumulation of a toxic end product, a dichloroethene (DCE) isomer (Holliger et al., 2003).

Table 8.1 organizes the biodegradation examples given above into four categories based on their ability to support growth and the extent of contaminant conversion.

Table 8.1 Classification of Example Biodegradation Reactions in a Bioremediation Matrix[a]

	Relationship of Biodegradation to Growth	
Extent of Biodegradation	Metabolism	Cometabolism
Mineralization		
Biotransformation		

[a]The potential for successful bioremediation is highest for metabolic reactions that lead to mineralization and lowest for co-metabolic substrates that undergo minor biotransformations.

These two factors have important practical implications. The potential to implement bioremediation successfully is greatest when biodegradation processes lead to mineralization and thus are likely to achieve contaminant detoxification. However, successful bioremediation can also sometimes be achieved by coupling biotransformation reactions that slightly modify contaminant structures with additional biodegradation processes that act on the biotransformation products. Anaerobic environments contaminated with highly chlorinated ethenes such as TCE or tetrachloroethene (PCE) are ideally suited for cleanup using coupled biodegradation processes. Dehalorespiring organisms that reductively dechlorinate PCE primarily to DCE are generally assumed to be present at contaminated sites (Fennell et al., 2001). Further biodegradation of DCE may occur if the necessary microorganisms and environmental conditions are present. If anaerobic conditions coincide with the presence of specific *Dehalococcoides* strains that are unique in their ability to grow via dehalorespiration of DCE and/or VC (Maymó-Gatell et al., 1997; Cupples et al., 2003; Sung et al., 2006), further reductive dechlorination of DCE to nontoxic ethene may occur. If DCE is exposed to aerobic conditions, oxidation to CO_2 could potentially occur. Wetlands may provide an environment in which mineralization of PCE to CO_2 could occur naturally through sequential anaerobic–aerobic biodegradation processes (Figure 8.1). Highly reduced conditions in the bulk soil are ideally suited for reductive dechlorination of PCE to DCE, and recently a wetland

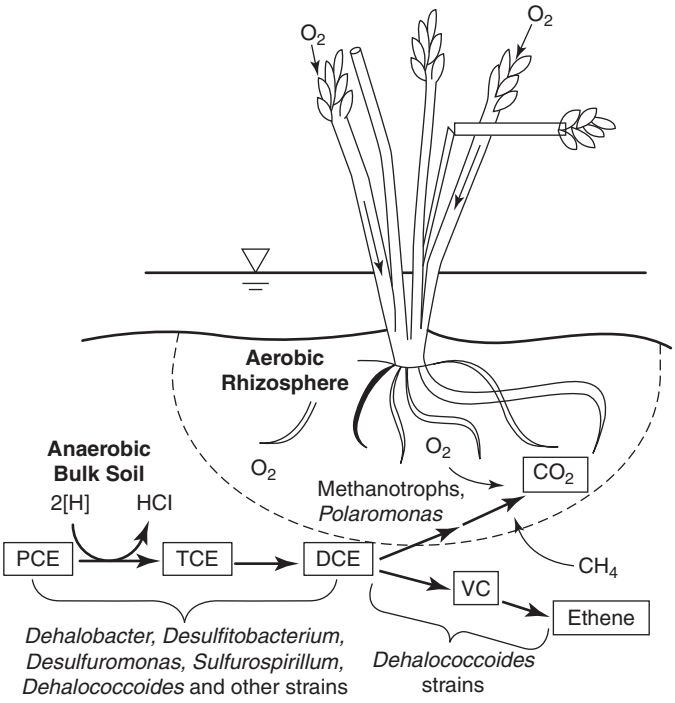


Figure 8.1 Potential pathways leading to complete detoxification of PCE in wetland environments, which typically contain anaerobic bulk soils and aerated zones within the rhizosphere. Certain strains within the genera listed can transform individual chlorinated ethenes metabolically via dehalorespiration or, in the case of *Polaromonas*, via oxidation. Some methanotrophs can oxidize lesser chlorinated ethenes co-metabolically.

microcosm was used to demonstrate that oxidation of DCE to CO_2 could be maintained in the rhizosphere, presumably by organisms that co-metabolized DCE using oxygen and plant exudates as growth substrates (Tawney et al., 2008). In natural wetlands, methane could also serve as a growth substrate for chlorinated ethene-degrading methanotrophs (Arp et al., 2001), which may associate with aquatic plant roots (King, 1994).

8.2.3 The Benefits of Contaminants that Serve as the Electron Donor or Acceptor

Bioremediation is facilitated by metabolic biodegradation processes. As discussed below, the size of the population mediating a biodegradation reaction influences the rate at which it occurs. Thus, metabolic biodegradation processes tend to be autocatalytic (i.e., biodegradation rates increase as the population grows on the contaminant). Contaminants that serve as growth substrates inherently select for populations that can carry out metabolic biodegradation reactions. In contrast, the growth substrates needed to maintain or grow populations carrying out co-metabolic biotransformation generally do not specifically enrich these populations and may also be utilized by microorganisms that do not contribute to contaminant removal. Both an electron donor and an electron acceptor must be provided to populations that mediate co-metabolic biotransformations because the contaminant does not fill either of these nutritional roles. In metabolic biodegradation reactions, the contaminant serves as either the electron donor or acceptor; therefore, generally only the complementary growth substrate must be available to ensure that biodegradation occurs.

Many organic compounds are highly reduced and therefore serve as metabolic electron donors in aerobic biodegradation reactions. Several constituents of refined petroleum compounds, including short-chain alkanes; benzene, toluene, ethylbenzene, and the xylene isomers (BTEX); and low-molecular-weight polycyclic aromatic hydrocarbons (PAHs), fall into this category. Therefore, an adequate supply of oxygen or another appropriate electron acceptor must be available for biodegradation of these contaminants to occur. Although some lightly chlorinated aliphatic and aromatic compounds can be utilized as electron donors by a few microorganisms, more frequently, chlorinated organic compounds contribute to growth by serving as the terminal electron acceptor in dehalorespiration processes (Fetzner, 1998; Holliger et al., 2003). H_2 or an organic electron donor is needed to sustain dehalorespiration. Anaerobic consortia can be sustained by providing lightly chlorinated aromatic compounds such as 2-chlorophenol (Becker et al., 2001, 2005) or 3-chlorobenzoate (Shelton and Tiedje, 1984; Becker et al., 2005) as the sole growth substrate. The populations that dechlorinate the aromatic compounds grow by carrying out dehalorespiration using electron donors produced by organisms that ferment the dechlorinated product. If the electron donor or acceptor needed to sustain a biodegradation process is not available in the environment or produced during biodegradation, an external source of the growth substrate must be provided. Various bioremediation strategies for providing electron acceptor and donors are described below.

8.2.4 Challenges Associated with Cometabolism

In many cases, cometabolism can be detrimental to cells because it diverts energy from growth and/or generates products that are highly reactive and, thus, toxic to cells.

For example, many aerobic biodegradation processes are initiated by oxygenases in reactions that consume reducing equivalents in the form of a nicotinamide adenine dinucleotide, NAD(P)H. This initial "investment" in NAD(P)H is subsequently recovered in the reactions that follow monooxygenation of a growth substrate. However, in cometabolic monooxygenation reactions, reducing equivalents are not replenished if the reaction product is not further metabolized (Arp et al., 2001). Oxygenases play a key role in the aerobic cometabolism of TCE, which generates short-lived products that can inactivate the enzymes and become incorporated into various cellular macromolecules. Inactivation of the enzymes catalyzing co-metabolic reactions and other cellular components can cause the rates of biodegradation and growth to decrease over time (Wackett and Householder, 1989). The cellular damage caused by co-metabolic reactions may also increase the amount of energy needed by cells for repair and other maintenance functions (Mars et al., 1996).

Co-metabolic transformations cannot be maintained without the substrate(s) needed to sustain the growth of the organism and, in many cases, to induce the enzyme. At the same time, the growth substrate will often compete with the cometabolic substrate for the enzyme. As a result of these competitive inhibition effects, toxic reaction products, and/or the energy demands of co-metabolic reactions, the maximal rate of a co-metabolic reaction may be 10 to 100 times lower than that of the growth-supporting substrate (Arp et al., 2001). Thus, numerous challenges must be overcome to manage microbial growth and contaminant biodegradation and implement bioremediation successfully based on co-metabolic reactions.

8.2.5 Common Strategies for Initiating Biodegradation

Wackett and Hershberger (2001) estimate that over 10 million different chemicals—representing unique combinations of the approximately 100 known organic functional groups and various carbon skeletons—have been described. Given the vast variety of organic chemical structures that have been released to the environment and the broad diversity of microorganisms that can act on these compounds, it is not surprising that biodegradation processes encompass a wide assortment of chemical transformations. However, the biodegradation of contaminants is frequently initiated by one of three general strategies: (1) oxidation by an electrophilic form of oxygen, (2) reduction by a nucleophilic form of hydrogen or a reduced metal, or (3) hydrolysis (Schwarzenbach et al., 2003). Reactions involving electrophilic oxygen or nucleophilic hydrogen do not occur abiotically because biochemical energy is needed to form these reactive species. The investment in biochemical energy usually pays off because these highly reactive species generally modify organic compounds in ways that make the parent contaminants less toxic, more polar (and thus easier to expel to the aquatic environment), and/or compatible with central metabolic pathways. However, as mentioned above, there are also examples of initial biotransformation reactions that form products that cannot be biodegraded further and/or are more toxic to the cells than the parent contaminant.

The distribution of electrons involved in the covalent bonds of an organic compound plays a key role in determining which of the initial biodegradation approaches is feasible and the site(s) at which the chemical reaction(s) can occur. Oxidations involving electrophilic oxygen are directed to the regions of a chemical structure with the most readily available electrons, which often is the π electrons in an aromatic ring or unsaturated bond or the nonbonded electrons of sulfur or nitrogen (Schwarzenbach et al.,

2003). Carbon–hydrogen bonds can also serve as the site of reaction with electrophilic oxygen in organic compounds that lack structural elements with greater electron density. Important groups of environmental contaminants that possess these electron-rich structural features include mono- and polyaromatic hydrocarbons, aliphatic hydrocarbons, TCE and other chlorinated ethenes, aniline and other aromatic amines, and certain pesticides and other compounds that contain thioether linkages.

The common form of oxygen in the atmosphere (known as ground-state or triplet oxygen) does not react directly with many molecules at ambient temperatures. This explains why spontaneous oxidation does not occur when organic contaminants and other organic materials are exposed to air. Oxygen is converted by oxygenase enzymes to a more energetic state (known as singlet oxygen) that can react with organic compounds. There are two major types of oxygenases. Monooxygenases frequently insert a single oxygen atom into an aliphatic or aromatic substrate, and the other oxygen atom is reduced by NAD(P)H to H_2O. Dioxygenases frequently insert two oxygen atoms into aromatic substrates. These reactions prepare or activate growth substrate compounds for ring cleavage reactions mediated by another group of dioxygenases, so that the aromatic ring carbons and electrons can be utilized by the organism. Hydroxylation of co-metabolic substrates may also be beneficial by making the compound more polar and thus more soluble in the surrounding aqueous environment. Like monooxygenase-catalyzed reactions, hydroxylation reactions catalyzed by dioxygenases also require NAD(P)H as a co-reactant. Some monooxygenase and dioxygenase enzymes, such as naphthalene dioxygenase and toluene dioxygenase, are very versatile and can carry out a variety of transformations in addition to the hydroxylations described above, as summarized by the University of Minnesota Biodegradation and Biocatalysis Database (http://umbbd.msi.umn.edu/index.html).

However, the presence of electron-rich regions within a contaminant structure does not guarantee biodegradation via an initial oxidation involving electrophilic oxygen. Bulky substituents can sterically hinder reaction with an oxygenase. Increasing substitution with carboxyl groups, nitro groups, halogens, or other electron-withdrawing substituents can also decrease the reactivity of a compound with electrophilic oxygen. Many contaminants with electron-rich features are also susceptible to other initial biodegradation strategies. For example, the amide and ester bonds in carbamates are susceptible to hydrolytic attack, as discussed below. Thus, biodegradation of the carbamate aldicarb can be initiated by an attack involving electrophilic oxygen at the thioether linkage (producing compounds with toxicity similar to that of the parent compound) or hydrolysis at the amide and ester bonds (producing compounds with reduced toxicity) (Goldman et al., 1990). Most chlorinated aliphatic compounds can be transformed by at least two of the three common initial biodegradation strategies. For example, the initial biodegradative attack on TCE may involve a cometabolic oxidation under aerobic conditions or reductive dechlorination under anaerobic conditions. Thus, environmental conditions and the physiological characteristics of the microorganisms in a given environment play an important role in determining which strategy is used to initiate biodegradation of a contaminant.

Molecular sites that are electron-poor (or oxidized) may be prone to an initial reduction reaction. These sites arise due to covalent bonding between atoms with different electronegativities. The electronegativities of some key atoms in hazardous organic

compounds relative to fluorine, which is the most electronegative atom in the periodic table and is assigned an electronegativity of 4.0, are: H (2.2) < C ≈ S (2.5) < Br (2.8) < N ≈ Cl (3.0) < O (3.5) < F (4.0).

The carbons bonded to halogens exhibit a positive partial charge and are susceptible to reductive dehalogenation. Similarly, the C atoms in carbonyl groups (R–C=O), the N atoms in nitro groups ($R–NO_2$), and the S atoms in sulfoxide (R–S(=O)–R′) and sulfone (R–S(=O)(=O)–R′) groups are oxidized and frequently serve as sites of reduction reactions. Other structural features that are often attacked through reductions include disulfide (R–S–S–R′) and azo (R–N=N–R′) groups.

Biological reduction of carbonyl groups and other functional groups that contain an atom with a partial positive charge due to a double bond with oxygen may involve a nucleophilic form of hydrogen such as that bound by NAD(P)H (Schwarzenbach et al., 2003). For example, several nitroaromatic compounds, such as nitrobenzene and trinitrotoluene (TNT), are susceptible to attack by NADPH-dependent nitroreductases, which catalyze the sequential reduction of nitro groups ($R–NO_2$) to the corresponding amines ($R–NH_2$) via nitroso (R–NO) and hydroxylamino (R–NHOH) intermediates (Fiorella and Spain, 1997). However, halogens and other bulky chemical moieties may sterically hinder attack by NAD(P)H. Thus, reduction of polyhalogenated compounds involves other reducing agents. Co-metabolic reduction of aliphatic compounds involves enzyme-bound forms of metallo-cofactors such as iron(II) porphyrins, corrinoids, or coenzyme F_{430} (Holliger et al., 2003). Metallo-cofactors are also key to the catalytic activity of reductive dehalogenases that transfer electrons to halogenated organics—the terminal step in the respiratory chain of organisms that grow via dehalorespiration.

The two most common types of reductive dechlorination reactions are shown for 1,1,2,2-tetrachloroethane (TeCA) in Figure 8.2. *Hydrogenolysis reactions* replace a halogen with a hydrogen and may involve saturated and unsaturated halogenated aliphatic compounds as well as halogenated aromatics. *Dihaloelimination reactions* are feasible only when at least one halogen is bonded to each of two carbon atoms linked by a single carbon–carbon aliphatic bond. One halogen is removed from each

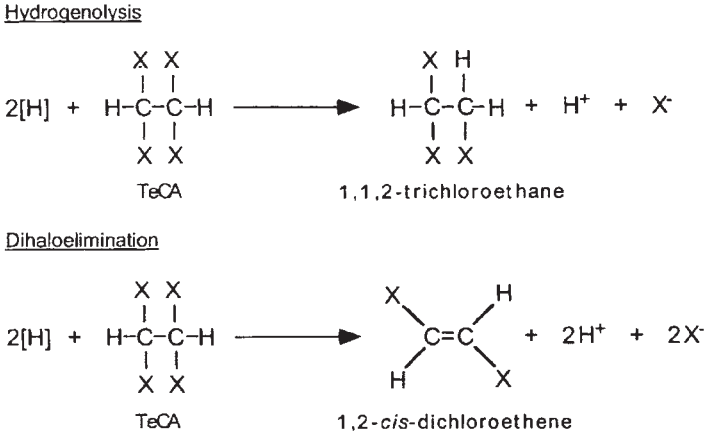


Figure 8.2 Two common types of reductive dehalogenation reactions (shown for 1,1,2,2-tetrachloroethane, TeCA). X = halogen.

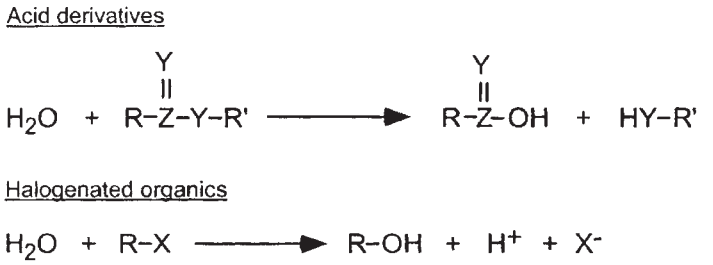


Figure 8.3 Model reactions for hydrolysis of acid derivatives and halogenated organics, where R and R′ = C or H; Y = O, NR, or S; Z = C, P, or S; and X = halogen.

carbon and simultaneously, the single bond linking the two carbons is replaced with a double bond.

Polar covalent bonds involving atoms with a partial positive charge are also often susceptible to initial hydrolytic attack in which water acts as the ultimate nucleophile and replaces a halogen or other functional group (termed a leaving group) with a hydroxyl group, as shown in Figure 8.3. Hydrolysis reactions are not redox transformations and do not require oxygen as a coreactant. Therefore, these transformations can occur under either aerobic or anaerobic conditions as long as organisms containing the necessary hydrolases are present. Hydrolases are commonly used by microorganisms to cleave the ester (R–C(=O)–OR′) and amide (R–C(=O)–N–R′R″) bonds in lipids and proteins. Many of these hydrolases have broad substrate ranges and are constitutively expressed. This suggests that hydrolysis of carbamates and other pesticides containing ester and/or amide linkages often occurs co-metabolically. There are also examples of hydrolysis reactions that are linked to growth. Hydrolysis of dichloromethane and other dihalomethanes by several pure and co-cultures yields formaldehyde, which can serve as the sole source of carbon and energy for these cultures (Leisinger et al., 1994). Hydrolytic dehalogenation of atrazine is carried out by a number of strains that use the pesticide as a sole source of nitrogen and convert its ring carbons to CO_2 (Wackett et al., 2002). Abiotic hydrolysis reactions can also transform atrazine and other contaminants in the environment. However, microbially mediated hydrolysis reactions occur at much faster rates than do the analogous abiotic reactions (Schwarzenbach et al., 2003).

8.3 BIODEGRADABILITY OF MAJOR GROUPS OF CONTAMINANTS

8.3.1 Hydrocarbons and MTBE

Hydrocarbons—compounds consisting of carbon and hydrogen alone—are common groundwater contaminants and often amenable to bioremediation. Releases of gasoline and other refined petroleum products represent a major source of hydrocarbon contamination. The BTEX compounds are of particular concern and regulatory importance because they constitute a significant fraction of gasoline and are relatively soluble and highly toxic (Seagren and Becker, 2002). Benzene is a known human carcinogen. PAHs containing from two to five fused aromatic rings are also of significant concern because of the mutagenicity and carcinogenicity of several of these compounds and

their tendency to bioaccumulate. PAHs are generated from the incomplete combustion of organic matter. Extensive PAH contamination is associated with coal gasification sites (manufactured-gas plants), as well as the production and use of the coal tar creosote, a wood preservative. Methyl *tert*-butyl ether (MTBE) is a fuel additive that was used originally as a replacement for tetraethyllead to increase the octane rating of gasoline and prevent engine knocking when leaded gasoline was phased out in the 1970s and 1980s (ASCE, 2004). The 1990 Clean Air Act Amendments led to the addition of increased amounts of MTBE to reformulated gasoline; however, MTBE is a problematic groundwater pollutant and its use as a fuel additive is being phased out, due to concerns about its potential carcinogenicity (Belpoggi et al., 1995), taste and odor problems at very low MTBE concentrations, and its tendency to migrate through the subsurface more rapidly than do hydrocarbon co-contaminants such as the BTEX compounds (Seagren and Becker, 2002).

BTEX Bacteria that can aerobically biodegrade BTEX compounds are indigenous at nearly all contaminated sites (Seagren and Becker, 2002). Aerobic biodegradation of a BTEX compound is initiated by one of many different monooxygenases and/or dioxygenases that can react with these compounds. Dioxygenase-mediated attack of the

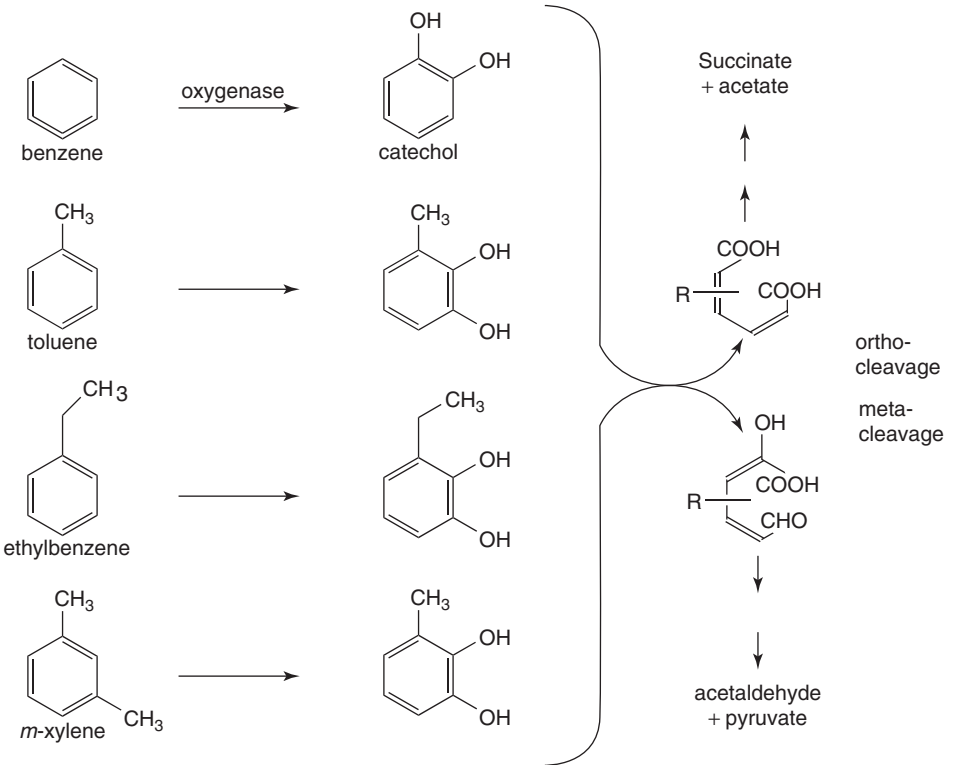


Figure 8.4 Biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) via initial oxygenase-catalyzed reactions that lead to the formation of catechols, which undergo ring cleavage and are ultimately converted to central metabolic pathway intermediates. Note that individual arrows frequently encompass multiple reactions.

aromatic nucleus of benzene yields catechol (1,2-dihydroxybenzene; Figure 8.4). The aliphatic side chains and aromatic rings of toluene, ethylbenzene, or the xylene isomers also serve as sites of initial attack by oxygenases, which generally lead to formation of substituted catechols. The presence of hydroxyl groups on adjacent carbons prepares the aromatic ring for further reaction with ring cleavage dioxygenases through the insertion of two additional oxygen atoms. Ring cleavage serves two important functions: (1) regeneration of the NAD(P)H that was invested in activating the ring for cleavage, and (2) generation of metabolic intermediates that are used in synthesis and energy generation. The aromatic ring may be opened between the hydroxyl groups, via the *ortho* cleavage (β-ketoadipate) pathway, or adjacent to the hydroxyl groups, via the *meta* cleavage (TOL) pathway. The products of both ring fission reactions are further degraded to form key intermediates in central metabolic pathways.

Field and laboratory studies have also demonstrated that biodegradation of BTEX compounds can occur under different anaerobic terminal electron-accepting processes (TEAPs), including Fe(III), nitrate, and sulfate reduction and methanogenesis (Seagren and Becker, 2002). Toluene is often the most readily degraded BTEX compound under anaerobic conditions (Gibson and Harwood, 2002; Seagren and Becker, 2002; ASCE, 2004). The anaerobic biodegradation of the other BTEX compounds has been observed at some contaminated sites, but not others. In particular, benzene is frequently recalcitrant or biodegraded only after lengthy lag periods. Thus, while growth of bacterial strains on benzene under denitrifying conditions has been observed (e.g., Coates et al., 2001), anaerobic benzene degradation appears to be a highly site-specific process.

Anaerobic bacteria use several different strategies to initiate the biodegradation of BTEX compounds; however, they all appear to direct the contaminants to formation of benzoyl-CoA as a central biodegradation intermediate, analogous to the formation of catecholic compounds during aerobic BTEX biodegradation (Gibson and Harwood, 2002). The CoA (coenzyme A) substituent is analogous to the dihydroxy groups in catecholic compounds in that it prepares the aromatic nucleus for subsequent (reduction) reactions that lead to destabilization and cleavage of the ring under anaerobic conditions. The initial attack on toluene involves the formation of a new carbon–carbon bond through the reaction of the methyl group with fumarate (Heider, 2007). The fumarate addition reaction initiates toluene biodegradation under a broad range of anaerobic TEAPs, and analogous reactions have been observed for other methylated aromatics (including *m*-xylene) and methylene groups in aliphatic compounds. Anaerobic ethylbenzene biodegradation is initiated by a dehydrogenation reaction, an oxidation that results in the hydroxylation of the aliphatic substituent but, unlike reactions mediated by oxygenases, uses water rather than molecular oxygen as the coreactant.

PAHs Under aerobic conditions, biodegradation of PAHs with two or three aromatic rings such as naphthalene, anthracene, and phenanthrene often occurs readily via reactions that are analogous to the biodegradation of the monoaromatic BTEX compounds. Monooxygenase- or dioxygenase-catalyzed reactions lead to the formation of catechols or *o*-phthalate (1,2-benzenedicarboxylate) intermediates that can be attacked by dioxygenases leading to eventual ring cleavage (Cerniglia, 1992; Kim et al., 2007). Similar reactions may also contribute to the biodegradation of four- and five-ring PAHs (Moody et al., 2004, Kim et al., 2007); however, the solubilities of these higher-molecular-weight PAHs are extremely low and limit the biodegradation

of these compounds. Bacteria use different strategies for increasing the limited bioavailability of PAHs, including the formation of biofilms on PAH crystals and production of biosurfactants that enhance their dissolution (Johnson et al., 2005). Evaluation of bioavailability and treatment strategies are discussed below. Utilization of naphthalene as the sole carbon and energy source has been demonstrated under denitrifying and sulfate-reducing conditions, and biodegradation of several other PAHs under these TEAPs has been observed. Under sulfate-reducing conditions, naphthalene is converted to 2-methylnaphthalene, which is further transformed through the addition of fumarate (as observed for toluene and *m*-xylene) (Heider, 2007).

MTBE The MTBE molecule contains both a stable ether bond and bulky methyl branching, structural features that are often resistant to biodegradation. Under aerobic conditions, cometabolism of MTBE can be mediated by organisms growing on short (C3–C5) normal and branched alkanes or other organic compounds that may be present as co-contaminants due to their presence in gasoline (Seagren and Becker, 2002). Aerobic growth on MTBE has also been observed. Metabolic and co-metabolic biodegradation of MTBE under aerobic conditions is initiated by a monooxygenase-mediated attack on the methoxy group and leads to the formation of *tert*-butyl alcohol (TBA) plus either formaldehyde or formic acid, depending on the degradation pathway. In some cases, TBA persists; however, TBA can putatively be funneled into central metabolic pathways via several routes (Müller et al., 2007) and can be used by some aerobic microorganisms as the sole source of carbon and energy. Nevertheless, MTBE and TBA frequently persist at contaminated sites (Seagren and Becker, 2002). This suggests that microorganisms that can biodegrade MTBE and/or TBA are not abundant in the environment. Another possibility is that the concentration of MTBE present at contaminated sites is below the minimum substrate level or threshold needed to sustain growth (Müller et al., 2007). There is evidence suggesting that substantial amounts of MTBE can be biodegraded anaerobically at a number of gasoline-contaminated sites, often after a lengthy lag period (Seagren and Becker, 2002; Kuder et al., 2005). However, in some cases, anaerobic biodegradation of MTBE does not proceed past TBA, which is an unacceptable bioremediation end product, due to its toxicity.

8.3.2 Chlorinated Aliphatic Hydrocarbons

Chlorinated aliphatic hydrocarbons (CAHs), including chlorinated methanes, ethanes, and ethenes, have a number of industrial uses. In particular, their widespread use as solvents (e.g., in the removal of grease from metal, clothing, and other materials) has led to frequent contamination of soil and groundwater through spills and improper disposal, particularly at military and industrial sites and dry-cleaning facilities. Contamination with CAHs is of concern because of their toxicity to humans and, in many cases, known or likely carcinogenicity.

As discussed above, polar carbon–halogen bonds may serve as the site of initial attack for each of the three major biodegradation mechanisms—hydrolysis, oxidation involving electrophilic oxygen, and reduction—although individual CAHs vary with respect to their susceptibility to transformation via a given mechanism. Hydrolysis is most commonly observed for CAHs with two or fewer chlorine substituents on a given carbon (ASCE, 2004). Hydrolytic attack of dichloromethane and 1,2-dichloroethane by some organisms allows them to use the CAHs as a source of carbon and energy, and longer halogenated alkanes are also subject to hydrolysis (Fetzner, 1998).

The selection of an oxidative or reductive biodegradation-based cleanup approach depends on the predominant redox conditions in the contaminated groundwater, the relative susceptibility of the contaminant(s) to oxidation and reduction reactions, the physiological capabilities of the indigenous microorganisms, and the availability of co-substrates, including electron donors and/or oxygen. For example, there are many challenges associated with the implementation of bioremediation strategies based on co-metabolic reactions. As discussed above, biodegradation of co-metabolic substrates occurs relatively slowly compared to metabolic processes due to competition with growth substrates for key enzymes, diversion of coreactants in metabolic reactions, and/or cellular damage caused by transformation product toxicity. Nevertheless, if the contaminated groundwater is aerobic and a potential source of carbon and energy (e.g., toluene or methane) is present, it may be reasonable to select a bioremediation strategy based on aerobic co-metabolism. Co-metabolic oxidation has also been used successfully to bioremediate TCE-contaminated groundwater through the careful addition of both oxygen and either phenol or toluene as the source of carbon and energy (e.g., McCarty et al., 1998). Only the most highly chlorinated aliphatic hydrocarbons (e.g., carbon tetrachloride, TeCA, and PCE) appear to be resistant to aerobic co-metabolic transformations.

On the other hand, all chlorinated methanes, ethanes, and ethenes can undergo reductive dechlorination reactions, although reductive dechlorination of more highly chlorinated CAHs (e.g., PCE and TCE) tends to occur at faster rates than does transformation of the less chlorinated analogs (e.g., DCE and VC). Thus, efforts to bioremediate anaerobic groundwater systems contaminated with highly chlorinated aliphatic hydrocarbons typically focus on promoting reductive dechlorination processes. Often, this involves the addition of electron donors, a practice known as *biostimulation*, due to the limited availability of suitable electron donors at most CAH-contaminated sites. In particular, biostimulation of reductive dechlorination reactions is an effective bioremediation practice for CAHs that can serve as terminal electron acceptors in dehalorespiration. Rittmann and McCarty (2001) provided a summary of CAHs that can serve as terminal electron acceptors, and their susceptibility to co-metabolic biotransformations and utilization as an electron donor under aerobic and anaerobic conditions. However, the biodegradation of CAHs is an active area of research, and new information on the metabolic roles that these compounds can fulfill is constantly emerging. For example, utilization of chloromethane and *cis*-1,2-DCE as electron donors under anaerobic and aerobic conditions, respectively, has been reported. Further, several chlorinated ethanes, including 1,1,1-trichloroethane, 1,1,2-trichloroethane, 1,1-dichloroethane, and 1,2-dichloroethane, are now known to serve as terminal electron acceptors for certain dehalorespirers.

8.3.3 Halogenated Aromatic Hydrocarbons

Several categories of halogenated aromatic hydrocarbons are of environmental significance, including polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). PCBs were widely used in electrical capacitors and transformers and dielectric and hydraulic fluids until the 1970s, when PCB use was banned in the United States. Spills and improper disposal practices led to widespread and persistent contamination of the environment with PCBs. The concern over PCBs is due primarily to their tendency to partition into organic matter and thus bioaccumulate in ecological food chains. Unlike PCBs, PBDEs break down with heat (Hooper and McDonald,

2000). In the process, they release bromine radicals that help quench combustion processes. These properties have led to their extensive use as flame retardants in plastics and textiles and their broad dissemination in the environment. PBDEs levels are also increasing rapidly in animal tissues and human breast milk, which is of concern due to the endocrine-disrupting action of some PBDEs.

PCBs Up to 209 distinct PCB molecules (or congeners) that differ with respect to the numbers and positions of chlorine substituents on the biphenyl backbone can be found (Figure 8.5). However, PCBs were typically produced and sold commercially as mixtures of 60 to 90 congeners under the U.S. trade name Aroclor. The Aroclor product numbers indicate the overall degree of chlorination in the mixture. For example, Aroclor 1242 contains 12 carbon atoms and 42% chlorine by weight, which corresponds to an average of approximately three chlorines per biphenyl molecule. Microorganisms utilize strategies similar to those involved in the biodegradation of CAHs and the BTEX compounds to biodegrade PCBs. However, individual congeners vary with respect to their susceptibility to various biotransformation mechanisms, which can complicate efforts to detoxify Aroclors under a given set of conditions. Under aerobic conditions, lightly chlorinated PCBs (generally those with three or fewer chlorines) are converted to dihydroxylated intermediates by dioxygenases, reactions that are analogous to the ring activation mechanisms in BTEX biodegradation. The dioxygenases typically attack the 2 and 3 positions (Figure 8.5) on the more lightly chlorinated ring and are hindered by chlorine substituents in these positions. The activated ring undergoes *meta* cleavage, as in toluene biodegradation, and leads to the formation of a chlorobenzoic acid, which is not transformed by most PCB-degrading bacteria but generally can be mineralized by chlorobenzoate-degrading populations. Other bacteria can dihydroxylate certain PCBs in the 3 and 4 positions via a similar biodegradation pathway. Bacteria that can aerobically degrade lightly chlorinated PCBs (either metabolically or cometabolically while growing on biphenyl or another primary substrate) appear to be fairly common in contaminated soils. Thus, under aerobic conditions, the composition of an Aroclor mixture is expected to shift toward more highly chlorinated congeners, which tend to persist under aerobic conditions. Conversely, the more highly chlorinated PCB congeners are better suited than the lightly chlorinated congeners for reductive dechlorination. Several

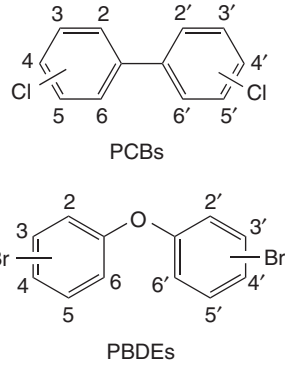


Figure 8.5 Structure and numbering of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs).

microbial reductive dechlorination processes that target chlorines in different positions have been identified (e.g., Bedard et al., 2005), although dechlorinations in the *para* and *meta* positions (relative to the biphenyl linkage) generally are dominant. Recently, a *Dehalococcoides* population and a related strain that can respire PCBs as terminal electron acceptors have been identified (Bedard et al., 2007; May et al., 2008).

PBDEs The nomenclature and number of PBDE congeners are the same as for the PCBs (Figure 8.5). Studies of the biodegradability of PBDEs have largely focused on the potential for dechlorination of the fully brominated decabromodiphenyl ether and other highly brominated congeners, due to their potential for biodegradation to less brominated congeners, which are more toxic and bioavailable (Gerecke et al., 2005; Robrock et al., 2008). Microbial reductive dechlorination of highly brominated PBDEs has been observed in both complex cultures and in pure dehalorespiring cultures maintained on chlorinated electron acceptors. Up to five bromines were removed by the dehalorespiring strains. At least two aspects of anaerobic PBDE and PCB biodegradation are similar. First, dechlorination of the more highly halogenated congeners occurred more slowly than did PBDEs containing fewer bromine substituents, presumably due to the reduced bioavailability of the more hydrophobic, highly brominated congeners. Second, removal of bromines occurred predominantly in the *para* and *meta* positions (relative to the ether linkage).

8.4 GENETIC CAPABILITY

Engineered bioremediation approaches generally focus on stimulating the natural potential of indigenous bacteria to biodegrade the contaminants at a site: for example, through the addition of a key cosubstrate such as an electron donor (biostimulation) or oxygen, as discussed below. For this approach to be successful, the native organisms must possess or acquire the genes that encode the enzymes catalyzing the biodegradation reactions. Adequate expression of these genes must also occur.

8.4.1 Assessing Genetic Capability

All remediation projects begin with site characterization. For bioremediation projects, site characterization also provides a critical opportunity to assess the genetic capability of a contaminated site. Preliminary laboratory treatability studies are traditionally conducted as part of a site characterization if there is any uncertainty about the suitability of the contaminant or site characteristics for biodegradation (Rittmann et al., 1994). These simple biodegradation assays are typically not needed to evaluate the potential for aerobic biodegradation of the BTEX compounds or other proven bioremediation scenarios that do not depend on unique organisms or conditions. However, treatability assays are needed to assess the potential for biodegradation of MTBE or benzene (under anaerobic conditions), which is highly site specific. Clearly, if biodegradation does not occur in the laboratory, the requisite genetic capability is probably lacking in situ. Conversely, demonstration of biodegradation under ideal laboratory conditions does not guarantee that the genetic capability is adequately expressed under field conditions. In particular, long lag periods during which substantial reductions in contaminant concentration are not observed may indicate that the indigenous microorganisms are not adapted to the

contaminant, and in situ biotransformation may not be occurring at a detectable rate (Becker et al., 2006). Despite its importance in controlling contaminant fate, it is currently difficult to predict if and when adaptive events will occur because adaptation may involve several interrelated mechanisms that create new genetic capability—leading to new biodegradation potential—or increase existing capabilities. These include: (1) mutation or genetic exchanges, (2) selective enrichment of a population that was able to biodegrade the contaminant before exposure occurred, or (3) induction or derepression of enzymes catalyzing key biodegradation reactions (Rittmann et al., 1994). In addition, microbial processes that alleviate environmental conditions that are not amenable to biodegradation can also contribute to the adaptation process. For example, adaptation to, and rapid biodegradation of, 3-chlorobenzoate did not occur in anaerobic sediment and digester sludge until endogenous substrates that were utilized by the microbial communities were depleted (Becker et al., 2006).

While treatability studies remain an essential tool in assessing genetic capability, they are increasingly being complemented by nucleic acid–based methods that directly assay complex microbial communities at contaminated sites for the presence and, in some cases, activity of specific functional genes that encode key enzymes in the degradation process. In particular, such tools are being used to assess the genetic potential at PCE- and TCE-contaminated sites. Populations that respire these chlorinated ethenes and reductively dechlorinate them to DCE are generally assumed to be present at these sites and include certain *Desulfitobacterium, Desulfuromonas, Dehalobacter, Dehalococcoides, Sulfurospirillum*, and *Geobacter* strains. However, rapid reductive dechlorination of DCE and VC does not occur at many sites and appears to require the presence of specific *Dehalococcoides* strains that can dehalorespire these lesser chlorinated ethenes using hydrogen as the electron donor (Ritalahti et al., 2006). Because 16S rRNA gene-based methods cannot distinguish between these DCE- and VC-respiring strains and *Dehalococcoides* strains that transform DCE and VC cometabolically, quantitative polymerase chain reaction (qPCR)–based methods that specifically target VC reductive dehalogenase genes are increasingly be used to assess sites for the potential for complete detoxification of PCE and TCE (Ritalahti et al., 2006; Cupples, 2008). Reverse transcriptase qPCR assays that quantify the expression of dehalogenase genes in *Dehalococcoides* strains offer even greater potential for describing the physiological state of these organisms, and thus the likelihood for successful bioremediation, under different conditions (Cupples, 2008).

8.4.2 Approaches for Increasing Genetic Capability

What methods can be used to increase the genetic capability at sites where it is deficient? If the contaminant is metabolized as a growth substrate by indigenous microorganisms at in situ concentrations, it should be possible to increase the biodegradation capacity by promoting selective enrichment of the contaminant-degrading population. Hence, at sites contaminated with chlorinated ethenes, biostimulation typically focuses on increasing hydrogen concentrations within the plume because DCE- and VC-respiring *Dehalococcoides* strains use hydrogen as the electron donor. For example, biostimulation frequently involves the addition of organic substrates that undergo a hydrogen-producing fermentation reaction. Biostimulation with substrates such as butyrate or propionate, which are fermented slowly and only at very low hydrogen concentrations, are most effective at selectively delivering the electron donor to

dehalorespiring populations because of their ability to utilize H_2 at lower concentrations (thresholds) than those of the H_2 thresholds of methanogens and many other hydrogenotrophs that are present at contaminated sites (Fennell et al., 1997).

If a contaminant-degrading population is not native to a site, it may be possible to add a laboratory-grown culture containing large numbers of these organisms through an engineered bioremediation process known as *bioaugmentation*. Processes that are highly site specific, such as biodegradation of MTBE or biodegradation of benzene under anaerobic conditions, may be good candidates for bioaugmentation if indicated by treatability studies. Similarly, bioaugmentation of sites with DCE- and VC-respiring *Dehalococcoides* strains may be necessary to achieve complete detoxification of PCE and TCE if molecular characterization of a site indicates that the key dehalogenases are lacking or not being expressed. However, a modeling study by Becker (2006) recently demonstrated that if native PCE-to-DCE dechlorinating populations are present, they may compete with *Dehalococcoides* populations for reducing equivalents and/or electron acceptors and reduce the effectiveness of bioaugmentation. In other cases, *Dehalococcoides* and other dehalorespiring populations may coexist and carry out complementary activities that enhance the rate and extent of reductive dechlorination. These results highlight the need to characterize the indigenous PCE-to-DCE dechlorinating populations at contaminated sites, in addition to testing for the presence of DCE- and VC-respiring *Dehalococcoides* strains before developing biostimulation- and/or bioaugmentation-based cleanup strategies.

If the contaminant is not biodegraded via a metabolic process, it may be possible to increase the genetic capability for biodegradation at the site through analog enrichment of the contaminant-degrading population. Analog enrichment involves the addition of compounds or primers that share key structural features with the contaminant and probably serve as growth substrates for the contaminant-degrading population and/or increase expression of key biodegradative enzymes. For in situ bioremediation applications, the primers should be nontoxic. In particular, primers have been used to enhance the biodegradation of several types of halogenated aromatic compounds, including PCBs (e.g., Bedard et al., 1998), halogenated dibenzo-*p*-dioxins and dibenzofurans (Ahn et al., 2005), and PBDEs (Gerecke et al., 2005).

8.5 OVERVIEW OF BIOREMEDIATION APPROACHES

Bioremediation strategies can be categorized based on the location of contaminant biodegradation and the aggressiveness of the remediation (Madsen, 1997). In situ bioremediation is performed with the contaminated material left in its natural or original position. Ex situ bioremediation involves the removal of contaminated material from its original position and its treatment in a bioreactor system. Both in situ and ex situ technologies can be applied to solid-, slurry-, and vapor-phase systems. The aggressiveness of in situ and ex situ bioremediation approaches ranges widely, but they can be divided into two broad classes (NRC, 1993; Madsen, 1997). At the passive end of the spectrum is *intrinsic* or *natural bioremediation*. This approach relies solely on the innate capabilities of naturally occurring microorganisms to degrade the contaminants in situ. In more aggressive bioremediation applications, actions are taken to modify the site or contaminated material to promote and enhance the biodegradative activities of microorganisms (e.g., via biostimulation or bioaugmentation). These technologies are typically referred to as *engineered* or *enhanced bioremediation* approaches.

8.6 KEY DESIGN TOOLS FOR BIOREMEDIATION TREATMENT SYSTEMS

Two key quantitative tools for the design of engineered in situ and ex situ bioremediation treatment systems and evaluation of intrinsic in situ bioremediation are the stoichiometry and kinetics of the pertinent microbiological reactions. Stoichiometry, which relates the quantities of reactants and products in the microbially mediated reaction, can be used to estimate the quantities of stimulatory materials that need to be delivered to a bioreactor or in situ treatment zone (Cookson, 1995). Kinetics, which describe the rate at which the reaction takes place, coupled with the desired cleanup rate, determine the rate at which the required stimulatory materials must be added to the contaminated material (Cookson, 1995). These data are used to size treatment process facilities and estimate the time and costs required to achieve the project goals (McCarty, 1988; Cookson, 1995).

8.6.1 Stoichiometry

Given an estimated amount of contaminant per unit mass of soil, or per unit volume groundwater, the quantities of reactants (e.g., electron donor and acceptor or nutrients) and products (e.g., biomass or CO_2) can be calculated using a balanced chemical reaction and information about the dominant mode of metabolism and availability of nutrients (Cookson, 1995). Balanced stoichiometric equations are also used to evaluate laboratory and experimental data.

A general thermodynamic approach to formulating a balanced reaction for the microbial metabolism of organic substrates was developed by McCarty (1972) and recently updated (Rittmann and McCarty, 2001). In this approach, electrons from the electron donor substrate are either coupled with the electron acceptor to generate energy or are used in biomass synthesis, and the fraction of electrons going to each process is estimated based on an energy balance (Bouwer, 1992). Detailed descriptions of this thermodynamic method are provided in the references cited.

Balanced stoichiometric reactions are shown in Table 8.2 for some of the contaminants and transformation processes presented in Table 8.1. The first step in applying

Table 8.2 Stoichiometric Relationships for Example Biodegradation Processes of Interest[a]

Reaction 1: Aerobic mineralization/metabolism of toluene

$$C_6H_5CH_3 + 2.66O_2 + 1.27HCO_3^- + 1.27NH_4^+ \rightarrow 1.27C_5H_7O_2N + 1.93CO_2 + 2.73H_2O$$

Reaction 2: Aerobic mineralization/co-metabolism of TCE via methanotrophs growing on CH_4

$$CH_4 + 1.04O_2 + 0.137NO_3^- + 0.137H^+ \rightarrow 0.137C_5H_7O_2N + 0.315CO_2 + 1.59H_2O$$

Reaction 3: Anaerobic biotransformation/metabolism of TCE to DCE

$$C_2HCl_3 + 0.47CH_3COO^- + 0.089NH_4^+ + 0.619H_2O \rightarrow 0.089C_5H_7O_2N + C_2H_2Cl_2 + HCl + 0.117CO_2 + 0.385HCO_3^-$$

Source: Adapted from Bouwer (1992).

[a] $C_5H_7O_2N$, an empirical formula for biomass. All compounds were considered to be in the aqueous phase, with the exception of CO_2, N_2, and CH_4, which were assumed to be gaseous. See Bouwer (1992) for other details.

these balanced reactions is to estimate the concentrations of contaminants and nutrients concentrations and then convert them into terms of mass (Cookson, 1995). Then the reaction stoichiometry can be used to estimate (1) the mass of each coreactant that should be added to the contaminated material to meet process requirements, and (2) the mass of each product expected. Application of the stoichiometric ratios for contaminants that serve as primary substrates is relatively straightforward. For example, aerobic mineralization of 1 kg of the electron-donor contaminant toluene (reaction 1) requires 0.93 kg of O_2 and 0.25 kg of NH_4^+ and produces 0.92 kg of CO_2 and 1.6 kg of cells. Similarly, reduction of 1 kg of the electron-acceptor contaminant TCE to DCE (Reaction 3) requires 0.21 kg of acetate and 0.012 kg of NH_4^+ and produces 0.92 kg of CO_2, 1.6 kg of cells, 0.74 kg of DCE, and 0.28 kg of HCl. Phosphorus requirements can be assumed to equal one-sixth of the requirement for nitrogen on a molar basis. As discussed below, addition of other nutrients is generally not necessary.

Application of the stoichiometry for contaminants that are biotransformed via co-metabolism is more complicated (McCarty, 1988). For example, co-metabolic transformation of TCE by methanotrophic bacteria growing on methane (reaction 2) has been shown to require between approximately 100 and 1000 kg of methane per kg of TCE oxidized. When this is coupled with the associated oxygen and nutrient requirements for reaction 2, it becomes clear that large quantities of chemical stimulants are required even for relatively small amounts of contamination. Unfortunately, high concentrations of methane reduce the TCE reaction rate, as discussed above.

8.6.2 Biotransformation Kinetics

Microbial kinetics allow us to describe the rates of biodegradation and identify the factors that may limit these rates and thus are key to understanding and implementing bioremediation successfully. Basic kinetic concepts are reviewed below following the approach of Rittmann et al. (1994) and McCarty (1988).

Kinetic Models for Primary Substrates The kinetics for removal of contaminants serving as primary substrates are related directly to biomass production:

$$\text{net biomass growth} = (\text{growth on primary substrate}) - (\text{biomass decay}) \tag{8.1}$$

where biomass decay is the consumption of cellular material due to cell maintenance, death, predation, and so on. The expression in equation (8.1) can be rewritten symbolically as

$$R_X = \frac{dX}{dt} = Y(-R_{su}) - bX \tag{8.2}$$

where R_X is the net rate of bacterial growth [$M_X L^{-3} T^{-1}$], X the biomass concentration [ML^{-3}], t the time [T], Y the true yield [$M_X M_S^{-1}$], R_{SU} the rate of substrate utilization [$M_S L^{-3} T^{-1}$], and b the first-order decay coefficient [T^{-1}]. The most commonly applied model for utilization of a dissolved, noninhibitory primary substrate is the Monod model:

$$R_{su} = \frac{dS}{dt} = -q_{max}\frac{S}{K+S}X \tag{8.3}$$

where q_{max} is the maximum specific substrate utilization rate [$M_S M_X^{-1} T^{-1}$], S the limiting substrate concentration [$M_S L^{-3}$], and K the substrate half-saturation constant [$M_S L^{-3}$]. This expression can be used to describe electron donor–and electron acceptor–limiting conditions. When $S \gg K$, Monod kinetics reduce to the first-order expression (zero-order with respect to S)

$$\frac{dS}{dt} = -q_{max} X \tag{8.4}$$

For very low substrate concentrations, $S \ll K$, and Monod kinetics reduce to a mixed second-order expression (first-order with respect to S):

$$\frac{dS}{dt} = -\frac{q_{max}}{K} SX \tag{8.5}$$

where q_{max}/K is often referred to as a second-order rate constant [$L^3 M_X^{-1} T^{-1}$]. When biomass concentration does not change significantly, a lumped, pseudo-first-order rate constant [T^{-1}] is sometimes used:

$$K_{B1} = \frac{q_{max} X}{K} \tag{8.6}$$

The rate at which the substrate is being utilized (dS/dt) given by equations (8.3)–(8.5) can be coupled with the stoichiometry described above (e.g., mass oxygen/mass toluene) to predict (1) the rate of oxygen supply needed to react with all of the oxygen-demanding material (i.e., the aeration rate), (2) the rate at which biomass will be produced (i.e., the specific growth rate), plus (3) the rate at which the nitrogen source must be added to support that biomass production. These are examples of three direct and important applications of stoichiometry and kinetics.

Microbial growth and substrate utilization rates can be reduced by a variety of inhibitors and inhibition effects. For example, the rate of biodegradation of primary substrates such as phenol, 4-chlorophenol, and toluene, which are self-inhibitory, is sometimes described using the Andrews (1968) kinetic model:

$$\frac{dS}{dt} = -q_{max} \frac{1}{1 + K/S + S/K_i} X \tag{8.7}$$

where K_i is the inhibition constant [$M_S L^{-3}$]. The Andrews model also reduces to first-order kinetics with respect to S when $S \ll K$.

The Monod and Andrews equations assume that only one substrate limits the overall transformation rate. However, the electron donor and acceptor may both be limiting, especially in situ. Control of the substrate utilization rate by two or more limiting substrates is frequently described using a multiplicative Monod model, which assumes that if two substrates are required and both are present at limiting concentrations, the concentration of both substrates will affect the overall growth rate. For example, if one limiting substrate is the primary electron donor and the other the primary electron acceptor, the substrate utilization rate for the electron donor is

$$R_{su}(d) = -q_{max}(d) \frac{S_d}{K_d + S_d} \frac{S_a}{K_a + S_a} X \tag{8.8}$$

where: $R_{su(d)}$ is the rate of electron donor utilization [$M_S L^{-3} T^{-1}$], $q_{max(d)}$ the maximum specific electron donor utilization rate [$M_S M_X^{-1} T^{-1}$]; S_d, S_a the electron donor and electron acceptor concentration, respectively [$M_S L^{-3}$]; and K_d, K_a the half-saturation constant for the electron donor and electron acceptor, respectively [$M_S L^{-3}$].

From the expressions above, it is clear that increasing the rate of substrate utilization, dS/dt, requires increasing the biomass concentration (e.g., via bioaugmentation or biostimulation). There is, however, a threshold substrate concentration, S_{min}, resulting in no net growth, which can be derived by substituting equation (8.3) for R_{su} in equation (8.2) and setting $dX/dt = 0$, giving

$$S_{min} = \frac{bK}{Yq_{max} - b} \tag{8.9}$$

A key consequence of the S_{min} concept is that under steady-state conditions, there is a minimum concentration, or threshold, to which a primary substrate contaminant can be reduced. As a result, if the contaminant concentration is initially greater than S_{min}, X and R_{su} can increase temporally according to equations (8.2) and (8.3), respectively. However, if the contaminant concentration is initially less than S_{min}, there can be no net growth of biomass and the contaminant acts as a secondary substrate, as defined above. Under these conditions, contaminant biotransformation cannot be modeled using equation (8.3), which applies to compounds that serve as a primary substrate. The reader is referred to Rittmann and McCarty (2001) and references therein for detailed descriptions of the biodegradation kinetics of compounds that are utilized as secondary substrates either because $S < S_{min}$ or because they are co-metabolically transformed.

8.7 ENVIRONMENTAL FACTORS LIMITING BIOREMEDIATION IN THE FIELD

The rates of biodegradation and microbial growth in the field are influenced by a variety of abiotic factors, including the environmental conditions and processes controlling the bioavailability of organic contaminants (Rittmann et al., 1994; Alexander, 1999).

8.7.1 Environmental Conditions

Environmental conditions that affect microbial kinetics include substrate and nutrient levels, temperature, pH, and moisture levels (Rittmann et al., 1994; Alexander, 1999). Bioremediation strategies frequently focus on modifying or controlling these factors when it is possible and economical to do so.

Primary Substrates If the concentration of a contaminant is too low to support growth (i.e., $S < S_{min}$), or cannot support growth for some other reason, it must be degraded as a secondary contaminant, as discussed above. On the other hand, high concentrations of a contaminant that is used as a primary substrate may be inhibitory or cause the complementary growth substrate and/or nutrients to become limiting (Alexander, 1999). For example, high concentrations of hydrocarbons that serve as an electron donor in soil or groundwater may rapidly consume the oxygen dissolved in the water or present in gas-filled pores. This is important because some contaminants are biodegraded only under aerobic conditions, while many contaminant biodegradation rates

are more rapid under aerobic than anaerobic conditions (McNabb and Dunlap, 1975). As a result, many bioremediation technologies focus on the addition of oxygen, as discussed below.

A number of electron acceptors other than oxygen are often present in the subsurface environment (e.g., sulfate, carbon dioxide, nitrate, manganese oxides, and iron oxyhydroxides) (McNabb and Dunlap, 1975; Ghiorse and Wilson, 1988), and many organic compounds can be biodegraded anaerobically. After oxygen is consumed, high levels of contamination can also quickly deplete these anaerobic electron acceptors, and natural rates of replenishment are likely to be very slow (Ghiorse and Wilson, 1988). Thus, the coupling of mass transport by groundwater flow and microbial reaction is likely to result in spatial gradients of electron acceptor concentrations (Bouwer, 1992). Based on the free energy released during respiration, a progression in redox conditions could occur along the groundwater flow path moving away from a contaminated zone, with the heterotrophic microorganisms predicted to use sequentially the following succession of electron acceptors: oxygen, nitrate, Mn(IV) and Fe(III), sulfate, and carbon dioxide.

Other Nutrients Chemotrophic microorganisms require macronutrients, minor and trace elements, and growth factors for the synthesis of cellular materials. If any cellular nutritional requirements are not met, biodegradation may stop; however, the focus is generally on the availability of N and/or P. Appropriate N and P doses can be calculated using stoichiometry, as described above, and in situ bioremediation applications have often included additions of inorganic nutrients (see, e.g., Lee et al., 1988 and Staps, 1990). Whereas many laboratory studies indicate that nutrient additions increase degradation rates, often little effect is observed in the field (Cookson, 1995). Potential explanations for this apparent discrepancy are discussed below.

Temperature Soil and groundwater temperatures fluctuate seasonally to a depth of approximately 10 m, below which the groundwater temperature is determined largely by the region's mean annual temperature (Kuznetsov et al., 1963). Temperature is a key factor affecting microbial growth and biotransformation rates (Alexander, 1999). In general, biodegradation rates decrease with temperature; however, temperature effects can be complex. For example, temperature influences hydrocarbon biodegradation by causing changes in (1) the physical nature and chemical composition of the hydrocarbons, (2) the rate of hydrocarbon metabolism by microorganisms, and (3) the microbial community composition (Atlas, 1981; Leahy and Colwell, 1990).

pH Nearly all groundwater falls in the pH range 6 to 9 (Freeze and Cherry, 1979), but soil pHs can be acidic in areas where sufficient rainfall occurs to leach bases from the soil or alkaline, primarily in arid and semiarid regions (Hartel, 1998). Soil and groundwater with low alkalinity may also become acidic due to contaminant biodegradation (e.g., through production of organic acids or HCl from reductive dehalogenation) (Figure 8.2). Fungi prefer acidic conditions (Maier, 2000), whereas bacterial biodegradation rates tend to be fastest at nearly neutral pHs (Alexander, 1999). Therefore, lime is commonly added to neutralize the pH where acidity is of concern (Cookson, 1995). Microbial oxidation of reduced sulfur compounds generates protons and can be used to lower the pH of alkaline environments (Madigan et al., 1997).

Moisture Inadequate moisture levels can severely restrict biodegradation in surface soils, which are subject to drying to suboptimal levels (Alexander, 1999), particularly when airflow through unsaturated soils is promoted via bioventing or biosparging (discussed below). For example, Dibble and Bartha (1979) found that the optimum moisture level for biodegradation of oily sludges was 30 to 90% of the soil's water-holding capacity. The optimum moisture level for a given situation is a function of the soil properties, contaminant characteristics, and oxygen requirements (Alexander, 1999). When moisture levels are high, there is less air-filled pore space and the soil soon becomes anaerobic, due to the slow rate of oxygen diffusion through water. However, degradation rates also decrease if the moisture levels become too low (Hinchee and Arthur, 1991).

8.7.2 Contaminant Availability

Bioremediation strategies designed to increase microbial activity through the addition of key substrates or modification of environmental conditions will not have a positive impact if the microorganisms cannot access the contaminant (i.e., it is not bioavailable). For example, as noted above, nutrient additions in the field often have little effect (Cookson, 1995). This could indicate that the distribution of nutrients in the field was ineffective, the existing supply of N and P was adequate and did not have to be supplemented, or another factor, such as contaminant availability was limiting the rate of biodegradation (Cookson, 1995; Alexander, 1999). In fact, a large fraction of contaminants in the environment appears to be unavailable for microbial degradation (Alexander, 1995). Thus, the overall biotransformation rate depends not only on microbial kinetics and the environmental conditions that affect them, but also on the physicochemical constraints that control contaminant bioavailability (i.e., the fraction of contaminant molecules that are dissolved and available to the microorganisms) (Ramaswami and Luthy, 1997). The appropriate remedial action needed to enhance in situ biodegradation can be selected only when the rate-limiting process has been identified correctly. Two key phenomena limiting contaminant bioavailability are their presence in the form of a non-aqueous-phase liquid (NAPL) and their sorption to solid surfaces.

A major challenge to successful bioremediation of hydrocarbons and CAHs is that they are often transported, used, and released to the environment as NAPLs, which have relatively low water solubility and can migrate through the subsurface as a separate phase (Seagren et al., 1993). This is problematic because NAPL contaminants, which may be present in various configurations, including blobs or pools, can serve as long-term sources of pollution as they gradually dissolve into the groundwater. At the same time, NAPLs can limit the bioavailability of contaminants as microbial substrates due to interphase mass-transfer limitations and partitioning into a NAPL, which can reduce the aqueous-phase concentrations, thereby decreasing biodegradation rates (Fu and Alexander, 1995; Labare and Alexander, 1995). When NAPL dissolution limits biodegradation, potential bioremediation approaches for enhancing the overall biotransformation rate in the subsurface include surfactant or cosolvent additions (Zhang et al., 1998).

Because of the large surface area present in subsurface porous media, significant sorption of microbial cells and, in some cases, of the contaminant may occur (Ghiorse and Wilson, 1988). The potential effects of sorption on biodegradation are complex and

depend on the characteristics of the contaminant, the solid surface(s), and the microorganisms (Rittmann et al., 1994). For example, if high contaminant levels are toxic to the microorganisms, sorption may positively influence biodegradation rates by reducing the aqueous concentrations (Apajalahti and Salkinoja-Salonen, 1984). Sorption can also reduce the biodegradation rate by reducing the aqueous concentration or due to interphase mass-transfer limitations (e.g., Zhang et al., 1998). If biokinetic rates are slow compared with NAPL dissolution or desorption, it may be possible to increase the overall biotransformation rate by alleviating a substrate limitation (e.g., electron acceptor or nutrient additions), improving environmental conditions (e.g., pH), and/or by increasing the amount of active biomass (e.g., via bioaugmentation) as discussed above. Nevertheless, Bosma et al. (1997) noted that biokinetics limit bioremediation in only a few cases; in most cases, full exploitation of the microbial biodegradative potential was prevented by mass-transfer limitations.

Biodegradation can also accelerate dissolution or desorption (i.e., it may result in a "bioenhancement" of interphase mass transfer). In the absence of microbial activity, contaminant levels in groundwater adjacent to a NAPL may approach or equal the maximum concentration that can occur in equilibrium with the contaminant in the NAPL. Microorganisms carrying out biodegradation near the NAPL–water interface decrease the aqueous concentration of the contaminant and thus create a driving force for more dissolution of the contaminant from the NAPL. This phenomenon has been examined theoretically (e.g., Seagren et al., 1993, 1994), and demonstrated in laboratory (e.g., Carr et al., 2000; Yang and McCarty, 2000; Seagren et al., 2002) and field studies (Essaid et al., 2003). Analogously, bioenhancement can also explain enhanced desorption of organic compounds from solids (e.g., Rijnaarts et al., 1990).

Effective bioremediation design also requires an understanding of how the distribution of the contaminant and the availability of substrate, nutrients, and electron acceptors to microorganisms are affected by mass-transport processes such as advection and dispersion (Sturman et al., 1995). For example, rapid pumping of water through a groundwater aquifer (known as *flushing*) can potentially accelerate NAPL dissolution and desorption by increasing the mass-transfer coefficient and the concentration gradient (e.g., Miller et al., 1990, Seagren and Moore, 2003). Care must be taken to avoid transporting the contaminant through the system (via advection) faster than it can be biodegraded by the microorganisms (Seagren et al., 1993, 1994). Further, increased flushing will increase the biodegradation sink only if it lowers the solute concentrations below toxic levels (Seagren et al., 2002).

The porous media (sediment, sand, gravel, etc.) in groundwater aquifers is characteristically heterogeneous. Advection and dispersion play important roles in the transport and biodegradation of microbial substrates in these heterogeneous media by affecting the mixing of contaminants, nutrients, electron acceptors, and cells capable of contaminant biodegradation. In particular, vertical dispersion is an important process for developing zones of mixing (Domenico and Schwartz, 1998) in situations including oxygen transfer across the water table (e.g., Borden and Bedient, 1986; Borden et al., 1986), NAPL pool dissolution (Johnson and Pankow, 1992; Seagren et al., 1994), and heterogeneities in hydraulic conductivity (e.g., Szecsody et al., 1994; Murphy et al., 1997). For example, Song and Seagren (2008) investigated the transport and biodegradation of dissolved naphthalene in a sand tank with two layers of contrasting hydraulic conductivities and limited sorption. The interactions between the mass-transfer processes controlling bioavailability and in situ biodegradation are complex. Therefore, a

systematic and quantitative approach based on analysis of dimensionless numbers that compare the rates of mass transfer processes and biodegradation was used to determine the overall rate-limiting process in the sand tank reactors. A detailed description of the use of dimensionless parameters as quantitative criteria for identifying the rate-limiting phenomena in environmental systems with biological activity is beyond the scope of this chapter, but the interested reader is referred to Ramaswami and Luthy (1997), Weiner et al.. (1999), and Song and Seagren (2008) for more information on this useful design tool. Under baseline conditions, dispersion was found to be the overall rate-limiting process in the sand tank reactors (Song and Seagren, 2008), which is probably a common situation for many readily biodegradable contaminants under natural conditions (Maier and Grathwohl, 2006). Accordingly, removing nitrogen and phosphorus limitations did not significantly impact biotransformation, because biokinetics were not the overall rate-limiting process. However, increasing dispersion by increasing advection alleviated the rate-limiting process and enhanced the overall biotransformation rate.

8.8 BIOREMEDIATION STRATEGIES

8.8.1 Site Characterization

Site characterization and treatability studies are performed to understand if it is necessary to engineer the system to alleviate limitations on biodegradation. An initial site assessment generally establishes the nature and the extent of contamination and obtains some general site geology and groundwater hydrology data (e.g., flow direction, hydraulic gradient, and the general nature of the aquifer) (Cookson, 1995). Three additional information-gathering steps are required for characterization of sites where bioremediation is being considered.

First, treatability studies must be conducted to evaluate the contaminant's potential for biodegradation and the range of conditions under which biodegradation may occur. A hierarchical approach for determining the type of treatability studies required is described by Rittmann et al. (1994). Second, environmental modifications that may be necessary for optimizing biodegradation must be delineated. This involves determination of (1) potential sources of carbon and energy for the microbes, (2) electron-acceptor availability and the redox condition, (3) existing microbial activity and potential toxicity, (4) nutrient availability, and (5) site temperature, pH, and moisture levels. Finally, the site characteristics that influence the control and operation of engineering processes that manipulate microbial processes in the subsurface must be characterized. Of particular importance is the capacity for the porous medium to transmit water (i.e., the hydraulic conductivity, K), or fluids in general (i.e., the intrinsic permeability, k), because subsurface characteristics are often modified via injection and/or extraction of either water or air, as discussed below. For engineered systems using groundwater circulation, K should be on the order of $\geq 10^{-4}$ cm/s; for systems using air circulation, the intrinsic permeability should be above 10^{-9} cm^2 (NRC, 1993). Other key hydrogeological data include groundwater velocity, soil grain size distribution and classification, porosity, bulk density, and heterogeneities in an aquifer. Heterogeneities are important because they can result in poor distribution of amendments and make it extremely difficult to predict contaminant fate and transport reliably (NRC, 1993; Cookson, 1995).

8.8.2 Intrinsic In Situ Bioremediation and Natural Attenuation

Intrinsic in situ bioremediation uses the natural biodegradative activities of native microorganisms to contain contaminants and prevent their migration away from the source (NRC, 1993; Rittmann and McCarty, 2001). This is done without engineering the site to enhance the process. However, the use of field tests and site-derived samples to document thoroughly the role played by native microorganisms in removing the contaminants is required. The site must also be monitored routinely to demonstrate the effectiveness of the intrinsic bioremediation for preventing contaminant migration, as discussed in a National Research Council report (NRC, 1993). Intrinsic bioremediation is most effective at sites where the velocity and direction of groundwater flow is fairly consistent, adequate levels of carbonate and other minerals can buffer pH changes, and the concentrations of electron acceptors (or donors) and nutrients are high. These properties are evaluated through site characterization. Intrinsic bioremediation can be used in combination with engineered bioremediation and is a component of the broader approach known as monitored natural attenuation (MNA). MNA refers to contaminant reduction via all types of naturally occurring physical, chemical, and biological processes, including biodegradation, dispersion, dilution, sorption, volatilization, and chemical reaction. Key aspects of the monitoring protocols at sites undergoing MNA include documentation of contaminant loss and other indicators of microbial activity (or footprints) coupled with mass balance analyses and solute fate-and-transport models (NRC, 2000; ASCE, 2004).

8.8.3 Engineered In Situ Bioremediation

Engineered in situ bioremediation strategies are designed to enhance the intrinsic biodegradation of contaminants via the input of stimulatory materials into the contaminated region (Rittmann et al., 1994). By supplying limiting substrates in such a way that no contaminant escapes biodegradation, a biologically active zone (BAZ) can be established. Therefore, success depends on the effectiveness of the system used to input the substrates designed to overcome the limitations discussed above. Correspondingly, the techniques can be broadly classified based on how the stimulating materials are added. Selection of a bioremediation technique depends, in part, on whether the contaminant is located above the water table in the *unsaturated* (or *vadose*) *zone*, where the pore space is filled primarily with air, or below the water table in the *saturated zone*, where water fills the pore spaces. Figure 8.6 shows where common in situ and ex situ engineered bioremediation approaches can be applied with respect to contaminant form and location in the subsurface.

Water Circulation Systems When contaminants are located partially or totally in the saturated zone (Figure 8.6), the stimulatory materials can be applied via water circulation systems (NRC, 1993; Rittmann et al., 1994; Rittmann and McCarty, 2001). A typical water circulation system involves the injection of dissolved limiting substrates and hydraulic control of the plume migration to force the flow of the contaminated water through the BAZ. Vertical or horizontal wells are the most direct method for adding stimulatory materials, but they are relatively expensive and prone to clogging. As shown in Table 8.2 (reaction 1), the amount of biomass produced during aerobic mineralization of toluene is larger than the original mass of toluene, and the CO_2 mass is similar to that of toluene. This localized bacterial growth and/or biogas production,

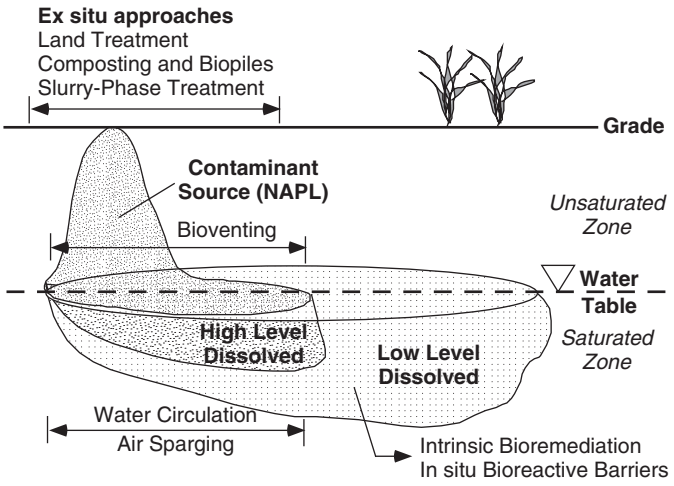


Figure 8.6 Overview of bioremediation approaches that could be applied to a contaminant source zone containing a light nonaqueous-phase liquid (NAPL) and the contaminant plume resulting from dissolution of NAPL into groundwater.

coupled with chemical precipitation, can potentially reduce the hydraulic conductivity and/or result in short circuiting of flow. Pulsed additions of stimulatory materials may help reduce bioclogging. Infiltration galleries can also be used to deliver stimulatory materials and are most effective when the contaminated zone is not too far below the water table. Groundwater is captured (or extracted) downgradient of the BAZ using wells or trenches. The extracted water is usually treated (e.g., using air stripping, biological treatment, or activated carbon) and reinjected in the aquifer.

Maintaining sufficient oxygen in the subsurface to support aerobic biodegradation (e.g., of petroleum hydrocarbons) is challenging using water circulation (Bouwer, 1992; NRC, 1993; Norris, 1994). The limited amount of oxygen ($\approx$8 to 10 mg/L) that can be delivered from the air into water, coupled with the high oxygen demand, results in the need to pump large quantities of water. For example, assuming 9 mg/L dissolved oxygen (DO), biodegradation of 1 kg of toluene requires 103,000 L of water, according to Table 8.2 (reaction 1). Use of pure oxygen can reduce the water required significantly (23,000 L of water assuming 40 mg/L of DO), but also increases costs. Hydrogen peroxide (H_2O_2), which disproportionates to produce oxygen ($H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O$), theoretically can further reduce the volume of water required due to the high solubility of H_2O_2 (3100 L of water, assuming 300 mg/L DO). However, H_2O_2 can be consumed by abiotic processes and can be toxic to microorganisms. If catalysts in the soil cause H_2O_2 disproportionation to occur too quickly, oxygen gas bubbles may also form, which reduces oxygen bioavailability and lowers aquifer permeability (Bouwer, 1992; Leeson and Hinchee, 1997). Alternative electron acceptors such as nitrate, which may be present as a cocontaminant, can also be used in water circulation (Bouwer, 1992). If chlorinated ethenes or other contaminants are used as electron acceptors, water circulation must provide adequate dissolved electron donors.

Air Sparging Air sparging uses air to supply oxygen for aerobic biodegradation. Air is a more efficient carrier than water for delivering oxygen to the BAZ [e.g., assuming

21% (v/v) oxygen in air at 1 atm pressure and 25°C, 3400 L air is required to degrade 1 kg of toluene] (NRC, 1993; Brown, 1994; Rittmann and McCarty, 2001). In air sparging systems, air is injected under pressure via vertical or horizontal sparge wells directly into the saturated zone, displacing water and creating a transient air-filled space. This enhances aerobic biodegradation of low-volatility contaminants by increasing oxygen transfer while promoting removal of high-volatility contaminants by volatilization. Contaminant-laden air arriving at the water table is collected using conventional vapor extraction technology, and a groundwater capture system can be used to prevent off-site migration of dissolved contaminants. Gaseous stimulatory amendments (e.g., methane) may be injected with air, and dissolved amendments may be added via water circulation systems. Air sparging is most effective in unconfined aquifers with homogeneous, high-permeability soils that are contaminated by aerobically biodegradable volatile organic compounds, with little or no NAPL present.

Bioventing The goal of bioventing is to provide air (or oxygen) using configurations and flow rates that ensure adequate oxygenation of the BAZ for aerobic biodegradation while minimizing production of offgases (NRC, 1993; Leeson and Hinchee, 1997; Rittmann and McCarty, 2001). It is a particularly cost-effective and efficient for bioremediation of hydrocarbons in the unsaturated zone. Air may be injected or drawn through unsaturated media via a vacuum (also known as soil gas extraction), but injection is typically used. Additional stimulatory materials may be provided in dissolved form or as gases (see the discussion of air sparging above).

In Situ Bioreactive Barriers In situ biobarrier systems are used to prevent further transport of a contaminant plume in the saturated zone (Rittmann and McCarty, 2001). Specifically, a BAZ is created in the path of the plume via the input of stimulatory materials. In some cases, hydraulic or physical controls (e.g., a funnel-and-gate system) may be needed to ensure that the plume passes through the BAZ. The stimulatory materials used to create the biobarrier can be added either in dissolved form via aqueous injection wells, infiltration galleries, or recirculating wells, or by placing slow-release materials (e.g., proprietary sources of electron donors or acceptors, mulch, wood chips, iron filings) in wells or trenches.

8.8.4 Ex Situ Bioremediation

Ex situ bioremediation strategies require excavation of the contaminated materials and are primarily applicable to sites with small, highly contaminated sources (Figure 8.6), or when a rapid cleanup is required (Rittmann and McCarty, 2001). The three main approaches—land treatment, composting/biopiles, and slurry-phase treatment—vary primarily in how the contaminated material is aerated and the degree of water saturation.

Land Treatment In land treatment, the contaminated materials are spread out in relatively thin layers, typically in a specially constructed above-grade treatment system or land treatment unit (LTU) (Cookson, 1995; Eweis et al., 1998). LTUs generally have controls for containing the contaminated materials, including a liner and leachate collection system and a cover to reduce volatile emissions. The contaminated material is tilled to aerate the soil and reduce mass-transfer limitations by mixing. Soil nutrient

and moisture levels are monitored along with pH and adjusted if necessary to further enhance biodegradation. The soil may also be amended with a bulking agent (e.g., wood chips, shredded bark) or organic material to improve porosity and permeability.

Composting and Biopiles In composting, the contaminated material is piled up rather than spread out in thin layers, which affects the degree of heat entrapment that occurs and the mode of aeration (Cookson, 1995; Eweis et al., 1998). In conventional composting, the contaminated material is typically mixed with an organic bulking agent (see the discussion of land treatment above) to improve airflow in the pile by increasing the porosity, and possibly a thermal source whose degradation results in an increase in pile temperature. Some materials, such as manure, may serve as both a bulking agent and a thermal source. Water is added as needed to control the moisture content. Three basic composting process designs are used: (1) windrows on an impervious surface, which are aerated by mechanical mixing at regular intervals; (2) static piles on an impervious surface, which are either aerated passively or by a forced-air system; and (3) in-vessel reactors, which are mechanically mixed and sometimes include forced aeration. In low-temperature composting, also known as biopiles or soil heaping, the contaminated soil, possibly mixed with a poorly degraded bulking agent, is placed in a static pile, aerated passively or by forced air, and irrigated to control moisture, pH, and nutrients (Cookson, 1995; Fahnestock et al., 1998).

Slurry-Phase Treatment Slurry-phase treatment is performed under water-saturated conditions (Cookson, 1995; Eweis et al., 1998). The contaminated material is pretreated (often to remove large materials) and mixed with water to form a slurry that can be mixed and aerated, thereby improving contaminant mass transfer, oxygen availability, and biodegradation rates. Other amendments (e.g., nutrients, neutralizing agents, microbial enrichment cultures, surfactants, dispersants) are added as required. Reactors are typically operated in a batch or semibatch mode and may be open or closed. Dewatering is used to separate the treated water and soil, which are treated further, reused, or disposed of.

8.9 CONCLUSIONS

The last several decades have greatly advanced our knowledge of microbiological processes affecting many contaminants and the physiologies of key microorganisms. This body of knowledge continues to grow because of the ever-increasing variety of organic compounds that are released to the environment and the enormous diversity in the microorganisms that can act on these contaminants. Currently, the biochemistry of some contaminants is well understood, and bioremediation of certain contaminants (particularly the BTEX compounds) is considered a mature technology. Nevertheless, major challenges remain before the full potential of bioremediation to detoxify many other important contaminants will be realized. Fundamental research aimed at understanding reaction mechanisms at the molecular level must be continued and paralleled by efforts to improve our ability to predict how the microbial ecology of a site and the presence of other contaminants and stimulatory amendments will affect the biodegradation of individual pollutants. Expanded application of bioremediation in the field will also

require improvements in process engineering. Areas of particular need include methods for determination of the rate-limiting processes, effective delivery of stimulatory materials, and management of contaminant source zones (especially those containing NAPLs). Finally, the selection of site remediation methods is currently based primarily on their capacities to improve local environmental problems. However, the remediation of contaminated sites may have much broader environmental impacts through the consumption of energy and other resources and the potential to transfer contaminants to other phases and/or locations. Thus, the sustainability of various treatment approaches must increasingly be considered when designing a bioremediation strategy.

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CHAPTER 9

Biosensors as Environmental Monitors

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9.1 INTRODUCTION

A biosensor is a two-component analytical device comprised of a biological recognition element that outputs a measurable signal to an interfaced transducer (Figure 9.1). Biorecognition typically relies on enzymes, whole cells, antibodies, or nucleic acids, whereas signal transduction exploits electrochemical (amperometric, chronoamperometric, potentiometric, field-effect transistors, conductometric, capacitative), optical (absorbance, reflectance, luminescence, chemiluminescence, bioluminescence, fluorescence, refractive index, light scattering), piezoelectric (mass sensitive quartz crystal microbalance), magnetic, or thermal (thermistor, pyroelectric) interfaces. This wide selection of interchangeable components has resulted in a similarly wide selection of biosensors focused here toward those related to environmental monitoring.

The detection of specific analytes of importance to environmental monitoring can be achieved with great precision using analytical techniques that center around mass spectrometry (MS), such as gas chromatography (GC)-MS, liquid chromatography (LC)-MS, liquid chromatography coupled to tandem MS (LC-MS^2), ion trap (IT)-MS, and quadrupole linear ion trap (QqLIT)-MS. With great precision, however, comes significant time, effort, and expense. Samples must be collected and transported to the obligatory confinements of the laboratory, and requisite preconcentration and cleanup steps must be performed prior to the sample being analyzed on an expensive, high-technology instrument by accompanying trained technical personnel. Considering that some percentage of the samples collected will be negative, either not being contaminated or containing the target analyte at concentrations too low to be detected, the adjusted cost on a per positive sample basis can be extensive.

Although biosensors cannot unequivocally replace the replicate accuracy and reproducibility of conventional analytical instrumentation, they can complement and supplement their operation through ease of sample preprocessing, which is often minimal to none, on-site field portability, simplicity and rapidity of operation, versatility, real-time to near-real-time monitoring capabilities, and miniaturization that has evolved down to a "lab-on-a-chip" format. Biosensors can therefore often find their niche as continuous monitors of environmental contamination or as bioremediation process monitoring and

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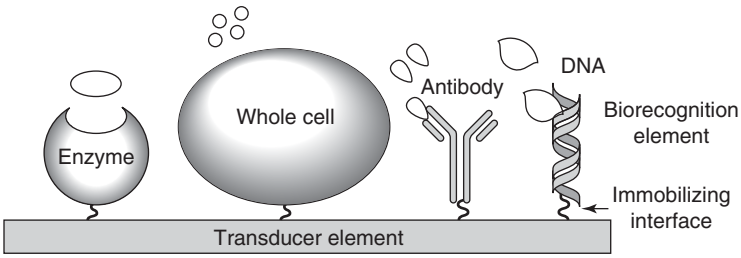

Figure 9.1 Anatomy of a biosensor. The interaction between the target analyte and the biorecognition element creates a signaling event detectable by the interfaced transducer element.

control tools to provide informational data on what contaminants are present, where they are located, and a very sensitive and accurate evaluation of their concentrations in terms of bioavailability. Bioavailability measurements are central to environmental monitoring as well as risk assessment because they indicate the biological effect of the chemical, whether toxic, cytotoxic, genotoxic, mutagenic, carcinogenic, or endocrine disrupting, rather than mere chemical presence as is achieved with analytical instruments. Despite their benefits, biosensors remain relatively unused in the environmental monitoring/bioremediation fields, due primarily to a lack of real-world, real-sample testing and standardization against conventional analytical techniques. Thus, although biosensors show significant promise, it is clear that more field validation studies need to be performed before regulatory agencies and other end users will gain sufficient confidence to adopt their routine use.

9.2 ENZYME-BASED BIOSENSORS

The historical foundation of the biosensor rests with the enzyme glucose oxidase and its immobilization on an oxygen electrode by Leland Clark in the 1960s for blood glucose sensing. Although the research emphasis of enzyme-based biosensors continues to be driven by more lucrative medical diagnostics, there has been a predictable application overlap toward environmental monitoring as well. Enzymes act as organic catalysts, mediating the reactions that convert substrate into product. Since enzymes are highly specific for their particular substrate, the simplest and most selective enzyme-based biosensors merely monitor enzyme activity directly in the presence of the substrate. Perhaps the most relevant examples are the sulfur/sulfate-reducing bacterial cytochrome c_3 reductases that reduce heavy metals. Michel et al. (2003) immobilized cytochrome c_3 on a glassy carbon electrode and monitored its redox activity amperometrically in the presence of chromate [Cr(VI)] with fair sensitivity (lower detection limit of 0.2 mg/L) and rapid response (several minutes) (Figure 9.2). When tested under simulated groundwater conditions, the biosensor did cross-react with several other metal species, albeit at lower sensitivities, and was affected by environmental variables such as pH, temperature, and dissolved oxygen, thus exemplifying certain disadvantages common to enzyme-based biosensors. Similarly operated biosensors for the groundwater contaminant perchlorate using perchlorate reductase as the recognition enzyme (detection limit of 10 μg/L) (Okeke et al., 2007), organophosphate pesticides using parathion hydrolase or organophosphorus hydrolase as recognition enzymes (detection

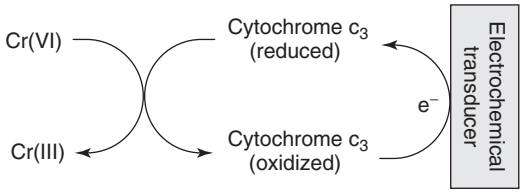

Figure 9.2 Enzymatic biosensor using cytochrome c_3 as the recognition element. Upon exposure to chromate [Cr(VI)], electrode-immobilized cytochrome c_3 reduces Cr(VI) to Cr(III). The current produced by the electrochemical regeneration of reduced cytochrome c_3 is proportional to the amount of oxidized cytochrome c_3 and, therefore, the Cr(VI) concentration.

down to low μM concentrations) (Trojanowicz, 2003), and environmental estrogens using tyrosinase as the recognition enzyme (detection down to 1 μM) (Andreescu and Sadik, 2004) have also been designed.

Another type of enzyme biosensor relies on enzyme activation upon interaction with the target of interest. For example, heavy metals in the form of cofactors—inorganic ions that bind to and activate the enzyme—can be detected based on this integral association. Metalloenzymes such as alkaline phosphatase, ascorbate oxidase, glutamine synthetase, and carbonic anhydrase require association of a metal ion cofactor with their active sites for catalytic activity, and can thus be used as recognition elements for heavy metals. Strong chelating agents are first used to strip the enzyme of all metal ion cofactors to form the inactive apoenzyme. Upon exposure to the sample, the apoenzyme binds any metal ions present and is reactivated, and this rate of reactivation can be related directly to the stoichiometric amount of metal complexed to the enzyme's active site. Alkaline phosphatase, for example, can be applied in this regard as a biosensor for zinc [Zn(II)] or ascorbate oxidase for biosensing copper(II) with detection limits down to very low part-per-billion levels (Satoh and Iijima, 1995). However, as various other metals as well as other sample cross-contaminants can act as cofactors and/or inhibitors of the metalloenzyme, selectivity becomes somewhat problematic. To enhance selectivity, molecular techniques such as site-directed mutagenesis or directed evolution can be used to genetically engineer or select for enzymes with superior specificity for the target, as has been accomplished with carbonic anhydrase and its selective biosensing of Zn(II) (Fierke and Thompson, 2001).

Alternatively, and more commonly, target analytes such as heavy metals can also inhibit enzyme activity, thereby diminishing the conversion of substrate to product. By monitoring subsequent Michaelis–Menten rate kinetics, a highly sensitive measurement of target can be obtained, often at picomolar detection limits, with little prerequisite sample processing. However, selectivity cannot be ascertained since the specificity of enzymes toward inhibitors is not target specific. Thus, inhibitor-based biosensors detect the global presence of heavy metals rather than identifying a particular heavy metal ion in a sample. The standard suite of enzymes used in these biosensors includes oxidases, urease, alkaline phosphatase, choline esterases, and invertase for the detection of arsenic, bismuth, beryllium, zinc, mercury, cadmium, lead, and copper. Note that alkaline phosphatase is also discussed in the preceding paragraph as an enzyme activation type of biosensor for Zn(II), which again exemplifies some of the cross-reactivity problems associated with enzyme-based biosensors. However, such disadvantages can cleverly be taken advantage of, as demonstrated by Kamtekar et al. (1995), to produce biosensors with broadened detection ranges, for example, using the activation feature

of alkaline phosphatase to detect Zn(II) while in parallel monitoring inhibition to detect other metals, although identifying metals separately is still impractical. Cholinesterases (acetylcholinesterase, butyrlcholinesterase) are other well-applied biosensor enzymes geared toward the detection of organophosphoros and carbamate pesticides/insecticides and tyrosinase (polyphenol oxidase) and peroxidase have been extensively applied for phenols. Other environmentally relevant inhibition enzymes include acid phosphatase for the detection of arsenic(V) and protein phosphatase 2A for algal microcystins (both nonselectively). By co-immobilizing several enzymes on the same transducer, biosensors capable of multiplexed sensing can be achieved. Additionally, as with the activated enzymes, the activity of inhibitory enzymes can be enhanced through genetic engineering, as has been accomplished with acetylcholinesterase, where site-directed mutagenesis yielded enzymes with 300-fold more sensitivity to the organophosphate target dichlorvos (Boubik et al., 2002). An extensive list of inhibition-based enzyme biosensors as they apply to environmental monitoring has been provided by Amine et al. (2006).

Biosensors can also incorporate nonenzymatic proteins or peptides as sensory elements. For environmental monitoring, this typically involves metal ion sensing and is accomplished either through the use of naturally occurring or engineered proteins. Metallothionein, for example, is a mammalian-derived metal-binding protein that has been incorporated into an optical biosensor for nondiscriminatory detection of cadmium, zinc, or nickel (Wu and Lin, 2004). Glutathione and phytochelatin proteins are also commonly used. Specificity is broad and interference or inhibition by other metals or sample constituents is problematic but can be partially addressed through genetic engineering to add, subtract, or replace amino acids to acquire improved metal-binding motifs, as has been done with phytochelatin (Bontidean et al., 2003). A review of peptide-based biosensors has been provided by Chow and Gooding (2006).

The most common means for signal transduction in enzyme-based biosensors are electrochemical transducers, as shown in Figure 9.2, where the biological recognition event is converted into an electrical signal and measured as either a current (amperometric) or a potential (potentiometric). Impedance can also be measured, usually as a change in conductivity in the medium as the target and receptor interact. The basic enzyme-based biosensor design relies on the redox nature of the enzyme and its requirement for a natural redox partner such as an electron-transfer protein or a small molecule co-substrate. The catalytic redox reaction occurring upon target substrate addition yields a current that is proportional, after calibration, to the amount of substrate added. Employing an electrochemical working electrode to monitor and measure this current forms the biosensor, with electron transfer between the enzyme and the electrode occurring either directly or through a mediator such as NAD, FAD, or ubiquinone. One of the most critical functional aspects of enzyme-based biosensors is the immobilization of the enzyme and/or mediator on the electrode surface, since this affects enzyme/mediator integrity and ultimately influences biosensor stability, longevity, and sensitivity. Immobilization techniques include adsorption, covalent attachment, entrapment in polymeric matrices such as sol–gels or Langmuir–Blodgett films, or direct cross-linking using polymer networks or antibody/enzyme conjugates. These methods have been expertly reviewed by Lojou and Bianco (2006). The recent integration of redox active carbon–based nanomaterials (nanofibers, nanotubes, nanowires, and nanoparticles) as transducers and their unique ability to interact with biological materials realizes promising advancements in enzyme biosensor design and sensitivity.

Optical transducers (absorption, reflectance, luminescence, chemiluminescence, evanescent wave, surface plasmon resonance) are also commonly employed in enzyme-based biosensors. These can be as simple as optically registering a pH change using a pH reactive dye; for example, bromcresol purple can be immobilized within an acetylcholinesterase-based biosensor to monitor pH changes related to this enzyme's activity upon exposure to pesticides. The hydrolysis of acetylcholinesterase releases protons (H^+), resulting in a decrease in pH, which in turn instigates a decrease in the absorption spectra of bromcresol purple. Andreou et al. (2002) have incorporated such a biosensor successfully on the distal end of a fiber optic cable for facile interrogation of water samples for pesticide residues. The integration of fiber optic cables among optical transducer methodologies is popular due to their ability to transfer signals over long distances, thus permitting sampling in inaccessible areas and remote sampling and monitoring in areas deemed too hazardous or harsh for personnel entry. Luminol is widely used as an electrochemiluminescent indicator, reacting with the acetylcholinestearease/choline oxidase hydrogen peroxide by-product to yield luminescent light signals that have also been used to quantify pesticide concentrations. Numerous other optical indicators/substrates are available [ruthenium(II) complexes, europium(III) complexes, porphyrins, amplex red, prussian white, fluoresceins, phenol red, FITC, etc.] and have been reviewed thoroughly by Borisov and Wolfbeis (2008).

9.3 WHOLE CELL–BASED BIOSENSORS

Whole-cell biosensors consist of two components: a bioreporter strain that functions as the detector of toxicity or a specific pollutant, coupled to a signal transducer that converts the response from the bioreporter to a detectable electric signal. Bioreporters are typically composed of a promoter (responsible for sensing or interacting with the target analyte) fused to a reporter gene (responsible for generation of the detectable biological signal) encoded on a plasmid or genetic construct, which is then transformed into a cell (Figure 9.3). The combination of a bioreporter with appropriate sensing technology in an integrated biosensor has great potential for environmental contaminant detection, with a multitude of possible pollutants monitored by various biosensors, depending on the reporter gene, promoter element, and detection method chosen.

Many types of reporter genes are employed in chemical detection, the most common being those that use luciferase (*luc* or *lux*) or green fluorescence protein (*gfp* and its derivatives) genes. The *luc* genes were first isolated from *Photinus pyralis* (firefly) and encode for luciferase, which catalyzes the two-step conversion of D-luciferin (which must be added to the reaction) to oxyluciferin, with light emission at 560 nm. Similarly, *lux* genes have been isolated from several bacterial sources (e.g., *Vibrio* spp. and *Photorhabdus luminescens*), all of which are encoded on a single operon in the order *luxCDABE*. Within this operon, *luxAB* encodes for the luciferase enzyme, which converts a long-chain aldehyde substrate (encoded by *luxCDE*) and a reduced flavin mononucleotide ($FMNH_2$) to FMN and a long-chain carboxylic acid, with light production at 490 nm. Biosensors that utilize the entire *luxCDABE* operon are self-contained, in that there is no need for substrate addition, although production and recycling of the long-chain aldehyde substrate requires energy expenditure by the cell. However, for biosensors that use *luxAB*, without *luxCDE*, the aldehyde substrate must be continually added to the reaction medium (as is also the case for *luc*-based luminescence),

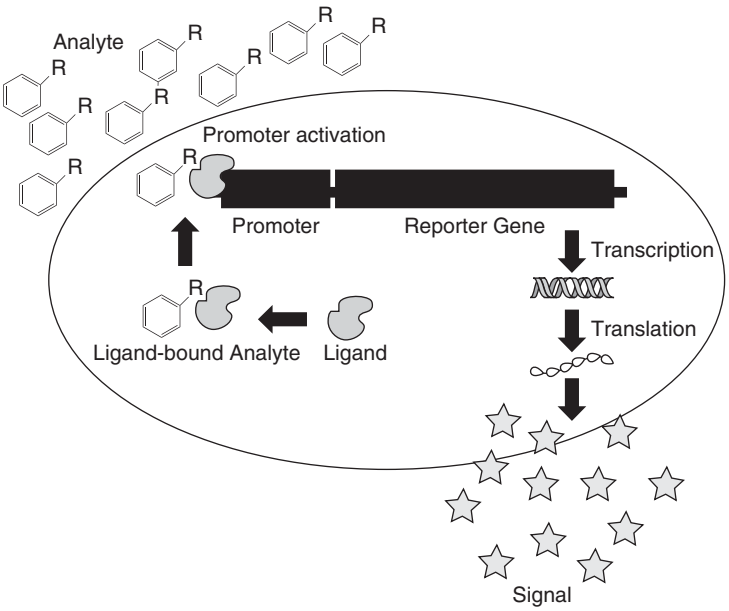

Figure 9.3 Bioreporter for the detection of environmental contaminants. The interaction of a target analyte with a regulatory ligand activates transcription of a reporter gene that ultimately yields a measurable signal. Genes for production of the regulatory ligand may be found on the chromosome or on the signal-producing genetic construct. The signal, monitored by an interfaced detector, is proportional to the amount of target chemical present, permitting analyte-specific detection from aluminum to zinc.

complicating their use in integrated biosensors. Bioreporters that use the green fluorescent protein (*gfp*) gene, isolated from the jellyfish *Aequorea victoria*, do not require substrates and encode a 238-amino acid protein that becomes activated by cyclization of a tyrosine (^{66}Tyr) residue. The protein fluoresces at about 508 nm upon excitation with ultraviolet or blue light. Derivatives of green fluorescent protein that have altered emission maxima are several, including blue-, red-, and yellow-shifted variants, allowing for detection of multiple contaminants simultaneously. Other fluorescent proteins, including red fluorescent protein (termed DsRed) with fluorescence emission at 583 nm, are also utilized; however, all of the fluorescence-based bioreporters require an external light source for excitation of the fluorescent protein product, also complicating their use as biosensors. Each reporter system has different light emission maxima, optimum temperature, and length of signal generation. For example, firefly luciferase (*luc*) has a temperature optimum at 25°C and is thermally inactivated above 30°C, whereas bacterial luciferase derived from *P. luminescens* (*lux*) is stable up to 42°C. Several excellent reviews on the use of *luc, luxAB, luxCDABE, gfp*, and *gfp*-derived reporter genes in bioreporters and biosensors are available (Hakkila et al., 2002; Keane et al., 2002; Purohit, 2003).

For contaminant detection, fusion of a reporter gene to an appropriate promoter is of critical importance because the promoter (or response element) is the portion of the biosensor that is actually sensing or interacting with the chemical contaminant. A multitude of promoters have been used, creating a range of bioreporters that may detect contaminants as general as anything that causes toxicity or as specific as those

that detect a single contaminant. There are numerous examples of bioreporters that detect toxicity, most of which contain a reporter gene fused to a strong constitutive promoter such that a decrease in signal indicates a toxic response. Bhattacharyya et al. (2005) utilized two such bacterial bioreporters (bioluminescent *Pseudomonas putida* and *Escherichia coli*), along with a naturally bioluminescent *Vibrio fischeri* (recently reclassified as *Aliivibrio fischeri*), to identify areas of a contaminated groundwater site in southern England that contained toxic substances. They also used two "catabolic" bioreporter strains for monitoring trichloroethylene (TCE) and compared data from all of these strains to chemical data, with the goal of gathering data to assess the bioavailability of contaminants in the groundwater. The three bacterial toxicity bioreporters differed in their assessment of the toxic potential for the samples, attributed to each strain being more or less sensitive to the primary pollutants in each sample, underscoring the benefit of using multiple bioreporters within biosensors.

A classic example of a functional whole-cell biosensor being used on environmental samples is the Microtox system, which utilizes a naturally bioluminescent *V. fischeri* for the detection of toxicity. This strain, which produces light constitutively, exhibits a decrease in bioluminescence proportional to the level of toxicity in the water sample. It has been tested with numerous classes of contaminants, including heavy metals, pesticides, chlorinated solvents, and many others. However, since this assay is based on a marine bacterium, it is less than ideal for toxicity testing of soil or freshwater samples. In addition to toxicity-sensing bacterial bioreporters, there are also yeast-based toxicity bioreporters, such as those constructed in *Saccharomyces cerevisiae* (Eldridge et al., 2007; Leskinen et al., 2005; Sanseverino et al., 2005). The bioreporter of Leskinen et al. (2005) contains a fusion of the GPD constitutive promoter to the *luc* gene, while that of Sanseverino et al. (2005) and Eldridge et al. (2007) contain a fusion of the bidirectional promoters GPD/ADH1 to the *luxCDABE* operon. Each of these reporters functions to detect chemical toxicity by measuring a decrease in bioluminescence and is used for sensitive determination of endocrine-disrupting potential. Endocrine-disrupting chemicals (EDCs; e.g., pesticides, plasticizers, pharmaceuticals, and synthetic hormones) have been recognized as waterborne contaminants that may cause reproductive dysfunction in humans and wildlife. Kolpin et al. (2002) performed a study in which they found low concentrations of EDCs in 40% of 139 U.S. streams tested.

The *S. cerevisiae*–based EDC bioreporter of Sanseverino et al. (2005), referred to as strain BLYES (Figure 9.4), contains two plasmids, pUTK404, from which the *P. luminescens* aldehyde synthesis genes (*luxCDE*) and the *Vibrio harveyi* flavin oxidoreductase gene (*frp*) are expressed from the yeast constitutive promoters GPD and ADH1, and pUTK407, from which the *P. luminescens* luciferase genes (*luxAB*) are expressed under the control of estrogen response elements (EREs). The human estrogen receptor (hER) is cloned into the yeast genome. When this strain encounters an estrogenic agent, the chemical is bound by the human estrogen receptor protein, forming a complex that binds to the EREs on pUTK407, thereby initiating transcription of the *luxAB* genes. This genetic expression, in concert with the *luxCDE* genes expressed from pUTK404, yields a quantifiable light signal, allowing users to discern the estrogen concentration in samples. 17β-Estradiol, for example, was detectable at a lower limit of 4.5×10^{-11} M within an assay time frame of less than 4 hours—significantly faster than the three days required using the widely applied colorimetric-based yeast estrogen screen (YES) assay (Routledge and Sumpter, 1996). The reporter of Eldridge et al. (2007), *S. cerevisiae* BLYAS, is an analogous assay used to measure androgen

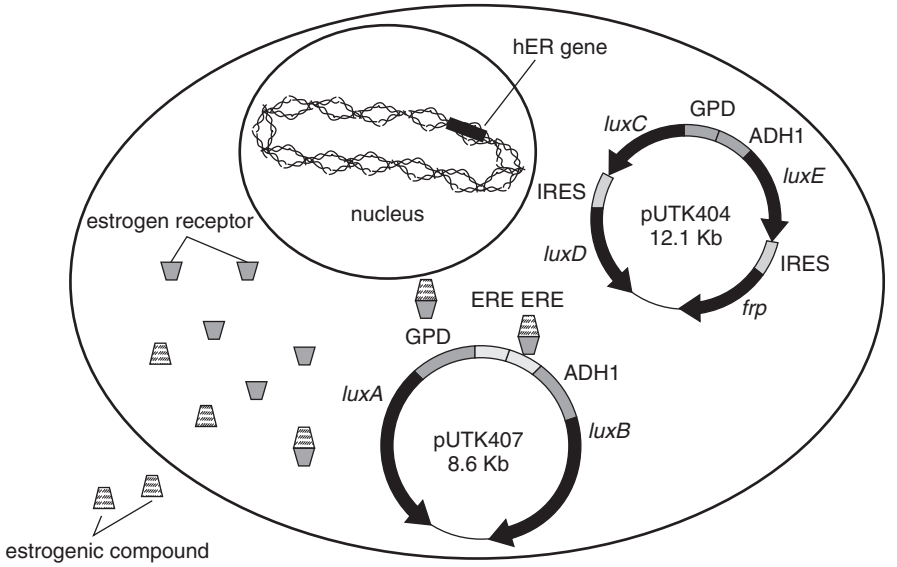

Figure 9.4 The *Saccharomcyes cerevisiae* BLYES *luxCDABE* bioreporter contains the human estrogen receptor (hER) gene on its genome and the plasmids pUTK404 and pUTK407, which enables the production of bioluminescence in the presence of estrogenic chemicals. (From Sanseverino et al., 2005, with permission.)

class chemicals and has been shown to detect, for example, testosterone within 4 hours at a lower detection limit of 2.5×10^{-10} M. Another *S. cerevisiae* bioreporter, strain BLYR, generates bioluminescence constitutively for measurement of general sample toxicity (Eldridge et al., 2007). When strains BLYES, BLYAS, and BLYR are combined into one assay, this suite of bioreporters can be used to detect substances that are potential endocrine disruptors in environmental samples in near real time, without the addition of exogenous substrates or cell permeabilization steps (Figure 9.5). Further, the ability to perform these assays fairly rapidly in multiwell microtiter plates permits the high-throughput screening necessary to test the estimated 87,000 existing and new chemicals mandated by the U.S. Environmental Protection Agency.

The goal of whole-cell biosensor development for bioremediation is the creation of a fully functional, reagentless biosensor that may easily be used in the environment based on sensitive detection by bioreporters that are stable over a long period of time with little decrease in performance. Sagi et al. (2003) have compared *gfp*-based bioreporters to *lux*-based bioreporters (*luxCDABE*), each of which was fused to two different promoters, *recA* and *grpE*, yielding *E. coli* bioreporters that respond to DNA-damaging agents and generally toxic agents, respectively. They demonstrated that *lux*-based bioreporters responded much more rapidly and were more sensitive to a model SOS inducer nalidixic acid in the short term, but that *gfp*-based bioreporters were nearly as sensitive if incubation times were increased to greater than 4 hours. Genotoxicity-sensing bioluminescent bioreporters (*recA-luxCDABE*) experienced an increase in signal intensity after only 30 minutes, whereas fluorescent bioreporters (*recA-gfp*) required at least 60 minutes, and in some cases 100 minutes prior to increased signal intensity. In addition, after 90 minutes, bioluminescent bioreporters responded to nalidixic acid concentrations as low as 0.2 mg/L, while after 200 minutes, fluorescent bioreporters had a "detection threshold"

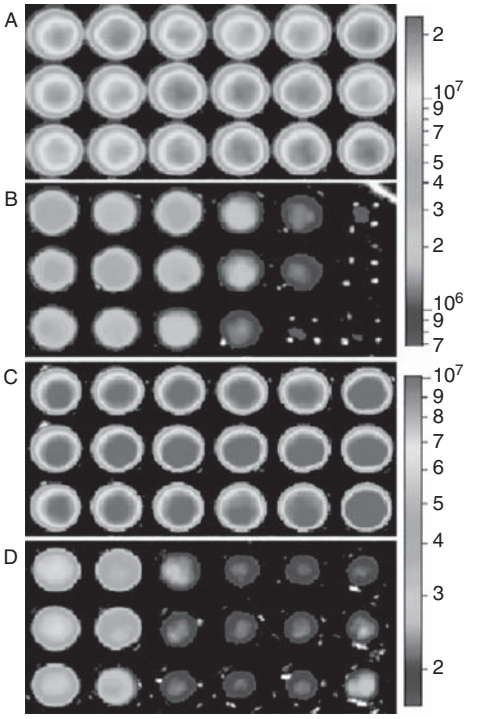

Figure 9.5 *S. cerevisiae luxCDABE* bioreporter assay in a high-throughput 96-well microtiter plate format imaged in real time with a Xenogen IVIS Lumina imaging system. Bioluminescence emission (as photon counts per second) by the bioreporters BLYR (A) and BLYES (B) in response to a range of 17β-estradiol concentrations, and bioreporters BLYR (C) and BLYAS (D) after exposure to a range of dihydrotestosterone concentrations. Each panel shows an 18-well exposure profile ranging from 1μM (top, left well) to 2.5 pM (bottom, right well) (1 : 10 dilutions were performed across each row and 1:2 dilutions down each column). Quantification of bioluminescence output from each well permits formulation of a standard curve and determination of estrogenic or androgenic equivalents present in the sample. (See insert for color representation.)

of 0.5 mg/L. However, they also determined that the products of *gfp* bioreporters, fluorescent proteins, were much more stable over time, remaining stable for greater than 7 hours. Two of these bioreporter strains (*E. coli* with *grpE-luxCDABE* and *E. coli* with *recA-gfp*, for bioluminescent toxicity and fluorescent genotoxicity detection, respectively) were immobilized together in a sol–gel matrix combining the advantages of both types of bioreporters in one assay. They reported that when immobilized separately, the two strains functioned similarly to their nonimmobilized counterparts, with the exception that the fluorescent bioreporter strain seemed to respond more quickly than demonstrated previously. Moreover, when immobilized together, the bioreporter strains had no interference with each other, demonstrating that in the future, arrays of bioreporters may be combined into whole-cell biosensors that detect a wide range of pollutants.

Bioreporters that detect specific contaminants are being used increasingly in environmental studies and have the distinct advantage over chemical measurements of assessing the bioavailability of chemical contaminants rather than just their presence. Keane et al. (2002) have reviewed a number of bioreporters for

the detection of environmental contaminants, including sensors for naphthalene, benzene–toluene–ethylbenzene–xylene isomers (BTEX), phenols and chlorinated compounds, and alkanes. They describe bioreporters that use *luc, luxAB, luxCDABE*, and *gfp* reporter genes fused to promoters that respond to a target chemical, some of which are immobilized for potential use in biosensors. For example, to monitor the herbicide 2,4-dinitrophenoxyacetic acid (2,4-D), Hay et al. (2000) fused a promoter ($tfdD_{II}$) required for the metabolism of 2,4-D to the *luxCDABE* operon and integrated this construct into the genome of *Ralstonia eutropha* with the detection of 2,4-D to below 2 μM in aqueous solutions and soil slurries. Subsequently, Toba and Hay (2005) have fixed this strain onto glass microfiber filter disks and incubated it in 2,4-D-amended soils. Disks were incubated in soils for 60 minutes and then placed into microtiter plates where bioluminescence was read. Immobilization onto glass microfiber disks resulted in decreased but detectable bioluminescence from the bioreporter; however, they report a strong correlation between soil 2,4-D concentration and the bioluminescence from the fixed bioreporter ($r^2 = 0.98$).

Verma and Singh (2005) have reviewed potential biosensors for heavy metal detection. As discussed, heavy metal contamination in the environment is of great concern because metals are recalcitrant and toxic in small concentration; therefore, monitoring for the presence of the most common metal contaminants—lead, chromium, cadmium, copper, zinc, and mercury—is necessary. They described numerous types of immobilized bioreporters, including that of Corbisier et al. (1999) for copper monitoring, which consists of *Alicaligenes eutropha* (also known as *Ralstonia eutropha*), with a fusion of the *copSRA* promoter to *luxCDABE*, suspended in two matrices for comparison, sodium alginate beads and seaplaque agarose. They determined that cells immobilized within agarose performed similarly to cells in solution, but bioluminescence from the reporters in all of the matrices tested had unacceptable detection limits. Leth et al. (2002) have coupled this bioreporter to fiber optics in both matrices and determined that detection of bioluminescence was possible but occurred in the μM range of Cu^{2+}. Obvious potential for the use of this type of biosensor to determine the bioavailable fraction of heavy metals in the environment exists if detection limits are brought within environmentally relevant values.

Whole-cell biosensors may even be used to assess the remediative potential of a population at a contaminated site. To date, the only field-deployed whole-cell biosensor for bioremediation has been *P. fluorescens* HK44 genetically modified with *luxCDABE* fused in a naphthalene degradation pathway, so that breakdown of polycyclic aromatic hydrocarbons could be monitored in field lysimeters that had been contaminated with naphthalene, anthracene, and phenanthrene (Ripp et al., 2000). This biosensor contained an alginate-immobilized *P. fluorescens* bioreporter (HK44) whose bioluminescence was detected via fiber optic cables and a portable photomultiplier tube in response to degradation of pollutants. Despite an initial reduction in cell numbers, bioremediation by this strain was detected successfully in soil samples as bioluminescence in response to the presence of volatile organic compounds in contaminated soils. HK44 cells were present in soils for more than 444 days, with detectable bioluminescence in biosensors after further pollutant exposure, demonstrating the long-term survivability of this biosensor for the bioremediation of polycyclic aromatic hydrocarbons.

Biosensors that use reporter elements other than fluorescence or bioluminescence exist. Banik et al. (2008) have developed a whole-cell biosensor based on a *Pseudomonas* sp., which is attached to a Clark type dissolved oxygen electrode, to measure

the change in oxygen concentration in response to degradation of *p*-nitrophenol (a highly toxic product of industrial processes that is a common environmental contaminant). The *Pseudomonas* strain chosen was shown to adapt to high concentrations of pollutant (up to 0.69 mM *p*-nitrophenol) and to be stable across relatively broad pH and temperature ranges (optimally 7.5 to 8.5 and 25 to 35°C, respectively, but *p*-nitrophenol degradation was detected above 35°C, with reduced signal intensity). This biosensor, which has a linear response range of 10 to 40 μM *p*-nitrophenol within 7 minutes of exposure, represents a quick and easy whole-cell biosensor that may be used in the future environmentally under a range of conditions for the detection of a highly toxic environmental contaminant. In addition, biosensors exist for the detection of biodegradation of organic contaminants. As reviewed by Lei et al. (2006), numerous strains (some singly and some as mixtures) have been utilized to measure biological oxygen demand (BOD) as an indicator of the presence of organic pollutants. Kumlanghan et al. (2008) have used a population of microorganisms, isolated from activated sludge, and immobilized in Ca-alginate, coupled to a Clark-type oxygen electrode, to create a biosensor for monitoring concentrated wastewater samples from a factory processing concentrated rubber latex. They used this biosensor for both off-line and online monitoring of wastewater from anaerobic reactor samples and compared BOD data to BOD_5 methods (the standard for BOD determination). They have determined that the biosensor measured BOD similar to measurement by BOD_5 methods, but was much faster (nearly immediately versus 5 days) and that there was good agreement between off-line and online measurements with the biosensor ($r^2 = 0.9989$). Although the authors state that the online biosensor may only be useful for the measurement of BOD from samples without high concentrations of organic pollutants (less than 300 mg/L BOD), this study demonstrates the potential utility of biosensors for continuous monitoring of wastewater treatment effluents.

Although only one fully integrated whole-cell biosensor has been deployed into the field for remote sensing of pollutants during bioremediation (Ripp et al., 2000), the future of environmental monitoring by whole-cell biosensors has great potential for the sensitive detection of contaminants so that target areas for bioremediation may be identified.

9.4 ANTIBODY-BASED BIOSENSORS (IMMUNOSENSORS)

Biosensors that use antibodies as recognition elements (immunosensors) are used widely as environmental monitors because antibodies are highly specific, versatile, and bind stably and strongly to target analytes (antigens). This high affinity for target, however, can also be disadvantageous since the target cannot easily be released from the antibody after the measurement has been made, resulting in many antibody-based biosensors being single-use disposable units [although release (regeneration) can be promoted by the addition of organic solvents or chaotrophic reagents, this requirement for a supplementary assay step is not optimal]. Additionally, the synthesis of antibodies and further testing and optimization of their target affinities can be a long, tedious, and expensive process, cross-reactivity with multiple analytes can occur, and antibody/antigen reactivity can be affected by environmental variables such as pH and temperature (see Hock, 2000 for a review). Nonetheless, antibodies can be highly effective detectors for environmental contaminants, and advancements in techniques such as phage display for the preparation and selection of recombinant antibodies with novel

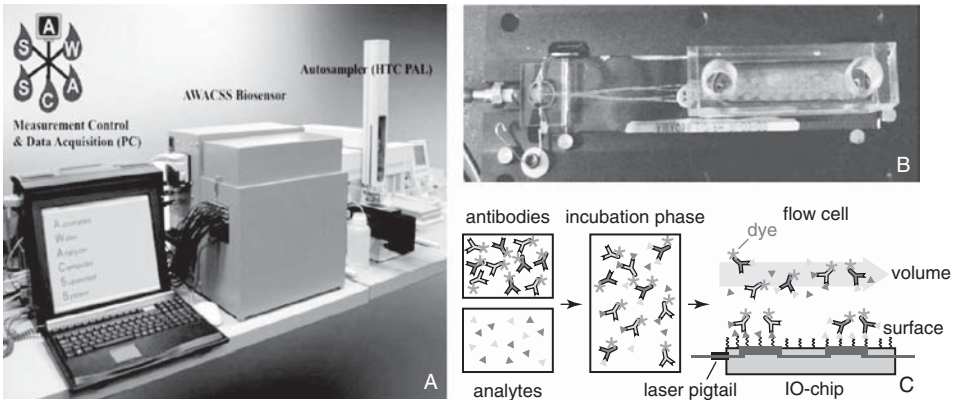

Figure 9.6 The AWACSS immunosensor (A) is capable of simultaneous multianalyte detection of up to 32 different target contaminants. Its detection methodology uses a semiconductor laser flow cell (B) to excite fluorophore-tagged antibody/target analyte complexes bound to the surface of a multisensor optical waveguide chip (C). (From Tschmelak et al., 2005, with permission.) (See insert for color representation.)

binding properties assures their continued environmental application. Perhaps the best introduction to antibody-based biosensing is the AWACSS (Automated Water Analyzer Computer Supported System) environmental monitoring system developed for remote, unattended, and continuous detection of organic pollutants for water quality control [and also its predecessor, the RIANA (River Analyzer)] (Figure 9.6) (Tschmelak et al., 2005). AWACSS uses an optical evanescent wave transducer and fluorescently labeled polyclonal antibodies for multiplexed detection of targeted groups of contaminants, including endocrine disruptors, pesticides, industrial chemicals, pharmaceuticals, and other priority pollutants, without requisite sample preprocessing. Antibody binding to a target sample analyte occurs in a short 5-minute preincubation step, followed by microfluidic pumping of the sample over the transducer element, which consists of an optical waveguide chip impregnated with 32 separate wells of immobilized antigen derivatives. As the antibody/analyte complexes flow through these wells, only antibodies with free binding sites can attach to the well surface (in what is referred to as a binding inhibition assay). Thus, antibodies with both of their binding sites bound with analyte will not attach to the surface and will pass through the detector. A semiconductor laser then excites the fluorophore label of bound antibodies, allowing for their quantification, with high fluorescence signals indicating low analyte concentrations and low fluorescence signals indicating high analyte concentrations. A fiber optic array tied to each well permits separation and identification of signals by the well, thereby yielding a simultaneous measurement of up to 32 different sample contaminants. The instrument has been used for groundwater, wastewater, surface water, and sediment sample testing with detection limits for most analytes in the ng/L range within assay times of approximately 18 minutes. Glass et al. (2004) have designed a similar but less refined benchtop flow-through immunosensor (KinExA) demonstrated to detect analytes successively based on a replaceable flow cell containing fluorescently labeled antibody. Their time of assay was approximately 26 minutes, with detection limits at picomolar concentrations. Another portable binding inhibition immunosensor

has been reported by Mauriz et al. (2007) for the detection of pesticides in river water, with an analysis time of approximately 20 minutes and sensitivity down to ng/L levels.

Although not as elaborate as the AWACSS, a multitude of other antibody-based biosensors have been applied as environmental monitors, traditionally serving as biosensors for pesticides and herbicides, but their target analytes have broadened considerably over the past several years to include heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), explosives (TNT and RDX), phenols, toxins such as microcystin, pharmaceutical compounds, and endocrine disruptors (see Farré et al., 2007 for a comprehensive review). Detection at part-per-billion or lower concentrations is the norm. For the most part, immunosensors use electrochemical (amperometric, potentiometric, conductimetric) or optical (evanescent wave, surface plasmon resonance, total internal reflection fluorescence, etc.) transducers to detect antibody/analyte binding. Either the antibody, the analyte, or an analyte derivative complementary to the antibody can be immobilized on the transducer surface, and various binding assays can be used to directly or indirectly monitor analyte concentration, typically using one of four assays: direct, competitive, binding inhibition, or sandwich. Direct binding assays employ a pre- or naturally labeled antigen/analyte that is detectable when bound directly to an immobilized antibody, and is applied more for clinical than for environmental monitoring. Competitive binding assays rely on the competition for a limited number of antibody binding sites between an immobilized or labeled analyte derivative and the target analyte. In binding inhibition assays, as shown in Figure 9.6C for the AWACSS biosensor, a labeled antibody and its complementary analyte are first incubated together and then exposed to immobilized analyte derivative, which binds up unbound antibodies, thus yielding an inverse analyte signal. In sandwich assays, the antibody and antigen are incubated together and then a secondary labeled antibody is added which recognizes another binding site on the analyte. Measurement of this label correlates to analyte concentration. Most environmental immunosensors use competitive or binding inhibition assays in conjunction with an optical label for ease of monitoring. Optical labels are usually fluorescent or chemiluminescent, or may even consist of quantum dots in newer biosensor applications. Examples of optical as well as electrochemical and other immunosensor formats and their analyte sensing capabilities have been well reviewed (Farré et al., 2007; Gonzalez-Martinez et al., 2007).

9.5 DNA-BASED BIOSENSORS

The foundation of nucleic acid–based biosensors relies on a transducer capable of monitoring a change in the nucleic acid's structure occurring after exposure to a target chemical. These structural changes are brought on either by the mutagenic nature of the chemical, resulting in mutations, intercalations, and/or strand breaks, or by the chemical's ability to covalently or noncovalently attach to the nucleic acid. Immobilizing the nucleic acid as a recognition layer on the transducer surface forms the biosensor, and detection of the chemically induced nucleic acid conformational change is then typically achieved electrochemically (i.e., a change in the current) or less so through optical or other means (see Fojta, 2002 for an excellent review).

Nucleic acid biosensors are generally nonselective and provide an overall indication of a potentially harmful (genotoxic, carcinogenic, cytotoxic) chemical or chemical

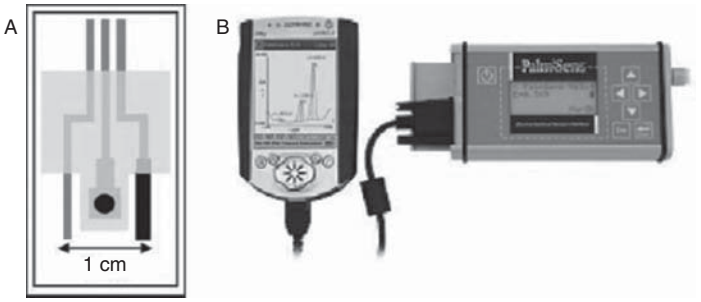

Figure 9.7 The screen-printed electrode (A) consists of three electrodes: a graphite working electrode in the center onto which the DNA is immobilized, and a silver reference electrode and graphite counter electrode on either side. The handheld potentiostat (B) on the right monitors variations in the electrochemical properties of the DNA, such as those occurring upon mutagenic chemical exposure. (From Lucarelli et al., 2003, with permission.) (See insert for color representation.)

mix in the test environment and, depending on the biosensor format, an estimate of concentration. A conventional example is illustrated in work by Bagni et al. (2005), where a DNA biosensor was used to screen soil samples for genotoxic compounds, using benzene, naphthalene, and anthracene derivates as model targets. Double-stranded DNA was immobilized on a single-use disposable screen-printed electrochemical cell operating off a handheld battery-powered potentiostat (see Sassolas et al. (2008) for a methodological review) (Figure 9.7). A 10-μL drop of a preprocessed and preextracted contaminated soil sample was placed onto the working electrode for 2 minutes, and resulting electrochemical scans, based on the chemical's propensity to oxidize DNA guanine residues, were measured. (Adenine moieties can be similarly redox reactive.) The magnitude of these "guanine peaks" in relation to a reference electrode was linearly related to their concentration in solution (i.e., the higher the concentration of the target chemical, the more damage is imposed on the DNA, and the lower the electrochemical measurement of the oxidation signal). In a very clever application of this DNA biosensor, the authors also applied it to the detection of PAHs in fish bile, using the accumulation of PAH compounds in live fish to monitor for water contamination events (Lucarelli et al., 2003). Hydrazine and aromatic amine compounds in fresh and groundwater, hydroxyl radicals in uranium mine drainage waters, herbicides such as atrazine, general toxicity events in wastewaters, industrially contaminated soils, and various other environmental sources have all been screened using DNA biosensors. Comparative studies in wastewater samples against more widely used whole-cell genotoxic bioluminescence inhibition biosensors (Toxalert) have indicated adequate correlations, but more substantial data will probably be required before the user community would consider replacing standard bioluminescent toxicity assays with DNA biosensors. However, the rapidity (only a few minutes to detect but sample processing is often necessary), sensitivity (typically down to low part-per-billion levels), ease of use, and cost-effectiveness of genotoxic DNA biosensors does hold great potential for screening environmental sites for toxic chemical intrusions or monitoring operational endpoints of bioremediation efforts.

Metals are also relevant detection targets, due to their various affinities for nucleic acid. Lead, cadmium, nickel, arsenic, copper, iron, chromium, and others have been detected through DNA biosensing, incorporating both single- and double-stranded

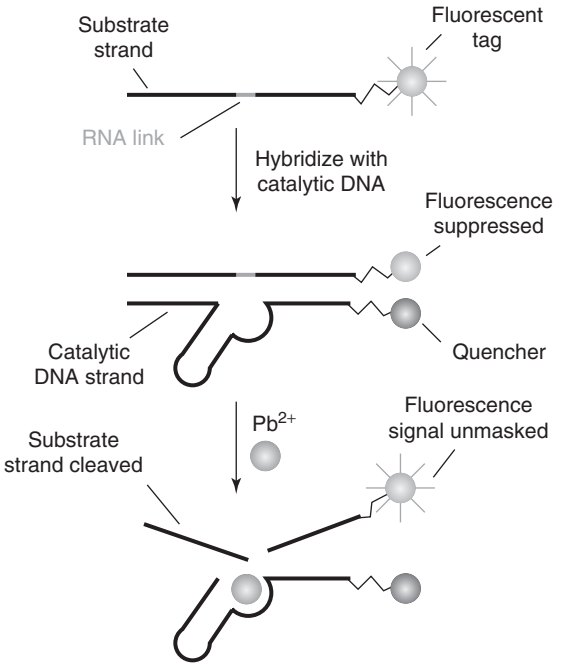

Figure 9.8 Catalytic beacon fluorosensor for lead (Pb^{2+}). In its uninduced state, fluorescence is suppressed due to close quencher proximity. Upon exposure to lead, the substrate strand is cleaved, thereby disassociating the fluorescent tag from the quencher to yield a quantifiable fluorescent signal. (From Borman et al., 2000, with permission.) (See insert for color representation.)

DNA as the sensing element, but again, nonselectively. Selectivity, though, has been demonstrated by several groups using deoxyribozymes (DNAzymes) or ribozymes (RNAzymes). These engineered catalytic oligonucleotides can mediate nucleic acid cleavages or ligation, phosphorylation, or other reactions. For example, a DNAzyme biosensor for lead uses a single-stranded DNAzyme absorbed to a gold electrode (Xiao et al., 2007). The DNAzyme incorporates a methylene blue tag at its terminus that is held distant from the electrode by pairing of a complementary oligonucleotide to the DNAzyme to maintain rigidity. Upon exposure to lead at concentrations as low as 62 ppb, the DNAzyme strand is cleaved, allowing the methylene blue tag to approach the transducer and transfer electrons, thereby instigating an electrochemical signal. A similar strategy employing a fluorophore-labeled DNAzyme and quencher-labeled substrate strand (thus mimicking the molecular beacon real-time polymerase chain reaction (PCR) amplification strategy where dissociation of the fluorophore away from the quencher results in optical emission) yielded a lead-specific "catalytic beacon" fluorosensor with nanomolar sensitivity that has been tested successfully in artificially contaminated fresh water (Lu et al., 2003) (Figure 9.8). Similar catalytic beacons for mercury, uranium, and copper are also available, with many more targets surely to come. A colorimetric DNAzyme-based biosensor for lead has also been demonstrated (Wei et al., 2008).

Nucleic acid can be manipulated similarly to create target-specific aptamers using a process called SELEX (systematic evolution of ligands by exponential enrichment).

By iteratively incubating nucleic acid with the desired target, one can select for oligonucleotide sequences (or aptamers) with the greatest affinity for the target. Kim et al. (2007) used SELEX to create an aptamer specific for 17β-estradiol and used it in an electrochemical biosensor (or aptasensor) to achieve detection of this important endocrine disruptor at levels as low as 0.1 nM. Predominant aptasensor development and application is in the clinical fields, but it is slowly and inevitably encroaching upon environmental sensing. An aptasensor for the cyanobacterial toxin microcystin (lower detection limit of 50 μg/mL) (Nakamura et al., 2001) and another for zinc based on fluorophore beacons (lower detection limit of 5 μM) (Rajendran and Ellington, 2008) have been reported.

9.6 BIOMEMS, BIOMIMETICS, AND OTHER EMERGING BIOSENSOR TECHNOLOGIES

BioMEMs and biomimetics clearly represent the rapidly approaching future of the biosensor. BioMEMs (biological microelectromechanical systems) are an assortment of biomicro, bionanotechnological, and microfluidic interfaces that form lab-on-a-chip, biochip, or micro-total analysis system (μTAS) biosensors. Their objectives are toward miniaturization, portability, redundancy, and a reduction in sample size, time of response, and cost. The majority of these biosensors serve biomedical rather than environmental causes, but they are slowly and inevitably being adapted for the environmental monitoring community. BioMEMs most often utilize optical transducers interfaced with enzyme, whole-cell, antibody, or nucleic acid–type receptors. Several recent examples should illustrate their various design and performance characteristics. Yakovleva et al. (2003) developed a microfluidic immunosensor flow cell for the detection of atrazine in surface water. Chemiluminescently labeled antibodies directed against atrazine were combined with artificially contaminated river water and microfluidically pumped at 40 to 50 μL/minute through a 42-channel 13 mm $\times$ 3 mm silicon microchip containing a functionalized antibody affinity-capture surface. Upon antibody/atrazine capture, a luminol substrate was added to mediate the chemiluminescent reaction which was monitored with a photomultiplier tube (PMT) suspended above the microchip. Although detection performance was hindered in crude river water samples due to matrix effects, atrazine detection down to the lowest contaminant level of 0.5 μg/L could be achieved in pre-diluted water samples within 10 minutes. Islam et al. (2007) have further improved on this sensing strategy by essentially integrating the PMT directly on the microchip flow cell to create a truly miniaturized biosensor referred to as a BBIC (bioluminescent bioreporter integrated circuit) (Figure 9.9). This 1.5 mm $\times$ 1.5 mm CMOS microluminometer was designed to capture and process bioluminescent signals emanating from immobilized whole-cell bioluminescent bioreporter bacteria. The BBIC converts the bioluminescently derived photodiode current into a digital signal, the frequency of which is proportional to the concentration of pollutant to which the bioreporter has been exposed. In water artificially contaminated with salicylate as a model pollutant, the flow-through BBIC responded within 30 minutes to part-per-billion concentrations. With its capacity to accommodate any of the many differently analyte-specific bioluminescent bioreporters, multiplexed sensing is well within its functional repertoire. Chang et al. (2005) developed a microfabricated lead (Pb^{2+}) microchip based on a functionalized molecular beacon DNAzyme integrated within a three-dimensional nano/microfluidic flow cell. Picoliter sample

Figure 9.9 BBIC microluminometer, here incorporated into a handheld probe; permits unattended monitoring of bioluminescence from adhered whole-cell *lux*-based bioreporters.

volumes are flowed through nanocapillary array interconnects 50 μm wide ×30 μm deep ×14 mm long. Lead present in the sample causes DNAzyme cleavage resulting in dissociation of the fluorophore away from the quencher, with the ensuing signal being detected via laser-induced fluorescence. A detection limit of 11 nM was achieved in a complex mixture of industrial electroplating sludge. As with the BBIC biochip, these DNAzyme microchips can be plug-and-play, being capable of accommodating any user-selectable DNAzyme based on current analyte monitoring needs.

BioMEMs also include microcantilever-based biosensors that translate a molecular recognition event into nanomechanical motion that is measured by induced bending in a microfabricated cantilever, similar on a macroscale to identifying a person on a diving board based on the deflection of the diving board by their weight. Optical or piezo-resistive transducers usually measure microcantilever deflections at nanometer-to-subnanometer ranges of motion, and due to their small size, several microcantilevers can be accommodated per transducer for multianalyte sensing. Alvarez et al. (2003) immobilized antibodies to the pesticide DDT on a microcantilever and demonstrated real-time detection at nanomolar concentrations. Dutta et al. (2007) functionalized a microcantilever with receptor proteins (estrogen receptor α and β) for endocrine-disrupting chemicals for sensing at nanomolar concentrations and demonstrated an immunosensor microcantilever assay for 17β-estradiol with similar detection limits. This group has also developed microcantilevers for metal ion sensing.

Biomimetics mimic the attributes of naturally occurring biological materials to synthetically recreate or enhance their properties. Molecularly imprinted polymers (MIPs) are one such example, and their application in biosensors (or more appropriately, in sensors, since the "bio" element has been removed) can deliver more robust, stable, and target-specific receptors. MIPs are essentially created by mixing the target analyte (or template) with a monomer. The monomers self-assemble around the template, and subsequent removal of the template leaves behind a highly cross-linked polymeric matrix containing cavities that fit "lock-and-key" with the template. Resulting MIPs then serve as analyte-specific synthetic receptors (or artificial antibodies or enzymes) that can be associated with transducers to form sensors. Dickert et al. (2000) synthesized MIP receptors for various PAH constituents, optically interrogated them with

a fluorescent sensor, and demonstrated detection of individual PAHs such as pyrene down to ng/L concentrations in artificially contaminated drinking water. More recently, Xie et al. (2008) molecularly imprinted the explosive 2,4,6-trinitrotoluene (TNT) onto the walls of silica nanotubes, thus implying an impending future for MIP nanosensors with exceedingly faster response times and increased sensitivity. Other environmentally relevant MIP sensors have been designed for various herbicides/pesticides (2,4-D, atrazine, phenylureas, CAT, DDT), aquatic toxins such as microcystin and domoic acid, and various heavy metals, with incorporation into a variety of optical, electrochemical, or piezoelectric transducer elements. See Ye and Mosbach (2008) for a review.

Biosensors based on the use of whole animals or their organs represent a very unique mode of sensing. Insect antennas, for example, are covered with highly sensitive and naturally tuned receptors called *sensilla* that respond to chemical, physical, and mechanical signals via electrical nerve impulses. By immobilizing the antenna or even the entire insect on a transducer and measuring these induced electrical impulses (or electroantennograms), a biosensor materializes. A multianalyte biosensor can be formed by adhering antennas from several different insects. The current targets for such biosensors are odorants such as those related to smoke (guaiacol and 1-octen) for early-warning fire detection or volatiles emanating from diseased plants, with detection limits in the part-per-billion range. Their parallel application for sensing volatiles associated with environmental contaminants and even non-odor-related compounds is a potential future prospect.

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CHAPTER 10

Effects of Genetically Modified Plants on Soil Microorganisms

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10.1 INTRODUCTION

Despite the ongoing debate on potential risks of the wide-scale application and commercial use of genetically modified (GM) crops, in particular in Europe, the number of countries planting GM crops increased to 23, comprising 12 developing countries and 11 industrial countries in 2007. Table 10.1 provides an overview of countries growing 50,000 ha or more of GM crops and the type of crop planted (modified according to James, 2007). The application of GM plants in agriculture and forestry (e.g., with the aim of increased pest resistance) could lead to a significant reduction in the release of pesticides into the environment. Therefore, the use of GM crops may be considered as environmental friendly. However, the overall impacts of GM crops on soil quality are poorly understood. Despite their importance for soil and plant health, the response of soil microbes to the large-scale application of GM crops has not been addressed adequately in many studies, simply due to the lack of appropriate methods to do so. It is assumed that changes in soil microbial community composition might occur directly, due to the biological activity of the transgene products, or indirectly,

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Table 10.1 Overview of Countries Growing Large Quantities of GM Crops

Rank/Country	Area (million hectares)	Biotech Crops
1. United States	57.7	Soybean, maize, cotton, canola, squash, papaya, alfalfa
2. Argentina	19.1	Soybean, maize, cotton
3. Brazil	15.0	Soybean, cotton
4. Canada	7.0	Canola, maize, soybean
5. India	6.2	Cotton
6. China	3.8	Cotton, tomato, poplar, petunia, papaya, sweet pepper
7. Paraguay	2.6	Soybean
8. South Africa	1.8	Maize, soybean, cotton
9. Uruguay	0.5	Soybean, maize
10. Philippines	0.3	Maize
11. Australia	0.1	Cotton
12. Spain	0.1	Maize
13. Mexico	0.1	Cotton, soybean

due to altered composition of root exudates, plant litter, or changes in agricultural practice (Kowalchuk et al., 2003). In view of the importance of soil microorganisms for soil functioning, geochemical cycles, and plant health, it is important to understand and predict potential effects of GMP plants. Although microbial ecology has developed enormously in the last two decades and new methods became available that allow the study of microbes in soil and in the rhizosphere independent of their ability to form colonies on solid nutrient media, it is still difficult to predict and assess effects of GM crops on soil microbiota, as agricultural soils are highly dynamic and changes in microbial community structure and function are a characteristic of soils of high quality. Changes in the microbial community composition in the rhizosphere of GM crops compared to the nonmodified cultivar need to be related to natural variation due to biotic and abiotic factors such as soil type, plant species, or cultivar. We are just starting to get a clearer picture of the factors that shape the structural and functional composition of soil microbial communities and to open the "black box" of soil.

In this chapter we address the two major questions related to the impact of GM crops on microbial life in soil:

1. Do altered root exudation patterns or released transgene products (e.g., those with antifungal or antibacterial activity) result in changes in the structural and functional composition of the soil microbial community in the vicinity of the roots which exceed variation due to natural factors?
2. Can transgenic plant DNA be taken up by naturally transformable competent soil bacteria and subsequently integrate stably into the bacterial genome? This question is often raised in view of the use of antibiotic resistance genes as marker genes in GM crops.

Although the discussion of studies addressing both questions is the focus of this chapter, we feel that an introduction to the particularities of the rhizosphere and the

methodological challenges to sampling and analyzing rhizosphere microbial communities is required for a better understanding of this research field.

10.2 THE RHIZOSPHERE

The habitat most likely to be affected by the transgene expression or altered root exudation is the rhizosphere. The *rhizosphere* is defined as the volume of soil adjacent to, and influenced by the plant root (Hiltner, 1904; Sørensen, 1997). In the proximity of plant roots, not only an enhanced abundance, but also metabolic activity of microbes that can utilize root exudates or deposits, is observed (Jaeger et al., 1999; Yang and Crowley, 2000; Brimecombe et al., 2001). Broeckling et al. (2008) demonstrated that root exudates are the mechanism through which a plant is able to regulate plant-associated and soil fungal communities. The composition and amount of root exudates differ among plant species (Neumann and Römheld, 2001; Bais et al., 2006). Root exudation patterns and root-associated microbial communities depend not only on the plant species but also on their developmental stage (Gomes et al., 2001,2003; Smalla et al., 2001; Berg et al., 2002; Costa et al., 2006a,b,2007) and on the nutritional status of the plants (Maloney et al., 1997) In addition, the soil type, climatic condition, pathogen exposure, or agricultural practices, such as tillage and crop rotation, were shown to influence the composition of microbial diversity in the rhizosphere (Latour et al., 1996; Westover et al., 1997; Lupwayi et al., 1998; Yang and Crowley, 2000; Marschner et al., 2001; Rasche et al., 2006a). Rhizosphere microorganisms, in turn, exert strong effects on plant growth and health by nutrient solubilization, N_2 fixation, or production of plant hormones (Raaijmakers et al., 2008). Soilborne pathogens can greatly reduce plant growth. Clearly, bacterial and fungal communities can be influenced by the plant, and vice versa. Due to the importance of plant–microbe interactions in the rhizosphere for carbon sequestration, ecosystem functioning, and nutrient cycling as well as for plant growth and health (Singh et al., 2004), it is crucial to understand the factors influencing the microbial communities in this habitat.

To evaluate potential changes of microbial communities in the rhizosphere of GM crops compared to the parental line, it is of utmost importance that baseline data are provided to relate potential changes to natural fluctuations. A relevant effect of GM crops should cause deeper changes in the microbial community than the commonly accepted changes due to factors such as plant growth stages, different cultivars, soil type, or climate.

10.3 EXPERIMENTAL DESIGN OF FIELD TESTS

The appropriate field design has been identified as one of the important prerequisites (Van Elsas and Smalla, 1996) for studies on the effects of the plant species, the cultivar, or the genetic modification on rhizosphere microbial communities. Field heterogeneities might be less important for fields that are frequently ploughed compared to forest stands, but randomized plot design is recommendable in any case. Figure 10.1 shows the field design for a field trial performed with GM potatoes with increased zeaxanthin content in their tubers. To evaluate potential impacts of the GM potato on

Monitoring potential effects of GM crops on soil microbial communities should be done during the stage of field releases
-post-market monitoring might be extremely difficult

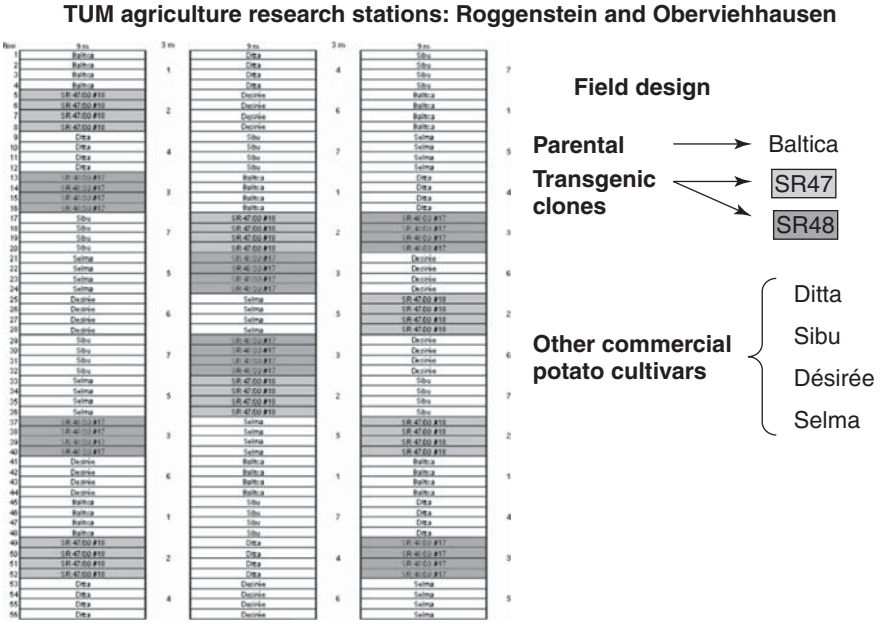

Figure 10.1 Design for a field trial performed with GM potatoes (Weinert et al., 2009a).

the structural and functional diversity of the soil microbiota in the context of natural variability among cultivars, the two transgenic lines were planted together with the parental cultivar and four commercial potato cultivars (Weinert et al., 2009a). As root exudation changes during plant growth development (Neumann and Römheld, 2001) the sampling time might be another critical factor of the experimental design. Thus, samples should be taken at different time points during plant growth development. In addition, pollen, entering the soil during flowering, might also affect the composition of the microbial community. Already slight differences in the development of the GM plant, the parental cultivar, and other cultivars might affect the composition of the microbial community in the rhizosphere (see Figure 10.2). In particular, when greenhouse-generated tubers were used, delayed emergence or earlier senescence had been observed (see Figure 10.3; Heuer et al., 2002). Thus, a compromise needs to be made between completely random sampling and selecting plants with comparable growth stages. Considering that soils often are the overriding factor shaping microbial communities in the rhizosphere, potential effects of GM plants in comparison to their parental lines and to other cultivars should be tested on different sites.

10.3.1 How to Sample the Rhizosphere

As the rhizosphere is defined as soil closely adhering to roots, the soil that remains attached to the roots of plants after vigorous shaking of the plant is usually treated

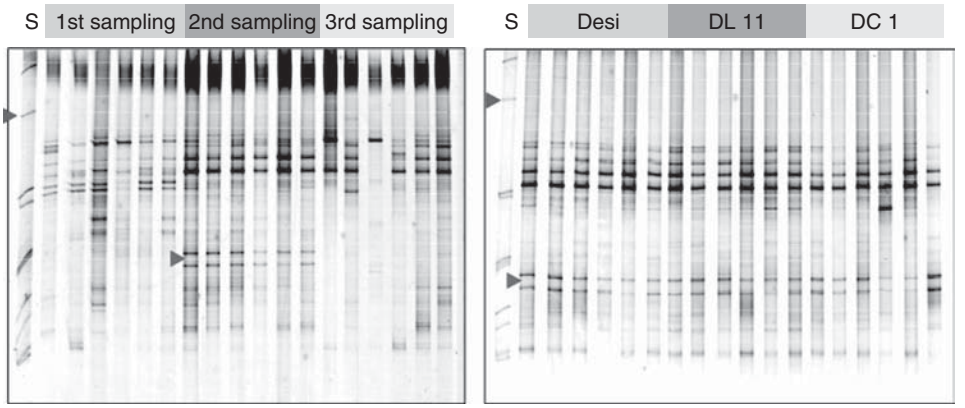

Figure 10.2 Comparison of DGGE bands of potato rhizosphere bacteria: left, seasonal shifts; right, comparison between GM potato (GM) and control (DC1) at the second sampling time.

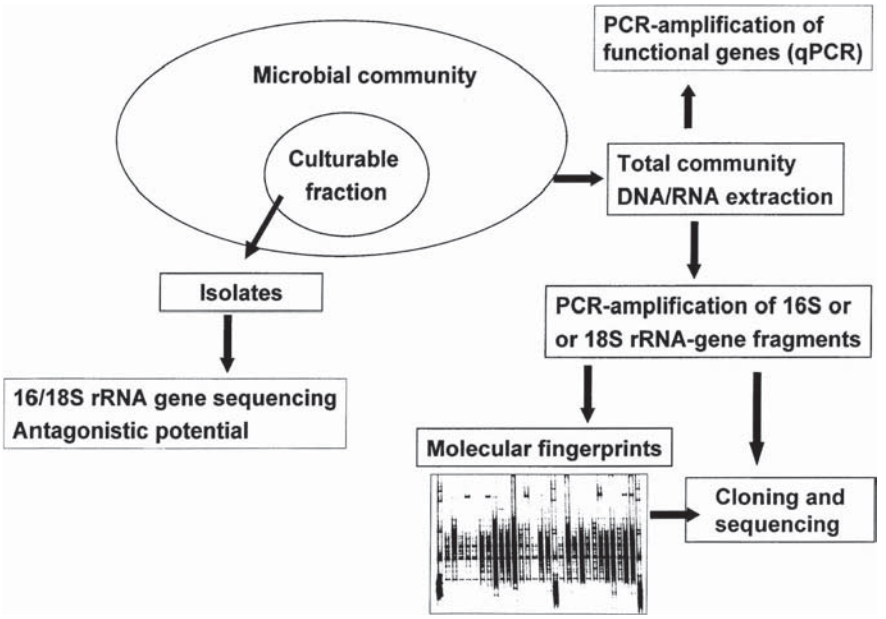

Figure 10.3 Process to monitor the fate of DNA from GM crops using construct-specific DNA extracted directly from the soil microbial community.

as rhizosphere soil. Both the soil type and soil moisture influence the amount of soil adhering to the root. Another critical point is which part of the root system should be sampled, considering the highly heterogeneous colonization patterns (Götz et al., 2006a). The use of subsamples of the entire root system and, depending on the size of the plant, composite samples might contribute to overcoming this variability. However, a picture of the type and abundance of microbes in the rhizosphere obtained by analyzing composite samples is certainly an underestimate of the existing diversity.

10.3.2 How to Analyze Microbial Communities in the Rhizosphere

Studies that aim to explore potential effects of GM crops on the microbial community composition have been performed at different levels of resolution which might influence the findings reported. In many studies cultivation-dependent methods were used to detect potential effects of GM crops on bacterial or fungal communities in the rhizosphere (Oger et al., 1997, 2000; Lottmann et al., 1999, 2000; Siciliano and Germida, 1999; Mansouri et al., 2002; Sessitsch et al., 2003; Götz et al., 2006b; Travis et al., 2007; van Overbeek and van Elsas, 2008; Weinert et al., 2009b). Whereas CFU counts are usually a rather insensitive method of detecting GM-related effects, in-depth phenotypic (e.g., antagonistic potential) and genotypic (16/18S rRNA gene sequencing; BOX fingerprints) characterization of dominant isolates enables a comparison with high resolution (Götz et al., 2006b; Weinert et al., 2009b). Another cultivation-dependent method used in various studies aiming to detect potential effects of GM crops is the community-level physiological profile (CLPP) by means of BIOLOG microtiter plates patterns (Heuer and Smalla, 1997b; Siciliano et al., 1998; Di Giovanni et al., 1999; Heuer et al., 2002; Blackwood and Buyer, 2004; Mulder et al., 2006). The bacterial cell suspension obtained from the rhizosphere is used to inoculate wells of a microtiter plate in which each well contains a different carbon source, nutrients, and a tetrazolium dye (Garland and Mills, 1991). Oxidation of the substrate is monitored periodically by measuring the concomitant reduction of the tetrazolium dye. Several studies showed habitat-specific and reproducible patterns of carbon source oxidation, so the method was used to discern temporal and spatial differences among microbial communities. However, the claim that BIOLOG substrate utilization patterns provide insight into the functional potential of the soil microbiota is incorrect. Although CLPP (BIOLOG) is a sensitive method, the data obtained need to be evaluated critically, as only a few fast-growing bacteria, mostly *Pseudomonas* and Enterobacteriaceae, contribute the substrate utilization patterns (Smalla et al., 1998).

A major problem of the traditional cultivation-based analysis is that only a small proportion of the bacterial populations from bulk or rhizosphere soil form colonies on solid nutrient media (Amann et al., 1995). Cultivation-based limitations can be overcome by analyzing fatty acid methyl ester (FAME; Frostegard et al., 1996), phospholipid fatty acid (PLFA; Zelles et al., 1995), or DNA or RNA extracted directly from rhizosphere and soil samples (van Elsas, 2000). To study spatial and temporal variation of rhizosphere and soil bacterial communities, multiple-sample analysis is essential (Muyzer and Smalla, 1998; Gelsomino et al., 1999). In most recently published studies, potential effects of GM crops on the soil microbiota were evaluated by analyzing ribosomal RNA gene fragments by denaturing gradient gel electrophoresis (DGGE; Muyzer and Smalla, 1998), terminal restriction fragment length polymorphism (T-RFLP; Liu et al., 1997; Osborn et al., 2000), single-strand conformation polymorphism (SSCP; Schwieger and Tebbe, 1998), or amplified ribosomal interspacer analysis (ARISA; Ranjard et al., 2001). The advantage of fingerprinting methods is that they allow comparison of the microbial community composition among a large number of samples. Profiles of multiple replicates can be run next to each other, allowing easy identification of bands that occur in all replicates or populations that are detectable only in replicates of certain treatments (see Figure 10.2). If such a block of replicates taken at one sampling time is followed by a block of replicates taken at a later stage of plant development, populations appearing or disappearing from this fingerprint are easily identified by eye. Similar to traditional cultivation approaches, where colonies are usually picked from

plates of dilutions giving 20 to 100 colonies for further characterization, the DGGE fingerprint of rRNA gene fragments obtained with bacterial- or fungal-specific primers are a fingerprint of the most dominant populations (Heuer and Smalla, 1997a; Muyzer and Smalla, 1998). However, as mentioned earlier, direct correlation between the intensity of a band and the cell number is not possible because bacteria harbor different numbers of 16S rRNA gene operons (Fogel et al., 1999; Klappenbach et al., 2000). Much less is known regarding how environmental factors affect less common colonizers in the rhizosphere: a general problem in microbial ecology recently termed "the tragedy of the uncommon" (Bent and Forney, 2008). The nested PCR approach of using taxon-specific primers in a first PCR followed by D/TGGE-PCR with bacterial primers allows us not only to reduce the complexity of the pattern but also to analyze minority populations (Heuer et al., 1997,2001,2002; Gomes et al., 2001; Milling et al., 2004). Biases recognized for the analysis of ribosomal gene fragments amplified from total community DNA or cDNA were reviewed by von Wintzingerode et al. (1997).

To link the exudation pattern of GM crops more closely to microbial community structure and function in the rhizosphere, stable isotope probing (SIP) has recently been introduced. In principle, plants are fumigated with ^{13}C-labeled CO_2, which result in a ^{13}C label in all plant assimilates. This label can be followed into the rhizosphere soil microbes. Thus, nucleic acids or lipids of microbes responding to these assimilates can be identified easily, as they carry a ^{13}C label. Although the application of SIP field-grown crops might be limited, this methodological approach will certainly be an important tool to study the effects of GM crops on the rhizosphere microbiota. Considering the advantages and limitations of the various methods, it is recommended that a polyphasic approach be used to study potential impacts of GM crops.

10.4 EFFECT OF GM PLANTS ON THE STRUCTURAL AND FUNCTIONAL COMPOSITION OF RHIZOSPHERE MICROBIAL COMMUNITIES

Plant breeding by traditional techniques, as well as transgenic modification, can affect the structural and functional diversity of rhizosphere microbial communities (e.g. by altered root morphology, physiology, and exudation). In particular, if a transgenic modification was made to improve the resistance of a plant toward bacterial or fungal pathogens by releasing transgene products such as cell wall–attacking enzymes or compounds such as chitinases, T4-lysozyme, or cecropine, a response of many soil microbes is likely, as most of these substances are not specific for a certain pathogen but have a broad host-range spectrum.

In this subchapter we discuss selected greenhouse and field studies performed to assess potential impacts of GM crops on belowground microbial diversity. In addition, we summarize briefly the findings of several case studies performed by our groups on the potential effects of GM potatoes on the microbial diversity in soil. For more comprehensive coverage on the contents of this chapter, the reader is referred to several published reviews (Azevedo and Araujo, 2003; Bruinsma et al., 2003; Kowalchuk et al., 2003; Dunfield and Germida, 2004; Lynch et al., 2004; Liu et al., 2005; Ikeda et al., 2006).

Effects of transgenic crops on the structural and functional composition of microbial rhizosphere communities are studied best at the stage of small-scale field releases. Greenhouse observations might deviate considerably from what is observed under field

conditions but have the advantage that parameters such as temperature, moisture, and light can be controlled. Field tests with GM plants growing together with their parental lines or isogenic controls in the same field are also ideal for the study of potential differences between the microbial rhizosphere community of the parental crop line and different transformant lines, because they make it possible to analyze sufficient replicates and to take samples at different stages of plant growth development and from different field sites. Usually, different transformation events are tested because in addition to pleiotrophic effects, they might show considerable variation in the level and stability of gene expression. In addition, it is recommended that different cultivars be included so as to obtain data on the natural variability of the microbial community in the rhizosphere of different cultivars grown at the same site and subjected to the same agricultural management practices (Dunfield and Germida, 2001; Milling et al., 2004).

Numerous studies have been published on the potential effects of transgenic crop plants on the composition of bacterial communities in the rhizosphere under field conditions (Baumgarte and Tebbe, 2005; Donegan et al., 1995, 1996; Lottmann et al., 1999, 2000; Siciliano and Germida, 1999; Dunfield and Germida, 2001, 2003; Heuer et al., 2002; Schmalenberger and Tebbe, 2002; Fang et al., 2005; Lamarche and Hamelin, 2007; LeBlanc et al., 2007; Devare et al., 2004; Becker et al., 2008; van Overbeek and van Elsas, 2008; Weinert et al., 2009a). Other groups have tried to evaluate potential impacts of GM crops on soil microbial communities under greenhouse or growth chamber conditions (Oger et al., 1997, 2000; Di Giovanni et al., 1999; Gyamfi et al., 2002; Mansouri et al., 2002; Sessitsch et al., 2003; Blackwood and Buyer, 2004; Brusetti et al., 2004; Fang et al., 2005).

The group of Y. Dessaux showed in several greenhouse studies that different plants (*Lotus, Solanum nigrum*) engineered to produce octopines select bacterial populations in the rhizosphere that utilize these substrates, and thus the abundance of octopine-utilizing bacterial communities was found to be increased significantly compared to the wild type (Oger et al., 1997,2000; Mansouri et al., 2002). Changes in the abundance of octopine-utilizing cultivable bacteria observed in greenhouse experiments seemed independent of the soil type and plant species.

One of the first studies that investigated the bacterial community composition in the rhizosphere and in the endorhiza of field-grown canola was performed by the group of J. Germida in Canada. By means of BIOLOG and fatty acid profiles, the authors demonstrated that the bacterial community composition of the transgenic line Quest (tolerant to the herbicide glyphosate) differed significantly from those of the two other canola cultivars (Siciliano et al., 1998). Differences were also observed in the composition of the bacterial isolates (Siciliano and Germida, 1999). However, in this study only one sampling time (flowering stage) was observed. In a follow-up study conducted at two field sites in Saskatchewan during the 1999 and 2000 field seasons, the microbial communities associated with a GM canola variety, a conventional canola variety, and a fallow soil were assessed six times during the field season. The bacterial communities were analyzed by different phenotypic and genotypic methods (FAME-, CLPP-, and T-RFLP). Although significant differences in the CLPP were detected between Quest and Excel in both years at different sampling times and at both sites, no significant differences were noted between the CLPP of soil samples taken at the pre-seeding stage (May 1999) or the overwintered stubble stage (April 2000). The microbial fatty acid composition of the rhizosphere community associated with the

conventional variety (Excel) was significantly different from the fatty acid composition of the community associated with the genetically modified variety, Quest. However, again no significant differences were noted between the fatty acid patterns of soil from unplanted fields and from plots that were planted with canola plants in the preceding year for either field site or field year. The differences were dependent on the sampling time; most of the differences were observed at the flowering stage. Based on their data set, the authors concluded that changes in the soil microbial community were temporary and dependent on the presence of transgenic plants.

In a study by Schmalenberger and Tebbe (2002), the bacterial communities in the rhizosphere of GM corn resistant to the herbicide glufosinate were compared to the isogenic cultivar and to the bacterial communities in the rhizosphere of sugar beets grown in the same field. DNA extracted from bacterial cell pellets was used as template DNA for PCR (polymerase chain reaction) amplification of different 16S rRNA gene fragments and subsequent analysis by SSCP. Neither the genetic modification nor use of the herbicide resulted in altered SSCP profiles. In contrast, SSCP patterns of bacterial communities from the rhizospheres of sugar beet were clearly different. In addition, a less pronounced but significant difference was also observed with rhizosphere samples from fine roots of maize plants collected 35 and 70 days after sowing.

Travis et al. (2007) reported that transgenic tobacco overexpressing a bacterial nitroreductase gene detoxified soil contaminated with 2,4,6-trinitrotoluene (TNT), and a significantly increased microbial biomass and metabolic activity were observed for microbial communities in the rhizosphere of transgenic tobacco compared with wild-type plants. This report showed that GM plants engineered for phytoremediation purposes can improve the structural and functional diversity of bacterial rhizosphere populations in polluted soils and thus represent an example for a positive impact of the GM plant.

10.5 STUDIES ON BT-EXPRESSING CROPS

The δ endotoxins (Cry protein) produced by *Bacillus thuringiensis* (Bt) strains have been exploited as microbial insecticides for more than half a century. Although microbial insecticides containing Bt products are regarded as an environmentally friendly alternative to chemical pesticides, they constituted only a minor proportion of the insecticides applied worldwide. With the introduction of different *cry* genes into crops, the situation changed drastically. Bt crops are now a major control measure to protect plants from insecticidal attack (Romeis et al., 2006) in many parts of the world. Thus, Cry1Ab-expressing maize is resistant to corn borer such as *Ostrinia nubilalis*, and Cry1Ab protects cotton from the cotton bollworm complex. The major advantage of insect-resistant GM crops is the drastically reduced need for insecticides. Plants expressing Cry proteins are the most frequently grown GM crops (James, 2007).

Several studies have investigated potential effects of Bt-expressing plants on the rhizosphere and soil microbiota. A comparison of the studies is rather complicated, as some were performed under greenhouse conditions whereas others investigated rhizosphere or bulk soil from field experiments. Very few studies included different cultivars or different soils. Moreover, the methods used to analyze potential effects differed in their level of resolution. Stabile isotope probing using a ^{13}C pulse-chase labeling method in combination with phospholipid fatty acids (PLFAs) was used in a study

by Wu et al. (2008) to assess the potential effects of Cry1 Ab expressed by Bt rice plants on the microbial community structure in the rice rhizosphere compared to the parental rice plants under greenhouse conditions. There were no detectable differences in ^{13}C distribution in rice roots and rhizosphere microorganisms at any point during rice development. Although a significantly lower amount of gram-positive bacterial PLFA and a higher amount of gram-negative bacterial PLFA were observed in the Bt rice rhizosphere than in control plants at all plant development stages, there were no significant differences in the amount of individual [^{13}C]PLFA between Bt and control rhizospheres at any growing stage. The authors concluded that the insertion of *cry1Ab* and marker genes into rice had no effect on photosynthate distribution in rice or the microbial community composition in its rhizosphere. In contrast, Brusetti et al. (2004) could show that bacterial community ARISA profiles obtained from the total DNA extracted after exposing soil to root exudates collected under hydroponic growth conditions clearly showed that the exudates of the transgenic Bt corn (event 176) led to a different bacterial community selection than that of the wild type. The authors speculated that Bt corn exudates differ in several ways from the nontransgenic plant, not just in the Cry protein. Based on greenhouse experiments performed with different soil types that were planted with Bt corn and a herbicide (Roundup ready)-resistant corn and the corresponding isogenic lines, Fang et al. (2005) concluded that bacteria in corn rhizospheres were affected more by soil texture than by cultivation of transgenic varieties. In a study by Blackwood and Buyer (2004), Bt corn was grown in three different soils, sampled 36 days after planting, and analyzed by PLFA and BIOLOG substrate utilization patterns. In this study, neither *cry*1ab expression nor the corn line had a significant effect on the PLFA and BIOLOG patterns, while differences in the PLFA patterns due to soil were significant.

Recently, Jung et al. (2008) evaluated the effect of *cry1AC* expression in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) on soil bacteria. Transgenic varieties of Chinese cabbage, which is one of the most important agricultural crops in Korea, were developed to reduce damage caused by diamondback moth (*Plutella xylostella)* and the use of chemical pesticides. The molecular fingerprints (16S DGGE) of the bacterial community in the rhizosphere of the transgenic cultivar and the conventional cultivar grown in two provinces in Korea in two years (2005, 2006) revealed no differences in the composition of the dominant bacteria. Devare et al. (2004) compared the effects of the pyrethroid insecticide tefluthrin or of the GM Bt corn expressing the Cry3Bb insecticidal protein active against corn rootworm (CRW) (*Diabrotica* spp.) on soil microbiota by determining the microbial biomass activity (N mineralization potential, short-term nitrification rate, and soil respiration) and by molecular fingerprints (T-RFLP) of 16S rRNA gene fragments amplified from total community DNA from soil samples taken from field experiments performed in 2001 and 2002. The field experiments consisted of four replicated plots planted with CRW Bt corn, three plots with non-Bt corn, and three non-Bt corn treated with tefluthrin. The analysis of samples taken at three time points during the growing season revealed that neither the Bt corn nor the insecticide had any significant effects on microbial biomass, N mineralization, or short-term nitrification rates. Although the insecticide depressed the microbial respiration, there was no difference in the microbial respiration activity between the Bt corn and the non-Bt corn samples. The molecular fingerprints revealed that bacterial species composition and relative abundance differed substantially between years but did not differ between

treatments. Based on their data, the authors concluded that the release of CRW Bt corn might pose little threat to the soil microbiota.

Although no effects of Bt corn were found in most studies, a study by Castaldini et al. (2005) performed under greenhouse conditions showed that transgenic Bt 11 and Bt 176 corn plant lines affected the early events of mycorrhizal establishment and the development of symbiosis by *G. mosseae*. The experimental model system used allowed them to monitor the formation of functional infective structures (i.e., entry points developing arbuscules), which were significantly reduced in Bt 11 and Bt 176 corn roots (by 72% and 67%, respectively) compared with non-Bt corn plants.

Compared with annual crops, trees might have more long-lasting impact on soil microbiota, as they grow with an increasingly widespread root system, often for several decades in the same ecosystems. White spruce (*Picea glauca*), which expresses Cry1Ab constitutively, is resistant to the spruce budworm *Choristoneura fumiferana* (Clemens). Lamarche and Hamelin (2007) found that Bt white spruce had no effect on N_2-fixing communities. In this study the diversity of the diazotroph bacterial community in the rhizosphere soil from GM and non-GM spruce was investigated using a cultivation-independent DNA-based approach. In addition, the rhizosphere of white spruce trees from adjacent stands was analyzed. Statistical analysis of sequences from clone libraries generated from *nifH* gene fragments amplified from total community DNA revealed that nitrogen-fixing communities did not differ significantly among GM and non-GM spruce. However, significant differences were found between diazotrophs associated with white spruce in natural stands (25 and 65 years old) and those associated with trees in a plantation, indicating that under natural conditions great differences in diazotroph communities of different sites were observed. Rhizosphere samples taken from the same plantation of white spruce 10 months earlier were also analyzed by a 16S rRNA gene-based approach (Le Blanc et al., 2007). In contrast to the *nifH* findings reported by Lamarche and Hamelin (2007), screening of more than 1700 clones of 16S rRNA gene fragments by restriction enzyme analysis and sequencing of 686 OTU (operational taxonomic units) from rhizosphere samples indicated significant differences in the rhizosphere microbial community composition of transgenic *cry1Ab, uidA*, and *nptII* carrying white spruce trees, the transgenic controls (containing only *uidA, nptI*), and respective control plants.

Planting of Bt corn will inevitably result in a release of Cry proteins to the soil from pollen deposition, root exudation, or during or after the harvest from decomposing root or plant residues. In several studies the fate of Bt toxin in soil was followed. Laboratory studies have shown that Cry proteins bind to organic or mineral compartments in soil and that soil-bound Cry proteins might be less accessible for proteolytic degradation but eventually maintain their insecticidal activity (Tapp and Stotzky, 1995; Crecchio and Stotzky, 1998, 2001). The fate of the CryAb protein was followed in different field studies (Hopkins and Gregorich, 2003; Zwahlen et al., 2003; Baumgarte and Tebbe, 2005). Whether the Cry1AB protein accumulates under field conditions when Bt corn is planted in subsequent years and whether the fate of the Cry protein is also influenced by the soil type was studied by Baumgarte and Tebbe (2005) in two agricultural field sites planted with transgenic corn in Germany. This study revealed that despite the detection of Cry1Ab protein in rhizospheres of MON810, the bacterial community structure was less affected by the genetic modification than by field site, plant growth stage, or field heterogeneities (Baumgarte and Tebbe, 2005). The data on the persistence of Cry1Ab protein indicated that the roots of maize stubbles are a major reservoir of the Cry1Ab

protein in soil, and thus further postharvest effects on nontarget organisms need to be considered.

Based on the use of CFU counts, BIOLOG substrate utilization patterns (both cultivation-dependent methods), and soil respiration, Mulder et al. (2006) observed that soil microbial communities responded differently to straw of transgenic corn GM-Novelis (MON 810) and GM-Valmont Event 176 compared to conventional corn. However, considering the experimental design and the biases of the methods used in the study, conclusions such as "Transgenic maize affect soil microbes" might be misleading and must be confirmed in longer-term field experiments including other cultivars, to address natural variability among different cultivars.

10.6 GM POTATOES

Next, the results of several studies performed in the frame of field release accompanying research in Germany will be discussed briefly. Two studies aimed to investigate the potential effects of GM potatoes expressing T4-lysozymes on rhizosphere microbial communities (Lottmann et al., 1999, 2000; Heuer et al., 2002). In contrast to many other transgenic plants, the gene product of this genetic modification is supposed to target bacteria (Düring et al., 1993), and it was shown by Düring et al. (1999) that plant-associated bacteria were indeed affected, in so far as the susceptibility of the transgenic potato plants to infections by *Erwinia carotovora* was reduced significantly. Furthermore, it was demonstrated that a detectable amount of T4 lysozyme was released from the roots (De Vries et al., 1999), causing bactericidal activity on the root surface (Ahrenholtz et al., 2000). The rhizosphere bacterial communities of two transgenic potato lines (DL4, DL5) that produce T4-lysozyme for increased resistance against *Erwinia carotovora* ssp. *atroseptica* were analyzed compared to the parental line and a transgenic control lacking the T4-lysozyme gene. Rhizosphere samples were taken at three plant growth developmental stages (principal growth stages 1, 6, and 9) from plants grown at two field sites during 1996–1998. The cultivation-dependent analysis of the bacterial communities was done by plating on R2A and determining the species composition and diversity based on fatty acid analysis of more than 2000 isolates (for eight samplings). Furthermore, the abundance and diversity of bacterial antagonists toward *Verticillium dahliae* and *Erwinia carotovora* ssp. *atroseptica* was determined. BIOLOG substrate utilization patterns were determined. The cultivation-independent analysis was based on the amplification of 16S rRNA gene fragments from DNA that was extracted from the microbial pellet with primers specific for actinobacteria and Alphaproteobacteria and Betaproteobacteria. The 16S rRNA gene fragments were analyzed either by molecular fingerprints (DGGE) or, for some samples, by cloning and sequencing. All methods revealed that environmental factors related to season, field site, or year influenced the bacterial rhizosphere community but not the T4-lysozyme expression (Heuer et al., 2002). While for DL5 no deviation of the bacterial rhizosphere community compared to the parental line and the transgenic control was found, the transgenic line DL4 showed differences at some sampling times, which were, however, not to be attributed to the transgene expression but probably to different growth characteristics. The abundance and diversity of bacterial antagonists isolated from the various potato lines also seemed not to be affected by the transgenic modification (Lottmann et al., 1999). The second study on potential effects of T4-lysozyme expression was continued with new transformant lines (DL10, DL11, DL12, DL13), which

had a higher expression level and were planted in the field (one site) in 1999 and 2000. The methodological approach was changed slightly, as in the meantime it was shown that T4 lysozyme can also affect fungi and several other eucaryotes by a nonenzymatic mechanism that involved disruption of membranes (Düring et al., 1999) and included the analysis of fungal rhizosphere communities by cultivation and molecular fingerprinting techniques. The results obtained with the new transformant lines confirmed very closely the observations of the first study. Although differences in the bacterial community patterns due to different plant growth development stages could be seen, no such differences were observed when the various potato lines were compared (see Figure 10.2). In contrast to the bacterial fingerprints, the fungal fingerprints showed a greater variability between replicates of the same treatment, and the seasonal changes seemed to be less pronounced (Smalla, unpublished). At the community level, no differences in the relative abundance of fungal rhizosphere populations could be revealed by molecular fingerprints (DGGE). However, effects might be hidden because the 18S rRNA gene fragments of different fungal populations often belonging to different taxa have the identical electrophoretic mobility in DGGE gels. A relatively large proportion of fungal isolates showed antagonistic activity against *V. dahliae*, and a high diversity was revealed by BOX fingerprints. However, no relevant differences could be observed with respect to the relative abundance and composition of fungal antagonists in the rhizosphere of the transgenic T4-lysozyme-expressing potatoes, the transgenic control, and the parental line. Because no relevant effects on the fungal rhizosphere communities could be shown based on the characterization of antagonistic fungal isolates and on the fungal DGGE fingerprints, a characterization of endophytic fungal isolates and DGGE fingerprints of endophytic fungal rhizosphere communities was done for the transgenic potato line DL11 and the parental line. Indeed, in this preliminary study on endophytic fungi, differences between the transgenic and the nontransgenic potato could be shown (Götz et al., 2006b). This result was not too unexpected because the T4-lysozyme is transported into the apoplast.

In another study, the effects of transgenic potatoes with a modified starch metabolism on soil microbial communities were analyzed (Milling et al., 2004). The aim of this study was to investigate potential effects of a transgenic potato line (SIBU S1), which was modified in its starch composition by antisense RNA on the composition of the bacterial and fungal diversity in rhizosphere and soil compared to the nontransgenic parental cultivar (SIBU) under field conditions. Furthermore, a second nontransgenic cultivar (SOLANA) was included in the study. The different cultivars and the transgenic line were planted in 1998, 1999, and 2000. Preexperiments using several screening techniques in 1998 and 1999 have shown that most of the differences occurred at the flowering stage of the plants. Therefore, detailed molecular analysis was performed only at the flowering stage in 2000 (Milling et al., 2004). The 16S- and 18S-rRNA gene fragment DGGE fingerprints from bulk soil samples did not show any significant differences between the two cultivars and the transgenic line. Similar results were obtained for the rhizosphere samples using the general alpha- and betaproteobacterial and fungal-specific primers, with the exception of the bacterial DGGE patterns obtained for the rhizosphere of SOLANA (EC60/2000). These patterns revealed that the relative abundance of one band was increased compared with the patterns of SIBU and SIBU S1, and the 16S rRNA gene sequence of the differentiating band showed the greatest similarity to *Enterobacter amnigenus*. When *Pseudomonas*-specific primers were used, relevant differences were found between the rhizosphere patterns of the transgenic

potato line (SIBU S1) and the parental cultivar (SIBU). However, clear effects of the cultivar SOLANA on the structure of the *Pseudomonas* community compared to SIBU were also detected.

In a recent study by Weinert et al. (2009a) effects of GM potato plants with enriched zeaxanthin content in their tubers on microbial communities in the rhizosphere were compared to natural variability among different potato cultivars. In this study two GM lines, their parental cultivar as well as four other potato cultivars were grown in randomized field plots at two sites and in different years. Comparing all seven plant genotypes, DGGE analysis revealed that different cultivars had a stronger effect on both bacterial and fungal communities than the genetic modification. Effects of the genetic modification were mostly detected at the senescence stage of the plants. The site was the overriding factor affecting microbial community structure compared to the plant genotype. In general, the fingerprints of the two GM lines were more similar to the parental cultivar and observed differences did not exceed natural cultivar variability.

10.7 HORIZONTAL GENE TRANSFER FROM GM PLANTS TO SOIL BACTERIA IS AN ISSUE OF CONCERN

Horizontal gene transfer (HGT) between bacteria is a natural process of great importance for bacterial adaptability and diversification. HGT is a major reason for the plasticity of bacterial genomes, ensuring their rapid adaptation in response to changing environmental conditions and enabling them to colonize new niches successfully. HGT between bacteria is driven by three major processes: transformation (the uptake of free DNA), transduction (gene transfer mediated by bacteriophages), and conjugation (gene transfer by means of plasmids or conjugative and integrated elements). Mobile genetic elements (MGEs) such as plasmids, bacteriophages, integrative conjugative elements, transposons, IS elements, integrons, gene cassettes, and genomic islands are the important vehicles in the latter two processes.

Natural transformation is supposed to be the most likely mechanism for horizontal transfer of transgenic genomic or plastid plant DNA to naturally competent bacteria (De Vries and Wackernagel, 1998; Gebhard and Smalla, 1998; Kay et al., 2002; De Vries et al., 2004). The uptake of DNA from lysed cells or decaying material, a situation in which the DNA might still be protected, is usually also considered to be a natural transformation (Nielsen et al., 2000a). Furthermore, spontaneous transformation or transformation by lightning (Cérémonie et al., 2004,2006) was also described for some bacteria which do not possess genes involved in natural competence. By means of marker rescue systems, *Acinetobacter* sp. BD413 or *Pseudomonas stutzeri* were shown to be able to take up plant DNA and to stably integrate stretches of transgenic plant DNA by homologous recombination or homology-facilitated illegitimate recombination (De Vries and Wackernagel, 1998, 2002; Gebhard and Smalla, 1998; De Vries et al., 2001). Recently, the naturally transformable *Acinetobacter* sp. ADP1 strain and its derivative BD413 were shown to belong to the newly described species *Acinetobacter baylyi* (Vaneechoutte et al., 2006).

Horizontal gene transfer by natural transformation relies on the uptake of naked DNA by competent bacteria from their surroundings and its subsequent integration into the bacterial genome of the transformed bacterium (Lorenz and Wackernagel, 1994). Thus, one prerequisite for transformation to occur is the persistence of transgenic

plant DNA in the soil environment. Different studies have shown that despite the ubiquitous occurrence of DNases free high-molecular-weight DNA could be detected and is persistent in different environments (Widmer et al., 1996, 1997; Nielsen et al., 1997a; Paget and Simonet, 1997; Gebhard and Smalla, 1999). Microbial activity was pinpointed as an important biotic factor affecting the persistence of free DNA in soil (Blum et al., 1997). Cell walls or other debris might play an important role in protecting DNA after cell death (Paget and Simonet, 1997; Nielsen et al., 2000a; Ceccherini et al., 2003). A more rapid breakdown of DNA was observed at higher soil humidities and temperatures to contribute to higher levels of microbial activity in soil (Widmer et al., 1996; Blum et al., 1997). Plasmid DNA adsorbed on clay particles was found not to be completely degradable even at high nuclease concentrations (Demanèche et al., 2001a). Since DNA can persist adsorbed on soil particles or protected in plant or bacterial cells, this DNA could be captured by competent bacteria colonizing in close vicinity. Monitoring the fate of DNA from GM crops is rather easy, as construct-specific DNA can be detected in DNA extracted directly from soil by PCR using primers annealing to the construct (see Figure 10.3). In combination with Southern blot hybridization, a sensitive and specific detection of construct-specific DNA in soil can be achieved.

Although it is supposed that natural competence is widespread among bacterial species (Lorenz and Wackernagel, 1994; Dubnau, 1999), the proportion of bacteria in natural settings that can become competent and the environmental conditions stimulating competence development are largely unknown. The molecular biology of natural transformation systems has been studied in great detail only for a rather limited number of bacterial species (reviewed by Dubnau, 1999). Natural competence was shown to be a genetically programmed physiological state permitting the efficient uptake of macromolecular DNA. Natural transformation is a tightly regulated process that requires elaborate machinery with more than a dozen of proteins involved. Transformability seems to be a property that is not shared by all isolates belonging to the same species, and transformation frequencies can vary up to four orders of magnitude among transformable isolates of a species (Sikorski et al., 2002; Maamar and Dubnau, 2005). It is important to note that also for species known to become naturally competent (e.g., *Bacillus subtilis* or *Pseudomonas stutzeri*), only a small proportion of isolates belonging to the species were shown to be naturally competent (Sikorski et al., 2002; Maamar and Dubnau, 2005). The stable integration of the DNA entering competent cells is most frequently achieved by homologous recombination, by homology-facilitated illegitimate recombination, or as autonomously replicating extrachromosomal DNA (Dubnau, 1999; De Vries and Wackernagel, 2002). In addition to differences in the DNA uptake processes, bacteria do not exhibit the same efficiency to integrate the incoming DNA by homologous recombination (Sikorski et al., 2002). Only a few reports exist on the development of competence state under environmental conditions (Nielsen et al., 2000b; Kay et al., 2002). Nielsen et al. (1997a, 1997b) showed that the addition of nutrients can stimulate the competence development of *Acinetobacter* ssp. BD413 in bulk soil. Competence development was reported for the plant pathogen *Ralstonia solanacearum* and the co-inoculated *Acinetobacter* sp. BD413 when colonizing tobacco plants. Even more striking, for two soil isolates, *Pseudomonas fluorescens* and *Agrobacterium tumefaciens*, natural transformation was observed in soil (Demanèche et al., 2001b). Several studies performed addressing this question failed to identify reproducibly certain taxa (Richter and Smalla, 2007; van Overbeek

et al., 2007). Different strategies were employed to test a range of phylogenetically different rhizobacteria for natural transformation.

In a study using both culture-dependent and culture-independent approaches, the prevalence and diversity of *bla* genes in soil bacteria, and the potential impact of a 10-successive-year culture of transgenic Bt176 corn carrying *bla*TEM as a marker gene, were assessed. The *bla* TEM gene confers resistance to ampicillin a β-lactam antibiotic, which is frequently used in medicine. The results indicated that resistance toward a broad spectrum of β-lactam antibiotics is widespread in soil bacteria, and even resistance toward a third cephalosporin generation was detected. These high resistance levels for a wide range of antibiotics are due partially to the polymorphism of *bla* genes, which occur frequently among soil bacteria. No significant differences were observed in bacterial antibiotic-resistance levels between GM and non-GM corn fields, although the bacterial populations were different.

Although the uptake of trangenic DNA by competent soil bacteria and its stable integration into the bacterial genome could be shown under laboratory conditions, the estimated frequencies are comparatively low and thus would be seen only if such a horizontal gene transfer event would contribute to selective advantage of the transformants. These observations have to be related to risks posed, for example, by spreading manure or sewage on agricultural farm soils. Recently it was shown that soil fertilization with piggery manures introduces not only antibiotic resistance genes into soil but that these genes are often carried on self-transferrable mobile genetic elements (Binh et al., 2007, 2008; Heuer and Smalla, 2007).

10.8 CONCLUDING REMARKS

Recent advances in molecular tools to study microbial communities have made it possible to open the black box of soil microbial communities partially. In particular, the analysis of 16S or 18S rRNA gene fragments amplified from community DNA by molecular fingerprinting techniques seems to be suitable for monitoring purposes and has been used in several studies. If potential effects of GMP are to be studied, this should be done at the stage of field testing when transgenic and parental plants and other cultivars are planted in the field side by side. In our opinion, it is not realistic to involve this type of analysis at the stage of postcommercialization monitoring.

Overall, the GM crops investigated so far seem to have little or no effect on soil microbial communities. The methods used, although suitable in different ways for the analysis of multiple sample numbers, were sensitive enough to detect changes due to environmental factors such as plant growth development, soil type, and year. Presently, molecular microbial fingerprinting techniques seemed to be most suitable. The use of group-specific primers helped to increase the resolution and to analyze less abundant but eventually important bacterial taxa. In the future, phylogenetic or functional arrays as well as high-throughput pyrosequencing might be an alternative technique. As suggested by Kowalchuk et al. (2003), the study of indicator organisms might be advantageous for the evaluation of potential effects on the soil microbial community due to either the transgene product and/or to altered root exudation or changes in agricultural practice. Still a methodological challenge is the development of tools that facilitate a better link of structure and function as an important step to a better understanding of the soil–microbe–plant system.

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CHAPTER 11

Anaerobic Digestion of Agricultural Residues

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11.1 INTRODUCTION

In the absence of terminal electron acceptors such as oxygen, sulfate, or nitrate, the methanogenic conversion of organic matter is an intrinsic characteristic of many ecosystems (Conrad et al., 1989). The process is mediated by complex microbial communities, which in a series of sequential and cooperative steps (Figure 11.1; Sekiguchi and Kamagata, 2004) achieve complete degradation of organic matter to a mixture of gaseous end products [biogas; 50 to 80% methane, 20 to 50% carbon dioxide, trace amounts of ammonia, hydrogen sulfide, and hydrogen; Warburton (1997)].

The biotechnological application of methanogenesis is known as *anaerobic digestion* (AD; Lettinga, 1995). It is an attractive proposition, resulting in the degradation of organic pollutants such as those found in wastes and wastewaters (e.g., animal manures, sewage, and industrial wastewaters) with the concomitant generation of methane (CH_4), a usable fuel. Since the end of the nineteenth century, AD has been used for the stabilization of wastes and wastewaters and thus is one of the oldest waste treatment technologies. The realization that the rates and extents of the natural microbiological reactions could be optimized, in more sophisticated engineered systems, led to the development, initially, of the Imhoff and septic tank systems; and subsequently, of temperature-controlled completely mixed bioreactors, which have been used widely for the stabilization of municipal sewage sludges and animal manures (Lettinga, 1995). More recently, the development of high-rate reactor designs in which biomass retention and liquid retention is uncoupled has accounted for a significant increase in the use of AD for the treatment of municipal and industrial wastewaters (Lettinga, 1995; Aiyuk et al., 2006).

The increased application of AD provides environmental and economic benefits by preserving fossil fuels and reducing polluting emissions while facilitating the implementation of cost-effective sanitation in developing countries (Aiyuk et al.,

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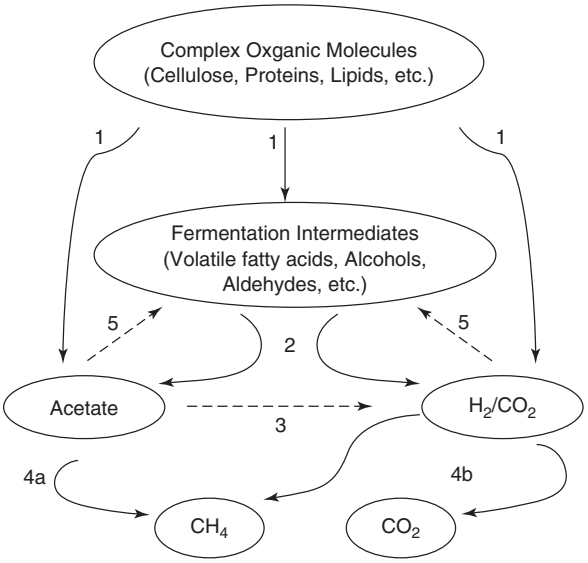

Figure 11.1 Carbon flow in methanogenesis: 1, hydrolytic/fermentative bacteria; 2, obligate hydrogen-producing bacteria; 3, homoacetogenic bacteria; 4a, acetoclastic methanogens; 4b, hydrogenotrophic methanogens; 5, fatty acid–synthesizing bacteria. (From O'Flaherty and Colleran, 2000.)

2006). Furthermore, the potential production of significant quantities of renewable energy, with a wide variety of end uses, suggests that AD technology has a bright future. In this chapter we discuss the current state of knowledge of the microbiology of AD and describe its use in stabilizing and producing energy from agricultural residues.

11.2 THE MICROBIOLOGY OF ANAEROBIC DIGESTION

Since knowledge of the ecology and function of methanogenic consortia is a prerequisite for control of AD processes, considerable effort has been made to understand the microbiology of AD using both culture-dependent and culture-independent molecular approaches. Through these analyses (e.g., targeting the 16S rRNA gene), a comprehensive view of the composition of methanogenic consortia is emerging (McHugh et al., 2003). Recently, several functionally important anaerobes that play key roles in the AD process have been cultivated. Furthermore, the ecophysiology of as yet uncultured organisms has begun to be elucidated (Collins et al., 2006; Narihiro and Sekiguchi, 2007).

Microorganisms from two biological domains, the bacteria and the archaea, carry out several interdependent, sequential, and complex biological reactions during methanogenesis. The various biological conversion processes in AD must remain balanced to avoid the accumulation of inhibitory intermediates such as volatile fatty acids (VFAs: e.g., propionate, acetate) (O'Flaherty et al., 1998a). For simplicity, four phases can be identified: hydrolysis, acidogenesis/fermentation, acetogenesis, and methanogenesis (Figure 11.1). Representatives of over 20 bacterial phyla have been detected in AD

sludges (Narihiro and Sekiguchi, 2007), including proteobacteria, chloroflexi, firmicutes, spirochaetes, and bacteroidetes. The classes Methanomicrobia, Methanobacteria, and Thermoplasmata, forming the archaea, are also typical phylotypes (McHugh et al., 2003).

11.2.1 Hydrolysis and Fermentation

Hydrolytic fermentative bacteria (including both facultative and obligatory anaerobic species) facilitate the enzymatic hydrolysis of complex organics such as proteins and polysaccharides (hydrolases: e.g., cellulase, amylase, protease, and lipase). Hydrolases may be secreted into the environment or be bound to the cell surface (Kaseng et al., 1992).

Polysaccharides are generally converted into simple monomeric or dimeric sugars. For example, starch is degraded into glucose units by a number of enzymes. Hydrolysis of cellulose by the cellulase enzyme complex yields glucose. Hemicellulose is biodegraded to a variety of monosaccharides, including galactose, arabinose, xylose, mannose, and glucose (Elefsiniotis and Oldham, 1994). The fermicutes are the best characterized carbohydrate degraders during AD (Sekiguchi and Kamagata, 2004). Recently, organisms from subphylum I of the chloroflexi, which are major populations in AD sludges, were isolated, and reports suggest that they may also play a key role in the primary degradation of carbohydrates and cellular materials (such as amino acids) in methanogenic digestion processes (Nahihiro and Sekiguchi, 2007).

Lipids are hydrolyzed into long- and short-chain fatty acids and glycerol moieties by lipases and phospholipases. Lipases catalyze the stepwise hydrolysis of the fatty acid–ester bonds in triglycerides to release the corresponding fatty acids and, eventually, glycerol. *Clostridia* and the micrococci appear to be responsible for most extracellular lipase production (Sekiguchi and Kamagata, 2004). Phospholipid metabolism by phospholipases results in the production of fatty acids and a variety of other organic compounds, depending on the substrate used (Elefsiniotis and Oldham, 1994). Proteins are broken down into amino acids, small peptides, ammonia, and carbon dioxide (CO_2) by proteases, mainly secreted by *Bacteroides, Butyrivibrio, Clostridium, Fusobacterium, Selenomonas*, and *Streptococcus* species (Sekiguchi and Kamagata, 2004).

The hydrolysis step is key for anaerobic digestion of agricultural residues, due to the high concentration of solids involved, and represents the overall rate-limiting step of the process (O'Flaherty et al., 2006). Further fermentation of the sugars, long-chain fatty acids, and amino acids by the hydrolytic and other, nonhydrolytic fermentative bacteria, results in the generation of a wide variety of fermentation end products, including acetic, propionic, butyric, and other short-chain fatty acids, alcohols, ketones, aldehydes, hydrogen (H_2), and CO_2 (Elefsiniotis and Oldham, 1994; Demirel and Yenigun, 2002).

11.2.2 Acetogenesis

Many products of acidogenesis are further oxidised to acetate, H_2, and CO_2 in a process referred to as *acetogenesis* (Stams, 1994; Schink, 2002), and mediated by the obligate hydrogen-producing acetogens (OHPAs). Under standard conditions, the oxidation of substrates such as propionate to acetate, H_2/formate, and CO_2 is an endergonic reaction (e.g., $\Delta G^{\circ\prime}$ for propionate oxidation is +76.1 kJ/reaction;

Table 11.1 Acetogenic, Methanogenic, and Sulfate-Reducing Reactions Involved in the Anaerobic Degradation of Organic Matter

Reaction Type (pH 7, 1 atm pressure, and all reactants and products at 1 M)	Free Energy, $\Delta G^{\circ\prime}$ (kJ)
Acetogenic	
Propionate: $CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2$	+76.1
Butyrate: $CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$	+48.1
Ethanol: $CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$	+9.6
Methanogenic	
Acetate $CH_3COOH + H_2O \rightarrow CH_4 + CO_2 + H_2O$	−31.0
$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	−135.6
Syntrophic reaction	
$2CH_3CH_2CH_2COOH + 4H_2O \rightarrow 4CH_3COOH + 4H_2CO_2$	+96.2
$+4H_2CH_4 + 2H_2O$	−135.6
	Net: −39.4
Sulfate-reducing	
$CH_3CH_2COO^- + \frac{3}{4}SO_4^{2-} \rightarrow HS^- + 4H_2O$	−37.7
$CH_3CH_2CH_2COO^- + \frac{1}{2}SO_4^{2-} \rightarrow 2CH_3COO^- + \frac{1}{2}HS^- + \frac{1}{2}H^+$	−27.8
$CH_3CH_2OH + \frac{1}{2}SO_4^{2-} \rightarrow CH_3COO^- + \frac{1}{2}HS^- + \frac{1}{2}H^+ + H_2O$	−66.4
$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	−38.1
$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	−47.6

Source: Adapted from Sekiguchi et al., (2001) and Schink (2002).

Table 11.1). It is only when the hydrogen partial pressure is lowered (e.g., by the presence of H_2- or formate-utilizing methanogens) that the reaction becomes exergonic (e.g., the $\Delta G^{\circ\prime}$ for propionate oxidation is −25.6 kJ/reaction in the presence of a H_2-utilizing methanogen). Therefore, OHPA bacteria must always grow in syntrophy with H_2-utilizing methanogens, sulfate-reducing bacteria (SRB), or homoacetogens, in order to facilitate interspecies hydrogen transfer and to gain energy from growth on the products of acidogenesis (Stams, 1994; Schink, 2002). Many syntrophic associations have been described in the literature, with optimum temperatures and pH levels for growth between 25 and 45°C and 6.3 and 8.5, respectively (Stams, 1994; Schink, 2002). Recently, important propionate-oxidizing syntrophic bacteria affiliated with the group *Desulfotomaculum* cluster I have been isolated and cultured from anaerobic wastewater processes. Interestingly, all these syntrophs lack the ability to dissimilatorily reduce sulfate, although other members of *Desulfotomaculum* cluster I are known to be sulfate reducers (de Bok et al., 2005; Imachi et al., 2006). The action of the OHPAs converts a range of fermentation intermediates to methanogenic substrates and thus provides a link between the initial fermentation stages and the ultimate methanogenic phase during AD (Stams, 1994; Schink, 2002).

11.2.3 Methanogenesis

The final step in anaerobic digestion is methanogenesis. Methanogens are strictly anaerobic archaea that can be subdivided into two groups: (1) hydrogenophilic or hydrogenotrophic species, which form methane by the reduction of CO_2 using H_2 as electron donor and (2) acetoclastic or acetotrophic methanogens, which generate methane by the decarboxylation of acetate (Table 11.1; Sekiguchi and

Kamagata, 2004). Methanogens can also utilize a limited number of other substrates to produce methane, such as methanol, methylamines, and formate (Ferry, 1999). Hydrogenotrophic methanogens reduce CO_2 via formyl, methenyl, and methyl intermediates, in association with specific coenzymes, to produce methane. Although approximately 70% of the methane produced during AD is via the acetoclastic pathway (Lettinga, 1995), only two acetoclastic genera, *Methanosarcina* and *Methanosaeta*, have been described to date, whereas nearly all known methanogenic species can produce methane from H_2/CO_2 (Ferry, 1999; Sekiguchi and Kamagata, 2004).

11.2.4 Other Microbial Groups Involved in AD

Sulfate-Reducing Bacteria In the presence of oxidized sulfur compounds, sulfate-reducing bacteria (SRB) can grow heterotrophically or lithotrophically on a range of different substrates and are important members of the microflora of a typical anaerobic digester (Oude Elferink et al., 1994). Interestingly, it appears that many species of SRB can grow acetogenically in the absence of sulfate, thus explaining the widespread occurrence of these organisms in anaerobic digesters treating wastewaters with low sulfate concentrations (Hao et al., 1996). The problems associated with anaerobic treatment of sulfate-containing wastewaters result from the ability of the SRB to utilize a range of substrates in the presence of sulfate and therefore interact competitively with the other bacteria involved in the process, resulting in the formation of hydrogen sulfide (H_2S) rather than methane. This competition is favorable to SRB based on thermodynamic considerations (Table 11.1), but the actual outcome under operational conditions is not straightforward. The main manifestations of SRB-related problems in AD include (O'Flaherty and Colleran, 2000):

1. Reduction in methane yield
2. Inhibition of many microbial groups involved in AD by H_2S (O'Flaherty et al., 1998b; O'Flaherty and Colleran, 1999)
3. Malodor; corrosion of piping and pumps
4. Need to scrub the biogas and effluents to meet discharge standards

Homoacetogenic and Fatty Acid–Synthesizing Bacteria The homoacetogenic bacteria may also play a significant role in the AD process, although their exact function remains unclear (O'Flaherty et al., 1998b; Ryan et al., 2004). Homoacetogens (mainly *Clostridium* and *Acetobacterium spp*.) are capable of both autotrophic and heterotrophic growth, generating acetate as the sole end product from either H_2/CO_2 or multicarbon compounds (Ryan et al., 2004). Fatty acid synthesizers, also belonging primarily to the genus *Clostridium*, produce lower fatty acids from acetate and/or ethanol when the concentration of hydrogen is high, thus reversing the reactions of the syntrophic bacteria. The activity of these organisms can be an indicator of reactor instability (Smith and McCarty, 1988).

Other, As Yet Uncharacterized Microorganisms The use of culture-independent methods (Collins et al., 2006; Narihiro and Sekiguchi, 2007) has recently highlighted the potential role of other, as yet uncultured groups of microbes in AD. Examples include the candidate bacterial phylum WWE1 (Choauri et al., 2005) and the phylum

Deferribacteres (Diaz et al., 2006). Uncultured archaeal lineages such as the nonthermophilic *Crenarchaeota* (Collins et al., 2004,2005) also seem to play an important role in the AD process, but the ecophysiology of these groups remains unknown and more research is required (Collins et al., 2005; Narihiro and Sekiguchi, 2007).

11.3 FACTORS INFLUENCING THE ANAEROBIC DIGESTION OF AGRICULTURAL RESIDUES

In addition to the operational parameters used and the process configuration, a number of environmental factors influence the microbiology and application of AD, including temperature, pH, the availability of nutrients and the presence of toxic components (Björnsson et al., 2000; Rajeshwari et al., 2000; Demirel and Yenigun, 2002; Aiyuk et al., 2006).

11.3.1 Temperature

The AD process is strongly influenced by temperature and is applied in one of three temperature ranges: psychrophilic (0 to 20°C), mesophilic (20 to 45°C), and thermophilic (45 to 75°C; Lettinga, 1995). Most full-scale anaerobic bioreactors used for biogas production from agricultural residues are operated within the mesophilic or thermophilic ranges. The acetogenesis and methanogenesis stages are, reportedly, more sensitive to temperature than either hydrolysis or acidogenesis (Kettunen and Rintala, 1997; Rebac et al., 1997).

Thermophilic AD is potentially attractive, since higher microbial growth rates result in comparatively higher levels of activity per unit biomass, and therefore higher organic loading rates may be employed (Rintala and Lepisto, 1997; van Lier et al., 2001). The results of a number of studies suggest that thermophilic AD is particularly suitable for the AD of high-temperature industrial effluents (e.g., canning factories and alcohol distilleries) and specific types of animal slurries (Dinsdale et al., 1997; van Lier et al., 2001).

Mesophilic processes require longer hydraulic retention times and are less efficient than thermophilic systems in killing pathogenic microorganisms (Bendixen, 1994). Despite this, mesophilic AD is by far the most commonly applied approach. This is because mesophilic AD is reported to be more robust and stable with respect to operational perturbations (Fang and Wai-Chung Chung, 1999; Pender et al., 2004). Also, if the feedstock requires heating, the efficiency of thermophilic operation must be offset against the energy requirement for heating, a very important factor in commercial AD for biogas production (Mahony et al., 2002).

Methanogenesis also occurs naturally at lower temperatures: for example, in soils and pond sediments (Conrad et al., 1989). Indeed, low-temperature methanogenesis is a significant contributor to global methane emissions to the atmosphere. Low-temperature or psychrophilic AD is of interest for the treatment of cold or low-strength industrial effluents (organic concentrations below 1 kg of chemical oxygen demand per cubic meter) for direct AD of domestic sewage and, importantly, to increase the net biogas yield from conventional AD applications (Rebac et al., 1997; Lettinga et al., 2001; Collins et al., 2006). Lowering the operational temperature of AD reactors has been reported to lead to a decrease in the maximum specific growth and substrate-utilization

rates, but may also result in an increased net biomass yield (van Lier et al., 1997). Furthermore, although most reactions in the biodegradation of organic matter require more energy to proceed at low temperatures than, for example, at 37°C, some reactions, such as hydrogenotrophic sulfate reduction, hydrogenotrophic methane production, and acetate formation from hydrogen and bicarbonate, actually require less energy (Lettinga et al., 2001). To date, research has shown satisfactory reactor performance and development of methanogenic activity during low-temperature AD of a wide range of wastewaters (Collins et al., 2006), animal manures, and slurries (Massé et al., 2007), indicating that low-temperature AD may become a widespread approach in the future.

11.3.2 pH

pH influences the utilization of carbon and energy sources, the efficiency of substrate dissimilation, the synthesis of proteins and various types of storage material, and the release of metabolic products from the cell during AD (Elefsiniotis and Oldham, 1994). The optimal pH for methanogens ranges between 6.8 and 7.2, while for acidogens it is lower (approximately 6; Moosbrugger et al., 1993). The growth rate of methanogens, and acetoclastic methanogens in particular, decreases sharply below pH 6.6. In a single-stage anaerobic treatment process, the pH is typically maintained at conditions more suitable for methanogens to prevent overgrowth of acidogenic bacteria, which can result in the accumulation of organic acids, the inhibition of methanogenesis, and process failure (Steffen et al., 1998). Indeed, falling pH accompanying accumulation of VFA is the main cause of toxicity and reactor failure in the AD process (Ahring et al., 1995). The toxicity of VFAs is also pH dependent, since only the nonionized forms are toxic to microorganisms. The concentrations of acetic, propionic, and butyric acids are considered to be the best indicators of the metabolic state of the most sensitive microbial groups in the anaerobic system and are therefore important in process monitoring (Ahring et al., 1995; O'Flaherty et al., 1998a).

11.3.3 Nutrients

The optimum carbon/nitrogen/phosphorus ratio for high methane yield is reported to be 100 : 3 : 1 (Rajeshwari et al., 2000). If the C/N ratio is high, there is a risk of nutrient deficiency, and a low buffering capacity will result in a more sensitive process, whereas if the nitrogen content is high, ammonia inhibition problems may arise. The digestibility of carbohydrate-rich wastes can be improved by mixing them with those containing high amounts of nitrogen to improve the C/N ratio (co-digestion; Ahring et al., 1992; Kaparaju et al., 2002; Macias-Corral et al., 2008).

The study of the effects of micronutrients on AD is a promising field of research (Gonzalez-Gil et al., 1999; Fermoso et al., 2008). Trace elements (i.e., sulfur, potassium, calcium, magnesium, iron, nickel, cobalt, zinc, manganese, and copper) are required for efficient AD and are usually present in sufficient amounts in most agricultural waste that are treated in anaerobic digesters (Steffen et al., 1998). Difficulties due to trace element limitation can arise, however, during AD of energy crops as the sole feedstock, unless supplementation is provided.

11.3.4 Substrate Constituents

The composition and anaerobic biodegradability of agricultural residues is variable, dependent on location and production process (Tables 11.2 and 11.3). There may also be a significant number of toxic or inhibitory compounds present in agricultural and food-processing residues, including:-

- High ammonia levels, pesticide residues, sheep dip, heavy metals, oil
- Bioagents (aflatoxins and antibiotics)
- Disinfectants (e.g., high levels of cresol and phenol, arsenic)
- Heavy metals

Although shock loadings of such compounds will undoubtedly cause problems for any biological treatment process, AD has been shown to be quite robust with respect to exposure to toxicants and recovery from inhibition (Colleran, 1992; Angenent et al., 2008; Nielsen and Angelidaki, 2008).

11.4 ANAEROBIC DIGESTION OF AGRICULTURAL RESIDUES

The major advantages of AD with respect to agricultural residues include the following (Colleran, 1992; Mahony et al., 2002):

- Organic waste stabilization (oxygen demand and odor reduction)
- Energy recovery as biogas
- Applicability to a wide range of feedstocks (animal manures and slurries, crop residues, food-processing wastes)
- Pathogen and weed seed destruction
- Inorganic nutrient recycling where field capacity for landspreading is available (inorganic fertilizer substitution)
- Potential for generation of a solid fibrous fraction (soil conditioner) and a high-value liquid fertilizer

Challenges associated with the installation of agricultural AD plants include the initial high capital costs, safety hazards with biogas production, and the need for a consistent, year-round demand for the energy produced (Mahony et al., 2002).

The AD of agricultural manures and wastes occurs on three levels: (1) small on-farm plants, treating individual farm arisings only; (2) commercial on-farm plants, where the farmer accepts and treats off-farm residues to increase biogas output and ensure commercial viability; and (3) centralized anaerobic digestion plants, treating waste arisings from a region, on a commercial, off-farm basis (Mahony et al., 2002).

11.4.1 On-Farm Anaerobic Digestion

On-farm AD of agricultural residues began during the energy crisis of the 1970s, when many countries provided financial incentives for renewable energy production and recycling. For example, a survey carried out in 1983 identified a total of 546 on-farm

Table 11.2 Characteristics and Operational Parameters of Agricultural Residue Digesters[a]

Feedstock	Total Solids (%)	Volatile Solids (%)	C/N Ratio	Retention Time (days)	Biogas Yield (m^3/kg VS)	CH_4 Content (%)	Inhibitors	Potential Problems
Swine manure	3–8	70–80	3–10	20–40	0.25–0.50	70–80	Antibiotics, disinfectants	Scum layers, sediments
Cow manure	5–12	75–85	6–20	20–30	0.20–0.30	55–75	Antibiotics, disinfectants	Scum layers
Poultry manure	10–30	70–80	3–10	> 30	0.35–0.60	60–80	Antibiotics, disinfectants	NH_4^+ inhibition, scum layers
Whey	1–5	80–95	n.a.	3–10	0.80–0.95	60–80		pH reduction
Fermentation slops	1–5	80–95	4–10	3–10	0.35–0.55	55–75		High acid concentration
Leaves	80	90	30–80	8–20	0.10–0.30	n/a	Pesticides	
Straw	70	90	90	10–50	0.35–0.45	n/a		Poor digestion
Garden waste	60–70	90	100–150	8–30	0.20–0.50	n/a	Pesticides	Poor degradation of cellulose
Food waste	10	80	n.a.	10–20	0.50–0.60	70–80	Disinfectants	Sediments

Source: Data from Steffen et al. (1998) and Mahony et al. (2002).
[a]n.a., not available.

Table 11.3 Sources, Composition, and Biodegradability of Anaerobic Feedstocks

Compounds	Sources	Examples	Anaerobic Biodegradability
Carbohydrates			
Sugars	Beet, corn	Breweries, distilleries, beet processing	Excellent
Starch	Beet, corn	Milk processing, potato chip processing	Excellent
Cellulose	Straw, grass, wood	Farmyard manure, harvest remains	Poor–good
Proteins	Animals and animal products	Milk processing, pharmaceutical industry	Excellent
Fats	Animals and animal products	Slaughterhouses, rendering plants	Excellent
Long-chain fatty acids	Fats, grease, oils, evaporation condensates	Rendering plants, oil mills	Excellent
Trace organic compounds	Pesticides, antibiotics, detergents	Pharmaceutical industry, manure	Poor
Inorganic material	Salts, food additives, silica gel (filtration)	Slaughterhouses, manure, food industry	Nonbiodegradable
Sand, grit	Stable walls and floors	Manure	Nonbiodegradable
Metals	Packaging material, process remains	Organic municipal solid waste, industry	Nonbiodegradable
Plastic	Packaging material	Organic municipal solid waste, industry	Nonbiodegradable
Heavy metals	Metal refining, batteries	Organic municipal solid waste, industry	Nonbiodegradable

Source: Data from Steffen et al. (1998) and Mahony et al. (2002).

AD plants in the European Union and Switzerland (Demuynck et al., 1984). The total working digester volume involved was 296,000 m^3, with a net biogas production of 33,000 metric tons of oil equivalent. About one-third of the on-farm plants were "do-it-yourself" units, and operational problems identified included pipe blockages, inefficient digester mixing, feedstock pump failure, gas leakage, and poor general plant maintenance. Inefficient gas usage also resulted in poor plant economics and was a major factor in the shutdown of many of these first-generation AD plants (Demuynck et al., 1984).

On-farm anaerobic digestion plants constructed since the late 1980s are of more efficient design, with improved operation and decreased maintenance costs (Lansing et al., 2008). The majority of on-farm plants are operated at mesophilic temperatures, with hydraulic and solids retention times of 20 to 30 days. Most on-farm plants use the biogas produced for space heating (domestic, piggeries, greenhouse) or for combined heat and power (CHP) production, with sale to a national or local grid of the electricity produced. In virtually all cases, the treated slurry is disposed of by landspreading without separation into a liquid and solid fraction (Mahony et al., 2002; Lansing et al., 2008).

11.4.2 Commercial On-Farm Anaerobic Digesters

Many on-farm digesters constructed during the past decade treat animal manures/slurries as the primary organic substrate, but also accept other organic wastes as co-digestion substrates (Lansing et al., 2008). These on-farm plants differ from centralized AD plants in being smaller scale and owned by an individual farmer rather than by a cooperative or utility. The treated waste is disposed of by landspreading. The biogas produced is generally used in CHP plants to provide electricity, which is sold to a national or local grid, and heat, which is used for on-farm applications (Raven and Gregersen, 2007; Lansing et al., 2008).

A more recent development has been the use of AD for biogas production from energy crops such as maize silage, grass silage and cereal grains. For example, in Germany there were over 3750 commercial on-farm biogas plants installed in 2007 (Costa-Gomez, 2008; Schattauer, 2008). The proportion of German biogas production from energy crops increased from <5% in 2003 to 15% in 2007, with the remaining feedstocks being made up of manures and food wastes. Increasingly, specialized AD plants designed to handle high-solids feedstocks (dry-fermentation), such as the DRANCO process, are increasingly operated using crops as the sole feedstock (Mata-Alvarez, 2002; Schattauer, 2008).

11.4.3 Centralized Anaerobic Digestion Plants

Centralized anaerobic digestion (CAD) plants (Figure 11.2) are generally operated by a private energy utility or by a local farmers' cooperative. CAD plants accept animal manures, together with other waste arising from local food-processing plants, abattoirs, breweries, and so on. Sewage sludge and the source-separated organic fraction of municipal solid waste (MSW) are also used occasionally as an additional organic substrate for CAD plants (Raven and Gregersen, 2007).

CAD plants are operated either mesophilically or thermophilically and at solid/liquid retention times of 12 to 20 days. Multiple digesters of about 2000 to 4000 m^3 are generally utilized (Figure 11.2). A pre- or postpasteurization/hygienization stage is

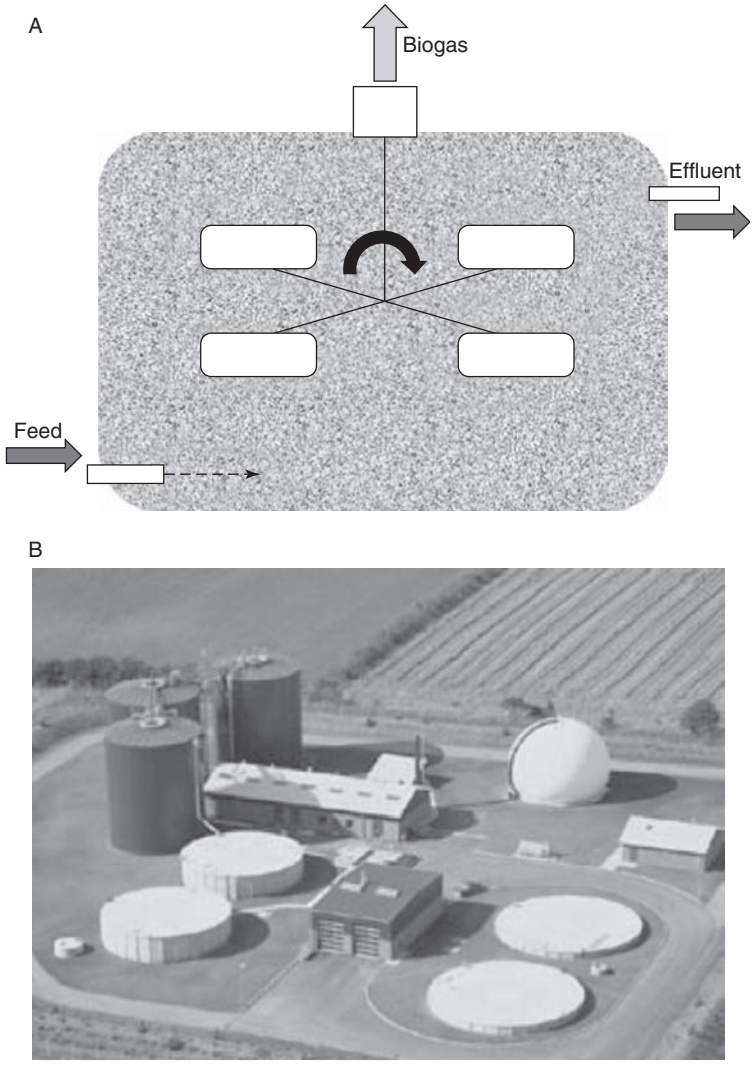

Figure 11.2 (A) Schematic diagram of a typical continuously stirred tank reactor (CSTR), here stirred mechanically with paddle mixers, but gas mixing by injection of biogas is also commonly employed. (B) Aerial view of Ribe centralized anaerobic digestion biogas plant in Denmark. (Image courtesy E. Colleran.) Three thermophilic CSTR digesters (1750 m^3 each) can be seen on the left, along with a spherical gas storage vessel, feedstock and digestate storage tanks, and reception area. The plant was commissioned in July 1990 and is owned by a cooperative involving local farmers, slaughterhouse owners, and a local power station. It treats approximately 301 metric tons of manure and 42 metric tons of industrial wastes (abbatoir and other) per day, at a retention time of 12 to 14 days. (From Raven and Gregersen, 2007.)

usually included to prevent fecal pathogen carryover in the treated waste (Raven and Gregersen, 2007). At least 50°C for a minimum of several hours is recommended (Bendixen, 1994). In Denmark, CAD plants that co-digest sewage sludge or household waste must incorporate a pre- or posthygienization treatment at 70°C for at least 1 hour (Christensen, 1995). Since not all of the feedstocks used in CAD plants require such

elevated-temperature treatment, different feedstocks may be subjected to different hygienization regimes to reduce the energy costs involved (Prisum and Norgaard, 1992).

The biogas produced is used to provide district heating with or without electricity generation in CHP plants, to generate steam or electricity for local industry, or increasingly, as vehicle fuel. The treated digestate is either landspread directly or separated into a liquid and a solid fraction. The solid fraction is usually composted prior to sale as a soil conditioner or horticultural product (Raven and Gregersen, 2007).

11.5 ORGANIC RESIDUES (FEEDSTOCKS) SUITABLE FOR ANAEROBIC DIGESTION

The agricultural and food-processing wastes commonly used in AD plants include (1) cattle and swine manures and slurries, (2) poultry manure (with or without litter), (3) abattoir wastes, (4) vegetable processing residues (e.g., from potatoes, sugar beets) and energy crops (maize, grass silage, cereals), (5) silage effluent, (6) dairy processing residues (e.g., cheese and yoghurt processing), (7) brewery residues, (8) fish oil and fish processing wastes, and (9) canning wastes and wastewaters. Feedstocks for CAD plants may also include sewage sludge and the organic fraction of municipal solid waste. Table 11.2 summarizes the chemical characteristics and potential biogas yields for some important AD feedstocks (Steffen et al., 1998).

The primary ingredients of the feedstocks are carbohydrates, proteins, fats, and long-chain organic acids. Although the majority of these organic compounds are readily anaerobically degradable, the presence of sand, grit, inorganic additives, heavy metals, and nonbiodegradable plastics may pose problems during the AD process (Table 11.3; Steffen et al., 1998). The lignocellulosic components of animal manures require long retention times, and digestion is usually incomplete. However, the stabilized, incompletely digested fraction is of value as a soil conditioner.

There are several advantages to using a mix of feedstocks. The primary advantage is the enhancement of the biogas yield per cubic meter of reactor, with consequent financial benefit to the plant operator, but co-digestion is also reported to result in more efficient digestion of some feedstocks (Ahring et al., 1992; Macias-Corral et al., 2008), perhaps due to co-metabolic or other synergistic effects. Furthermore, when organic wastes and residues are mixed with manure, the result will normally be the relatively cheap and environmentally sound recycling of these wastes as fertilizer.

Pretreatments, including, for example, removal of excess sand, grit, and long straw and maceration of animal manures containing bedding and crop residues, may be advantageous for some feedstocks (Steffen et al., 1998). Since AD requires a relatively narrow pH range to proceed (6.8 to 8.0; Steffen et al., 1998), feedstocks should ideally be within this range and some pH control may be necessary. Since acidification of carbohydrate-rich wastes is rapid at ambient temperatures, it is recommended that feedstocks be fed to the digester as soon as possible to maximise biogas yield and avoid process disturbance (Ahring et al., 1992).

11.6 ANAEROBIC BIOREACTORS FOR AGRICULTURAL APPLICATIONS

The main requirements for an AD plant include minimization of mechanical and electrical equipment requirements, effective insulation and use of corrosion-resistant

materials, simple design and automatic operation, and equipment fail-safe devices and environmental controls (Warburton, 1997; Ward et al., 2008). There are three main types of AD reactor configuration: *batch reactors* are filled with the feedstock, left for a period (the hydraulic retention time), and then emptied; in *one-stage continuously fed systems* (either plug-flow or completely mixed), all the methanogenic reactions take place in one reactor; and in *two- or multistage continuously fed systems*, hydrolysis/acidification and acetogenesis/methanogenesis are separated. Solid waste digesters are also divided into wet or dry types. *Wet reactors* are those with a total solids value of 16% or less, while *dry reactors* have between 22 and 40% total solids, and those that fall between wet and dry are considered semidry (Mata-Alvarez, 2002; Ward et al., 2008). Dry reactor technology is used primarily with municipal solid waste, vegetable wastes, or energy crops.

One-stage, plug-flow, and completely mixed systems such as the continuously stirred tank reactor (CSTR) are most common for AD of agricultural residues (Warburton, 1997; Raven and Gregersen, 2007). The principle of plug-flow design is a one-step, once-through biomethanation system with a predominantly horizontal flow, which may be mechanically assisted (Figure 11.3).

Whereas plug-flow digesters are commonly utilized in on-farm plants, CSTRs are employed primarily for larger CAD plants (Figure 11.2). Efficient mixing is important to ensure that digestion is complete; to prevent short-circuiting; to maximize pathogen removal, to ensure uniform heat transfer, and to prevent sedimentation of silt in the reactor. Several methods of mixing are commonly used, including gas mixing, where biogas is collected, compressed, and injected back into the digester, usually through lances, promoting mixing from the gas bubbles (Warburton, 1997).

Multistage AD plants attempt to separate the hydrolysis/acidification processes from acetogenesis/methanogenesis to improve the stability of AD compared to one-stage systems, particularly when digesting easily hydrolyzable feedstocks (Mata-Alvarez, 2002; Parawira et al., 2008). Instability can be caused by fluctuations in organic loading rate, heterogeneity of wastes, or excessive inhibitors. Multistage systems provide some protection against a variable organic loading rate, as the more sensitive methanogens are buffered by the first stage. Thus, the material passing from the first stage to second

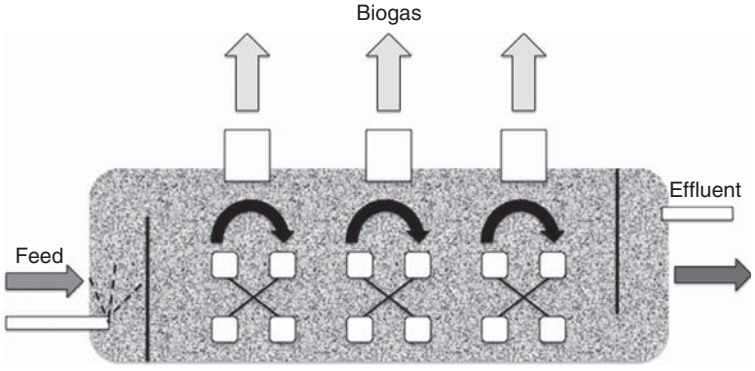

Figure 11.3 Schematic diagram of a typical on-farm, plug-flow anaerobic digester, with paddle mixers to avoid sedimentation and enhance biogas production. Unmixed systems are also commonly employed.

has become homogenised and therefore more stable (Mata-Alvarez, 2002; Parawira et al., 2008). However, multi-stage digesters are more expensive to build and maintain, but are generally found to have a higher performance than single-stage digesters (Ward et al., 2008).

Although the main process stage is the AD tank(s), a range of preliminary and postdigester units and a range of structures are necessary, particularly for CAD plants. These include (1) appropriate waste reception and loading facilities; (2) mixing devices; (3) gas storage and handling equipment, including pipework, valves, and flare stack; (4) electricity-generating equipment, powered by ignition engines converted to run on methane, gas turbines, and electricity generators; (5) boilers and heat exchangers to provide heat for the digester; (6) appropriate storage for digestate, (7) control and monitoring equipment, and (8) odor control equipment (Warburton, 1997; Mahony et al., 2002).

For all AD plants, digester capacity is related directly to the design retention time, the operating temperature, and the quantity of material processed. Increasing retention times within the reactor releases more biogas and cuts down postdigestion methane release. However, excessive retention times are not economical. Therefore, installed capacity becomes an economic balance between the capacity cost and biogas production (Mahony et al., 2002). In large-scale CAD plants, reactors should be multiple units to (1) ensure continuity of treatment if a reactor malfunction occurs or maintenance is required, and (2) reduce the visual impact of the plant by the use of smaller reactors. The number of reactors for a given capacity will depend on the optimum financial solution, local planning restrictions, and operational factors (Warburton, 1997; Ward et al., 2008).

11.7 PRODUCTS OF ANAEROBIC DIGESTION

11.7.1 Biogas

The biogas production is relatively constant in CSTR digesters, with a mean production of 1 m^3 of biogas per cubic meter of working volume of digester per day from mesophilic digesters treating animal manure (Demuynck et al., 1984; Raven and Gregersen, 2007). However, the biogas production can be significantly higher under thermophilic conditions or where mesophilic co-digestion with food processing wastes is practiced. Biogas is collected using either floating or fixed covers (Prisum and Norgaard, 1992; Raven and Gregersen, 2007).

The potential biogas production and energy output from various types of animal waste is summarized in Table 11.4. Many food-processing wastes (e.g., fish oils, blood, distillery slops) yield considerably more biogas per unit volatile solids. Up to one-third of the biogas energy may be needed to sustain the heat in the digester, while surplus biogas can be burned to generate heat either on site or piped elsewhere (Cantrell et al., 2008). If a combined heat and power (CHP) plant is used, all of the biogas produced is generally consumed to provide both heat and electricity. High-quality biogas can be exported to the distribution network to replace natural gas. However, a gas cleaning process is first required to remove carbon dioxide and trace contaminants. Purified biogas can achieve a methane content of 95%, which compares favorably to the typical natural gas methane content of around 85%. It is also possible to use the clean gas

Table 11.4 Biogas Production and Energy Output Potential from One Metric Ton of Various Fresh Feedstocks

Feedstock	No. Animals to Produce 1 Metric Ton/Day	Dry Matter Content (%)	Biogas Yield (m^3/metric ton feedstock)	Energy Value (MJ/m^3 biogas)
Cattle slurry	20–40	12	25	23–25
Pig slurry	250–300	9	26	21–25
Laying hen litter	8000–9000	30	90–150	23–27
Broiler manure	10,000–15,000	60	50–100	21–23

Source: Adapted from information from the Energy Technology Support Unit; Warburton (1997), and Mahony et al. (2002).

to run motor vehicle engines, which need prior conversion to gas use (Cantrell et al., 2008).

11.7.2 Digestate

The recycling of livestock waste to land is environmentally appropriate and constitutes a valuable source of macro- and micronutrients. If manure is the major feedstock in AD plants, the macro- and micronutrient content of the digestate is similar to untreated manures and slurries. Although AD does not significantly alter the nitrogen/phosphorus/potassium (NPK) content of livestock wastes, it does affect the chemical form of nitrogen, in particular. The content of ammonium-nitrogen is generally 20% higher in digested than in untreated cattle slurry (Nielson et al., 1997). Consequently, the replacement of synthetic fertilizer by landspreading of digestate maximizes plant nitrogen uptake and minimizes the risk of nitrate leaching to ground or surface waters (Nielson et al., 1997).

In CAD plants, the digestate is often separated into solid (fiber) and liquid fractions (Chiumenti et al., 1991). Energy use is between 0.01 and 0.6 kWh/m^3. The fiber produced from AD is bulky, contains a low level of plant nutrients, and is an ideal soil conditioner. In the majority of European CAD plants practicing digestate separation, the fiber fraction is aerobically composted to provide a stable and marketable peat moss substitute. Screw and ram presses may be able to produce up to 60% dry solids material (Chiumenti et al., 1991), which is suitable for combustion, often in combination with wood chips, municipal solid waste, or sewage sludge, in incinerators for electricity generation and district heating. The liquid fraction is either locally landspread or marketed as a liquid fertilizer for amenity areas such as golf courses. Due to its high water content, the liquid also has irrigation benefits and can be used for "fertigation" on agricultural land (Chiumenti et al., 1991).

11.8 FUTURE PERSPECTIVES FOR ANAEROBIC DIGESTION OF AGRICULTURAL RESIDUES

Significant advances are being made toward opening the microbiological "black box" of the AD processes. Important anaerobes have recently been cultivated and characterized

(Narihiro and Sekiguchi, 2007), but there remains much to discover with respect to the huge number of anaerobes as yet uncultured. In this regard, the application of culture-independent methods to assess the in situ ecophysiology of methanogenic consortia is proving very valuable (Collins et al., 2006). An improved understanding of the microbiology underpinning AD should allow both improved management of existing plants and facilitate its application to a broader range of scenarios.

The generation of biogas as vehicle fuel appears to be a very promising future direction for the AD of agricultural residues (Björnsson and Mattiasson, 2007). Due to the increased demand for land for the production of biomass for food, chemicals, materials, and energy, it is important to prioritize production systems, processes, and products that are efficient in terms of land area used and environmental impact (including greenhouse gas emissions). Based on these criteria, biogas vehicle fuel along with second-generation vehicle fuels based on lignocelluloses will be much more efficient than the majority of biofuels currently being produced. Indeed, biogas has a far better performance with regard to both area efficiency and life-cycle emissions, and it is therefore a strong potential candidate for becoming one of the most sustainable vehicle fuels in the near future (Björnsson and Mattiasson, 2007).

Finally, a number of emerging areas with respect to anaerobic biotechnology are apparent, which may have significant impacts for the AD of agricultural residues. For example, there is increasing interest in developing low-carbon, renewable, hydrogen-producing technologies. Hydrogen can be produced through dark anaerobic fermentation using carbohydrate-rich residues, while methane can then be produced in a methanogenic second stage. A two-stage AD system for production of hydrogen and methane from agricultural residues was recently proposed by Martínez-Pérez et al. (2007), who also reported that very significant amounts of energy could be produced from land not currently utilized for agriculture (set aside) in the UK. Recent developments regarding the potential of microbial fuel cells to produce electricity directly from waste and wastewater could also suggest a future direction of the technological development of the AD process (Logan et al., 2006), and much interesting microbiological and process information is likely to emerge from this area.

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CHAPTER 12

Anaerobic Biodegradation of Solid Waste

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12.1 INTRODUCTION AND OBJECTIVES

Solid waste is a residual of human activity that must be managed in every community. As with domestic wastewater, proper treatment and disposal of solid waste is required for both human and environmental well-being. The improper management of waste can result in fire hazards, breeding grounds for rodents and insects, groundwater contamination, and air pollution, including emissions of greenhouse gases.

Solid waste is comprised of a mixture of many components, as described later in the chapter. Several waste components contain biodegradable organic matter, and an understanding of the biological reactions involved in solid waste decomposition is relevant to proper waste management. Landfills receive over half of all solid waste generated in the United States, and the biologically produced methane that is generated in landfills is recovered for energy at over 400 landfills. However, fugitive methane emissions also make landfills an important source of greenhouse gas emissions (USEPA, 2008).

The objective of this chapter is to provide an overview of solid waste generation and the biological reactions involved in its decomposition. The emphasis will be on anaerobic processes that reflect decomposition in landfills and in engineered treatment alternatives. The generation and composition of solid waste is described in the following section. This is followed by a description of a modern landfill in which solid waste decomposes under anaerobic conditions. We then describe the reactions involved in the anaerobic conversion of solid waste to methane (CH_4) and carbon dioxide (CO_2) and the application of these biological reactions to landfills. Information on the microbiology of solid waste decomposition is presented and we conclude with a discussion of engineered processes (i.e., anaerobic digesters) for solid waste treatment.

12.2 SOLID WASTE COMPOSITION AND MANAGEMENT

Solid waste is typically divided into a number of categories based on the source or point of generation. Categories include municipal solid waste (MSW) as well as commercial,

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industrial, agricultural, and construction and demolition (C&D) waste. For this chapter, solid waste excludes wastes that are defined by regulation as hazardous, as hazardous wastes have unique characteristics and are managed in a different infrastructure.

In the United States, MSW has been defined by the U.S. Environmental Protection Agency (EPA) to include wastes from residential, multifamily, commercial, and institutional (e.g., schools, government offices) sources. However, this definition excludes many materials that are frequently disposed with MSW in landfills, including ash from MSW combustion, residuals that are generated from potable water treatment, biosolids that are generated during biological wastewater treatment, C&D waste, and nonhazardous industrial process wastes (USEPA, 2007). Examples of the latter category include solid wastes from the food-processing industry and paper mills.

Waste generation and composition will be presented based on U.S. data because there are well-defined data sets. Waste generation and composition are similar throughout the developed world, and the reader is referred to Loughlin and Barlaz (2006) for a discussion of global waste management. It is difficult to compare waste composition data across countries because the waste included varies from only residential waste at one end of the spectrum, to all waste managed in the municipal system (e.g., C&D, nonhazardous industrial) at the other end of the spectrum. This complexity is exacerbated by the fact that some definitions include high-moisture-content residuals from water and wastewater treatment. These high-weight streams dilute the composition of more traditional dry solid wastes, such as paper and plastics.

In 2006, the EPA estimated MSW generation of 228 million megagrams in the United States, which is equivalent to 2.09 kg MSW/(person-day). This does not mean that each person disposes 2.09 kg of MSW at home each day, but that the activity of the average person, including the products they purchase and the waste they generate during the day, will average 2.09 kg or perhaps more based on other studies (Simmons et al., 2006). The manner in which this waste is managed is illustrated in Figure 12.1.

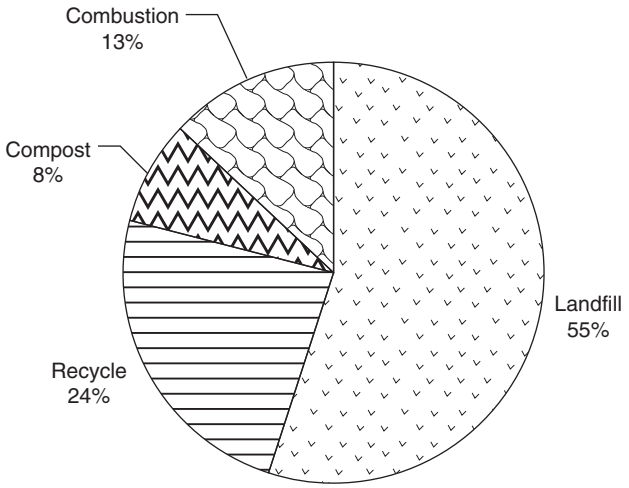

Figure 12.1 Waste management in the United States. Combustion represents waste that is burned, with recovery of the resulting energy as steam or electricity. (Adapted from USEPA, 2007.)

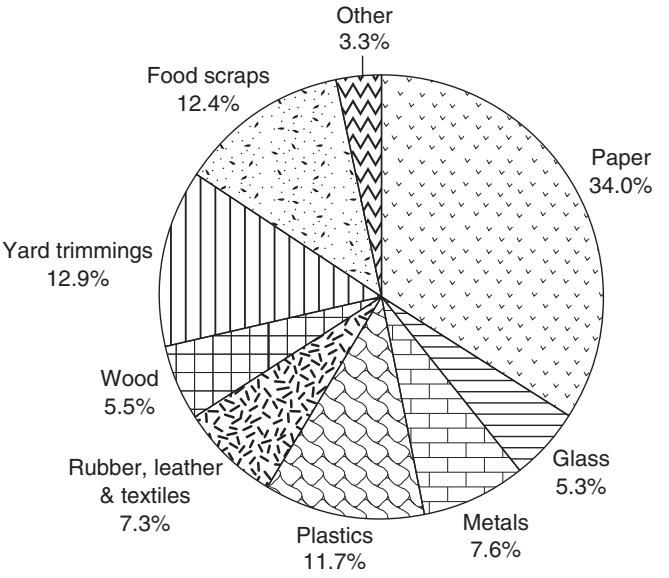

Figure 12.2 Composition of MSW in the United States. (Adapted from USEPA, 2007.)

Information on waste composition is needed to estimate the amount of biodegradable organic carbon and to estimate the amount of recyclable material. The composition of MSW in the United States is presented in Figure 12.2. It is important to recognize that the composition in Figure 12.2 represents MSW as generated and for several reasons is not representative of the waste that is buried in landfills. First, as illustrated in Figure 12.1, some waste is diverted for composting and recycling. Second, many other types of waste are buried in landfills. Table 12.1 shows an estimate of the composition of MSW actually discarded in landfills after correction for waste that is composted and recycled. The more detailed composition data presented in Table 12.1 serve to illustrate the range of biodegradable (e.g., paper), recyclable (glass, plastics, paper), and nondegradable (e.g., plastics, rubber) materials is MSW. C&D waste also contains biodegradable organics, including lumber (33.2%) and cardboard (8.0%) (Jang and Townsend, 2003).

Waste may also be characterized by the composition of the major organic components, which is most useful toward understanding its biodegradation. Cellulose and hemicellulose are the principal biodegradable components of MSW, while the other major organic component, lignin, is at best only slowly degradable under methanogenic conditions (Colberg, 1988). Reported cellulose, hemicellulose, and lignin concentrations in residential refuse range from 28.8 to 54.3%, 6.6 to 11.9%, and 12.1 to 28% of dry weight, respectively (Barlaz, 2006). Data on the cellulose, hemicellulose, and lignin concentrations in municipal waste components are summarized in Table 12.2.

Although the precise composition of waste buried in landfills varies, it is well established that waste contains large concentrations of cellulosic materials. Paper is the major contributor to the cellulose and hemicellulose content of waste, with additional contributions from wood, yard debris, and food waste.

Table 12.1 Composition of Discarded Municipal Solid Waste[a]

Waste Component	Percent Wet Mass	Waste Component	Percent Wet Mass
Organics	37.8	Durable goods	14.0
Tires	1.5	Appliances/electronics	2.7
Textiles	4.2	Carpet	1.6
Wood (non-C&D)	7.3	Misc./bulky items	9.6
Food waste	17.7		
Yard trimmings	7.1	Metal	1.4
Misc. organics	0.2	Steel cans	0.5
		Other ferrous	0.1
Paper	23.8	Aluminum cans	0.5
Newspaper	0.9	Other nonferrous	0.4
Office paper	4.3		
Mixed paper	2.9	Residues	2.2
Glossy paper	0.9		
OCC/kraft bags	5.7	Glass	4.9
Composite/misc.	9.3		
Plastics	15.8	Total MSW	100
HDPE containers	1.0		
PET containers	0.9		
Other containers	0.4		
Film/wrap/bags	3.1		
Other plastics	10.4		
Appliances/electronics	2.7		
Carpet	1.6		
Misc./bulky items	9.6		

[a]Data were calculated from the mass of each component generated minus the amount recovered for recycling and composting as reported by the USEPA (2007).

Table 12.2 Chemical Composition and Ultimate Methane Yield of Solid Waste Components

	Cellulose (%)	Hemi-cellulose (%)	Lignin (%)	Methane Yield (mL/dry g)[a]
Newsprint	48.5	9	23.9	74.3
Office paper	87.4	8.4	2.3	217.3
Corrugated cardboard	57.3	9.9	20.8	152.3
Coated paper	42.3	9.4	15	84.4
Branches	35.4	18.4	32.6	62.6
Grass	26.5	10.2	28.4	136.0
Leaves	15.3	10.5	43.8	30.6, 56.8
Residential food waste	55.4	7.2	11.4	300.7, 152.9, 207.0
Hardwood lumber	40.47	19.58	23.55	Not measured
Softwood lumber	41.95	20.29	27.39	Not measured

[a]Data are based on measurements in 2-L reactors that were operated to maximize methane production (Eleazer et al., 1997). Additional values represent samples tested in subsequent studies.

12.3 INTRODUCTION TO LANDFILLS

As 55% of MSW and many other solid wastes are disposed in landfills, a basic understanding of the design of a landfill is helpful. In the United States, the design and operation of landfills is regulated by Subtitle D of the Resource Conservation and Recovery Act, the New Source Performance Standards of the Clean Air Act, and related state regulations. Landfills have evolved from open dumps to highly engineered facilities designed to contain waste and separate it from the environment, capture contaminated water that contacts the waste (leachate), and control gas migration. A landfill site is typically excavated and lined with a system that includes layers to (1) minimize the migration of leachate to the groundwater, and (2) collect leachate for treatment. A common system used to restrict leachate migration consists of a 0.67- to 1-m-thick clay layer with a hydraulic conductivity of no more than 10^{-7} cm/s overlain with a geomembrane (GM). The GM is typically 1.5-mm-thick polyethylene. A drainage layer that contains a high-permeability material such as sand or gravel is placed above the liner to promote leachate collection. This layer has perforated pipes embedded to remove leachate from the landfill. A protective barrier is then installed above the leachate collection system to shield it from the equipment used to place and compact refuse. Waste may then be placed above the protective barrier, and it is covered daily to minimize wind blown refuse, odors, and the attraction of disease vectors. Daily cover alternatives include a 15-cm soil layer, spray-on foams, and synthetic materials that are rolled over the waste at the end of the working day. Once refuse has reached the design elevation, a final cover is applied. The final cover will include, at a minimum, a layer of low-permeability soil designed to minimize stormwater infiltration overlain by a layer of soil that will support vegetative growth. The final cover frequently includes a drainage layer and a GM beneath the vegetative layer. Vegetation serves to minimize erosion of the soil cover and to promote evapotranspiration. A typical cross section of a landfill is illustrated in Figure 12.3.

12.4 ANAEROBIC DECOMPOSITION OF SOLID WASTE

As described above, cellulose and hemicellulose are the major biodegradable components of MSW. Thus, to understand the biodegradation of waste in landfills, it is important to understand the general pathway of anaerobic biodegradation which is a complex process that requires the coordinated activity of several trophic groups of microorganisms (Madigan et al., 2003). Common electron acceptors in anaerobic systems such as NO_3^- and SO_4^{2-} are present in limited quantities or are quickly consumed in decomposed municipal refuse, leaving organic compounds and CO_2 as the dominant electron acceptors.

The first reaction is the hydrolysis of polymers (carbohydrates, fats, and proteins), which yields soluble sugars, amino acids, long-chain carboxylic acids, and glycerol. Fermentative bacteria then convert these hydrolysis products to short-chain carboxylic acids (primarily acetate, butyrate, propionate), ammonia, CO_2, and H_2. Alcohols and other carboxylic acids are also formed. Next, fatty acid–oxidizing bacteria oxidize such products as propionate and butyrate to acetate, CO_2, and H_2. Oxidation of propionate and butyrate is only thermodynamically favorable at very low H_2 concentrations (Zinder, 1993). Thus, the bacteria that oxidize propionate and butyrate only function

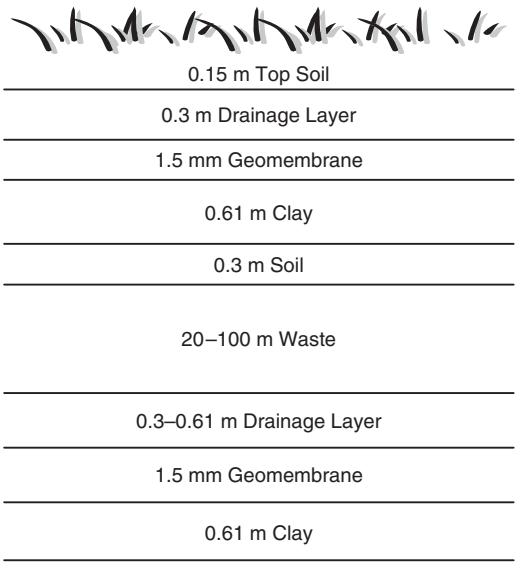

Figure 12.3 Typical cross section of a modern landfill permitted to receive nonhazardous solid waste.

in syntrophic association with an H_2 scavenger such as a methanogen or a sulfate reducer to allow for efficient interspecies hydrogen transfer. The significance of the homoacetogenic reaction, the production of acetate from CO_2 and H_2, has not been established in the landfill ecosystem.

The terminal step in the conversion of complex polymers to methane is carried out by the methanogenic archaea. The most common methanogenic substrates are acetate and CO_2 plus H_2. Most methanogens have a pH optimum around 7 (Zinder, 1993). Should the activity of the fermentative organisms exceed that of the carboxylic acid degraders and methanogens, there will be an imbalance in the ecosystem. Carboxylic acids and H_2 will accumulate and the pH of the system will fall, thus inhibiting methanogenesis. The general scheme of anaerobic substrate biodegradation and microbial community relationships illustrated in Figure 12.4 applies to anaerobic ecosystems as diverse as the rumen (e.g., cows, termites), wetlands, rice paddies, anaerobic digesters, and landfills. Typical metabolic reactions occurring during waste decomposition are summarized in Table 12.3.

It has long been thought that the majority of methane was generated directly from acetate by aceticlastic methanogens (*Methanosarcina* or *Methanoseata* spp.). However, research has suggested that syntrophic acetate oxidation, where acetate is first converted to H_2 and CO_2 by fermentative bacteria and then to CH_4 by methanogens, is significant under conditions similar to those found in landfills. For example, the dominant CH_4 generation pathway shifts from aceticlastic methanogenesis to syntrophic acetate oxidation under high ammonia concentrations (Schnurer and Nordberg, 2008), and it has been suggested that acidic or sulfate-limited conditions may cause a similar shift (Hattori, 2008). Similar to propionate and butyrate oxidation, H_2 concentrations must be very low for acetate oxidation to be energetically favorable.

Typically, sulfate concentrations in landfills are minimal and methane is the major electron sink. A possible exception to this is landfills in which significant quantities of

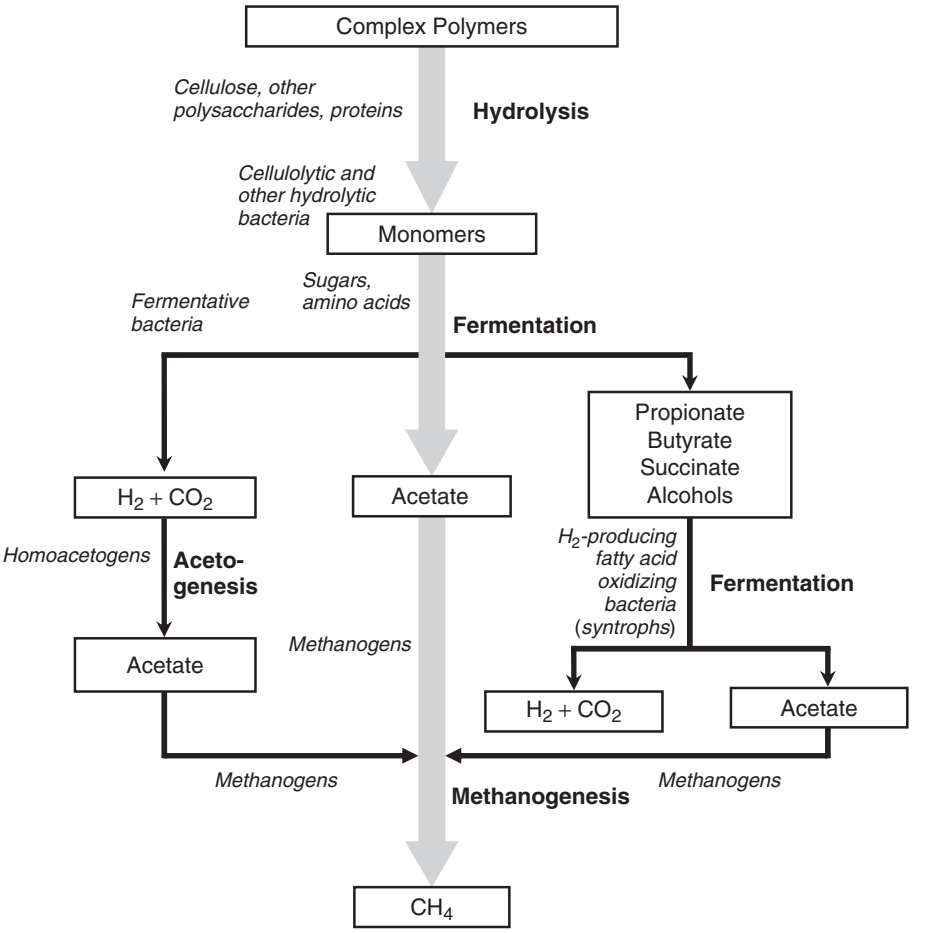

Figure 12.4 Overall process of anaerobic decomposition showing the manner in which various groups of fermentative anaerobes act together in the conversion of complex organic materials to methane and carbon dioxide. (From Madigan et al., 2003.)

Table 12.3 Primary Acetogenic and Methanogenic Reactions During Refuse Decomposition

Reaction			$\Delta G_0'^a$ (kJ/reaction)
Acetogenic reactions			
(1) $4H_2 + 2HCO_3^- + H^+$	$\rightarrow$	$CH_3COO^- + 4H_2O$	−104.6
(2) $CH_3(CH_2)_2COO^- + 2H_2O$	$\rightarrow$	$2CH_3COO^- + H^+ + 2H_2$	+48.3
(3) $CH_3CH_2COO^- + 3H_2O$	$\rightarrow$	$CH_3COO^- + HCO_3^- + H^+ + 3H_2$	+76.1
Syntrophic acetate oxidation			
(4) $CH_3COO^- + 4H_2O$	$\rightarrow$	$4H_2 + 2HCO_3^- + H^+$	+104.6
Methanogenic reactions			
(5) $4H_2 + HCO_3^- + H^+$	$\rightarrow$	$CH_4 + 3H_2O$	−135.6
(6) $CH_3COO^- + H_2O$	$\rightarrow$	$CH_4 + HCO_3^-$	−31.0

Source: Adapted from Stams et al. (2003) and Hattori (2008).
[a]Gibbs free energy for the reaction at pH 7; values taken from Thauer et al. (1977).

gypsum board has been buried. Substantial sulfate reduction to hydrogen sulfide can occur concurrent with methanogenesis in decomposing refuse (Fairweather and Barlaz, 1998). Other potential inorganic electron acceptors, such as manganese (Mn^{4+}) and iron (Fe^{3+}), are probably present in concentrations that are not significant relative to the quantity of biodegradable organic matter.

12.4.1 Decomposition in Landfills

Landfills represent a complex and unique anaerobic ecosystem and are involved in the global cycling of organic carbon. Landfills serve as a repository for biogenic carbon that is biodegraded to CH_4 and potentially recovered for energy. While the residence time of the waste is essentially infinite, the system is poorly mixed, and substrate inputs vary in both quantity and quality. Methane may be recovered from landfills for use as an energy source, as occurs at an estimated 445 U.S. landfills (LMOP, 2007). When CH_4 is recovered for beneficial reuse, it results in avoided emissions from power plants that rely on fossil fuels for energy production. However, as a result of CH_4 that is not collected, landfills are estimated to be the second-largest source of anthropogenic CH_4 emissions in the United States (US EPA, 2008).

Landfills also represent a significant sink of biogenic carbon. Not all of the biogenic carbon that is buried in landfills degrades and that which does not degrade is said to be *sequestered*. Virtually all of the lignin that is buried in a landfill is sequestered, as is some of the cellulose and hemicellulose. Although cellulose and hemicellulose are intrinsically biodegradable under anaerobic conditions, they do not degrade completely, as some are protected by lignin and are not bioavailable. In addition, waste decomposition in a landfill is by no means complete, as environmental conditions are often suboptimal. Estimates of the fraction of MSW components that do biodegrade under even the most favorable optimal conditions range from 5% carbon sequestration of office paper to 42% sequestration for newspaper (Barlaz, 1998). Based on the composition of waste presented in Table 12.2 and published carbon sequestration factors, we estimate that at least 0.11 kg of carbon are sequestered per dry kilogram of refuse buried in a landfill (Staley and Barlaz, accepted for publication).

The burial of solid waste in a landfill initiates a complex series of chemical and biological reactions that has been described in a series of phases (Barlaz et al., 1989a). In the first phase of decomposition (aerobic phase), the O_2 entrained in the refuse at burial is depleted, large quantities of CO_2 are produced, the refuse temperature increases from the waste heat of aerobic decomposition, and the system becomes anaerobic, as there is no mechanism for depletion of the entrained oxygen in a modern landfill. (Note that in open dumps with shallow waste and little cover, some oxygen replenishment and aerobic biodegradation may occur.) In the second phase (anaerobic acid phase), there is an imbalance between the activity of the hydrolytic and fermentative bacteria, which convert cellulose and hemicellulose to soluble intermediates, and the acetogenic and methanogenic microorganisms, which work together to convert these intermediates to CH_4 and CO_2. Due to this imbalance, short-chain carboxylic acids accumulate, the pH decreases, and there is little solids decomposition. During the acid phase of decomposition, the leachate contains a high chemical oxygen demand (COD) that is attributable to carboxylic acids. Because these acids are biodegradable, the highest BOD and COD concentrations in the leachate will be measured during this phase (Kjeldsen et al., 2003).

Methane production begins in the third phase of decomposition (accelerated methane) and increases exponentially due to the accumulation of soluble substrate (i.e., carboxylic acids). Carboxylic acid concentrations decrease with corresponding decreases in the leachate COD and BOD and a pH increase. In the fourth phase of decomposition (decelerated methane), the accumulation of carboxylic acids is depleted and the rate of CH_4 production is dependent on the rate of cellulose and hemicellulose hydrolysis. In this phase the BOD/COD is relatively low because dissolved organic matter that is degradable is consumed as rapidly as it is produced. Some COD is present in the leachate, but it is mostly due to recalcitrant compounds such as humic and fulvic acids (Christensen et al., 1994).

The four phases of refuse decomposition described above have been defined on the basis of both field and laboratory-scale data and are summarized in Figure 12.5. In theory, over long periods of time, refuse will decompose until all degradable organic matter is exhausted. These hypothetical phases of decomposition have been described (Kjeldsen et al., 2003).

Environmental conditions in the landfill will have a significant impact on the rate of refuse decomposition. Studies on factors affecting refuse decomposition have been summarized (Barlaz et al., 1990; Komilis et al., 1999). The factors that have most consistently been shown to affect the rate of refuse decomposition are the moisture content and pH, and it is generally accepted that refuse buried in arid climates decomposes more slowly than refuse buried in regions that receive greater than 50 to 100 cm of annual precipitation. Whereas high concentrations of carboxylic acids are often considered to be inhibitory in anaerobic ecosystems, laboratory studies suggest that the microbial communities resident in waste can acclimate to at least of 20, 5, and 15 g/L of acetate, butyrate, and propionate, respectively (Wang et al., 1997).

Landfill operation has evolved over the past several decades. Initially, landfills were operated to minimize water infiltration and therefore decomposition. This is a relic of the days when landfills did not include leachate collection systems and leachate generation often resulted in groundwater contamination. With the advent of leachate collection and treatment, there has been increased interest in the operation of landfills to maximize waste decomposition and CH_4 production. This is done by the recirculation of leachate and sometimes other liquids through the waste. Landfills operated to enhance decomposition are referred to as bioreactor landfills and there are several reviews of this practice (Reinhart et al., 2002; Benson et al., 2007).

12.4.2 Stoichiometry of Waste Decomposition

The volume of gas that can be produced during anaerobic biodegradation can be calcuated as (Parkin and Owen, 1986).

$$C_nH_aO_bN_c + \left[n - \tfrac{a}{4} - \tfrac{b}{2} + 3\left(\tfrac{c}{4}\right)\right]H_2O \rightarrow \left[\tfrac{n}{2} - \tfrac{a}{8} + \tfrac{b}{4} + 3\left(\tfrac{c}{8}\right)\right]CO_2 + \left[\tfrac{n}{2} + \tfrac{a}{8} - \tfrac{b}{4} - 3\left(\tfrac{c}{8}\right)\right]CH_4 + cNH_3 \qquad (12.1)$$

Caution must be employed in the application of equation (12.1), as it should only be applied to the biodegradable components of solid waste. A general formula for solid waste that includes cellulose, hemicellulose, lignin, and plastic should not be

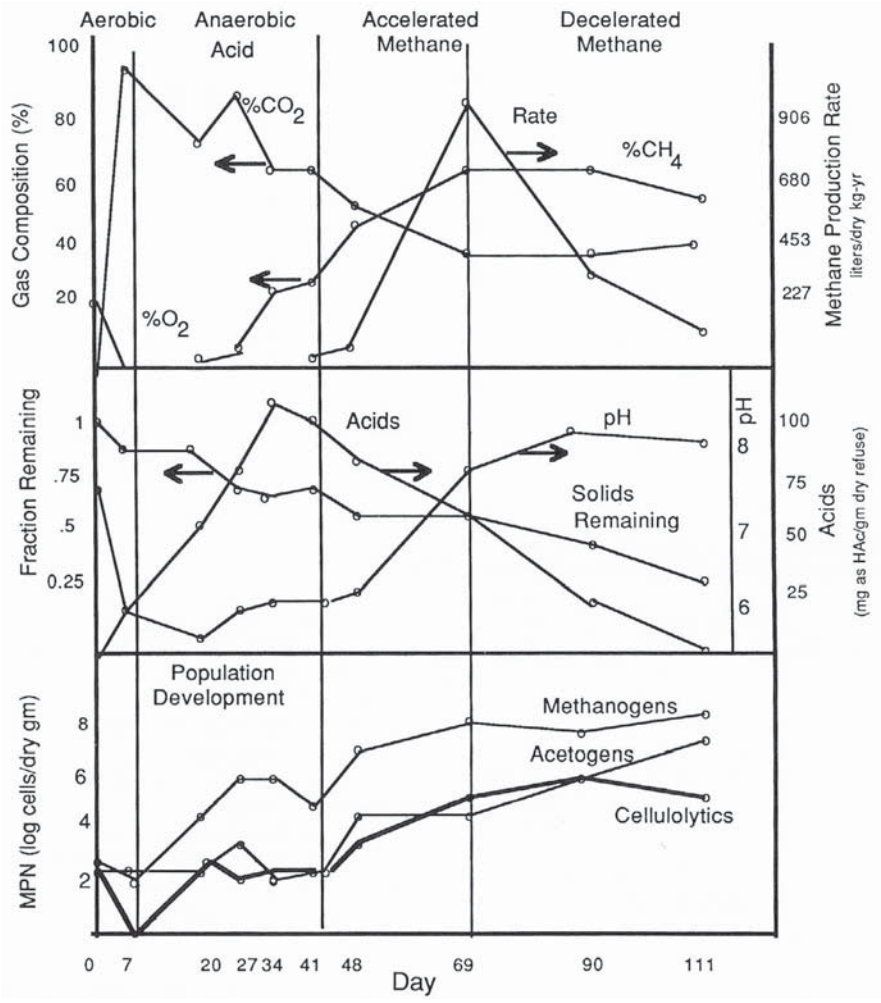

Figure 12.5 Summary of trends observed in refuse decomposition with leachate recycle in a laboratory system. The acids are expressed as acetic acid equivalents. The solids remaining are the ratio of the weight of cellulose plus hemicellulose removed from a container divided by the weight of cellulose plus hemicellulose added to the container initially. Methanogen MPN data are the log of the average of the acetate- and H_2/CO_2-utilizing populations. (From Barlaz et al., 1989a.)

used in equation (12.1), as the lignin and plastic contain organic carbon but are not biodegradable. The application of equation (12.1) to cellulose results in

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow 3nCO_2 + 3nCH_4 \tag{12.2}$$

Equations (12.1) and (12.2) represent the net reactions of the coordinated activity of the trophic groups described in Figure 12.4 and suggest the production of 414.8 and 424.2 mL of CH_4 per dry gram of cellulose and hemicellulose decomposed, respectively. The measured methane yields for the major biodegradable components of waste

are presented in Table 12.2. Note that the measured values are well below the theoretical yields that would be calculated from equations (12.1) and (12.2) because even under optimal conditions, not all of the cellulose and hemicellulose will biodegrade.

12.5 LANDFILL MICROBIOLOGY

Landfills represent a rich but relatively unexplored anaerobic ecosystem. A more complete understanding of landfill microbiology may lead to more sustainable management practices by reducing greenhouse gas emissions and improving CH_4 recovery for beneficial use (e.g., heating, electricity generation). This unique ecosystem may also harbor organisms that possess useful traits for biotechnological applications.

The dearth of information on landfill microbiology is due in part to the difficulty associated with its study. Solid waste contains varying fractions of paper, plastic, metals, food scraps and other components, and particle sizes within this agglomeration of materials range from millimeters to meters. Methods that have been used to culture microorganisms from refuse and to extract nucleic acids from refuse are described in the first part of this section, followed by information on the microbial ecology of landfills.

12.5.1 Methods for Microbial Isolation and Nucleic Acid Extraction from Solid Waste

Accurate assessments of microbial ecology and function in solid waste are predicated on obtaining cells or extracted nucleic acids that are representative of the community sampled. Both culture-based and molecular techniques have been used to study landfill microbiology. Culture-based techniques typically involve separation of live cells from the sample followed by incubation in the laboratory using selective growth media to determine if specific microbial groups or species are present (Hilger and Barlaz, 2006). In contrast, molecular methods do not require growth of microorganisms since nucleic acids can be extracted directly from the sample. Regardless of whether culture-based or molecular methods are used, the heterogeneous composition dictates that refuse samples be processed to reduce particle size and ensure some degree of uniformity.

The culturing of microbes from refuse has typically been performed by suspending a sample in growth media (Fielding et al., 1988), water (Luton et al., 2002), or a phosphate buffer (Barlaz et al., 1989b; Westlake et al., 1995; Pourcher et al., 2001). After suspension, the mixture is typically homogenized by blending. An additional step of hand squeezing (Barlaz et al., 1989b) is then used to generate a particulate-containing liquid fraction, and further separation of larger particulates can be achieved by squeezing the refuse mixture through a fine mesh material (Pourcher et al., 2001; unpublished data). The resulting liquid, or supernatant, is then used as a basis to culture microbes.

It has been estimated that less than 1% of all microbial species in nature have been characterized using culturing techniques (Rondon et al., 1999). This is due to microbial diversity and the difficulty in culturing many microbes in a laboratory. Compared to culture-based techniques, the use of molecular techniques is advantageous since they allow for analysis of uncultured organisms and offer higher resolution. Another

advantage of molecular methods is that in situ conditions of the microbial community can be measured or inferred. For instance, DNA levels of specific populations can be used to estimate that population's biomass since most microorganisms harbor a single copy of their genome per cell. On the other hand, the metabolic contribution of specific microbial groups to the total population can be assessed by monitoring the fraction of RNA contributed by the specific population to the total community RNA. However, there are limitations in the use of molecular techniques, and they are discussed extensively elsewhere (Stams et al., 2003).

Most studies employing molecular techniques have used leachate rather than the solid fraction of refuse to evaluate microbial diversity. This is probably due to the relative ease with which leachate can be collected and processed for DNA or RNA extraction compared to processing and extraction of the solid material (refuse). For example, commercial kits and many laboratory protocols used to extract nucleic acids require sample masses of 0.25 to 1 g with lower and upper limits of roughly 0.1 and 10 g, respectively. This range is governed by a number of factors, including available tube size, reagent volume/sample mass ratio, and cell lysing efficiency. Such small masses, along with the large particle sizes found in refuse, often preclude direct placement of refuse into extraction tubes. Another issue is the difficulty of getting representative DNA or RNA from a heterogeneous matrix such as refuse, leading to biases that can yield a measured microbial community structure that differs from the true microbial diversity.

The potential issue with using leachate rather than solids for nucleic acid extraction is that the microbial communities found in leachate and solids can differ. This has been demonstrated in a study comparing the microbial community structures in solids and leachate samples from the same landfill, using molecular methods (Staley et al., unpublished data). The results show that (1) dominant bacteria diversity is greater in solids in well-decomposed refuse; (2) although the archaeal diversity is similar in both phases, different populations were detected; and (3) the relative abundances of microorganisms are substantially different between solids and leachate. Thus, whether leachate or solid material should be used for molecular analysis will depend on the study objectives.

Methods to process solid refuse samples for recovery of nucleic acids have recently been developed and compared. Direct sample processing methods, where cells are lysed and nucleic acids are released directly into the sample matrix, have been used more frequently than indirect methods, where cells are separated from noncellular debris prior to lysing. The direct methods assume that material placed in extraction tubes is representative of the ecosystem sampled; however, this assumption is tenuous for refuse. After extensive review of existing protocols, seven methods (four direct methods, three indirect methods) were evaluated using a molecular fingerprinting method: terminal restriction fragment length polymorphism (T-RFLP).

T-RFLP creates a unique signature of the microbial community by grouping polymerase chain reaction–amplified nucleic acids into operational taxonomic units (OTUs). Although an OTU may represent multiple organisms, each OTU is unique to the ecosystem sampled. Thus, if different processing methods are used on the same sample, one would expect the same OTU signature for all methods. Any measured differences in this OTU signature would indicate that one method is biased against another. One way to evaluate methods is to compute the total number of OTUs under the assumption that the method with the most OTUs recovers the highest number of

species and thus is least biased. Microbial community structure estimated based on T-RFLP can be evaluated using a common ecological statistic, Jaccard's coefficient (Magurran, 2004), to indicate which methods were most similar to the total measured microbial diversity. The Jaccard coefficient is based on the presence or absence of a species within a sample and can be expressed as

$$J = \frac{\text{number of OTUs in common between two samples}}{\text{number of OTUs in both samples}} \tag{12.3}$$

Table 12.4 shows the number of OTUs and community similarity (based on Jaccard's coefficient) recovered from accelerated methane phase refuse using each of the seven methods. The Bacteria and Archaea domains were compared and it was found that an indirect or cell separation method that involves blending refuse in phosphate (PO_4) buffer followed by hand squeezing of the mixture through a mesh material proved to be the least biased method for recovering nucleic acids. After hand squeezing, the supernatant (liquid) fraction is then centrifuged and the resulting pelletized material is used for nucleic acid extraction. A similar method is typically used to harvest live cells for culturing. Another indirect method where a detaching agent (methylcellulose) is added to the PO_4 buffer performed equally well but proved more cumbersome than using PO_4 buffer alone. The use of hand squeezing also is advantageous since a larger sample size can be consolidated into a small pellet for DNA or RNA extraction. For a more complete discussion of molecular techniques and their applications to anaerobic microbiology, the reader is referred to Madigan et al. (2003) and Stams et al. (2003).

Table 12.4 T-RFLP Results Showing the Effect of Sample Processing Methods on the Number of OTUs[a] and Microbial Community Structures in Methane-Phase Decomposed MSW

	Bacteria		Archea	
Method[b]	OTUs Recovered	Similarity[c]	OTUs Recovered	Similarity[c]
Direct[d]				
1: Phosphate buffer	28	0.85	16	0.84
2: Freeze–thaw[e]	19	0.52	15	0.74
3: Sodium dodecyl sulfate[f]	19	0.55	15	0.79
4: Dry and grind[g]	6	0.15	15	0.74
Indirect[h]				
5: Phosphate buffer	27	0.85	18	0.95
6: Methylcellulose[f]	27	0.85	17	0.89
7: Methylcellulose + Tween 80[f]	26	0.79	17	0.84

[a] OTUs, operational taxonomic units.
[b] Samples are blended in phosphate buffer except method 4.
[c] Similarity is Jaccard's coefficient (Magurran, 2004) relative to total measured diversity. The higher the number, the more similar the method is to total measured diversity.
[d] A subsample of material was used for nucleic acid extraction after processing.
[e] Three freeze–thaw cycles performed prior to blending in phosphate buffer.
[f] This chemical was added to the phosphate buffer prior to blending.
[g] Material dried at 75°C, then ground into a powder.
[h] Blended material squeezed through mesh material; supernatant used for nucleic acid extraction.

12.5.2 Microbial Ecology of Landfills

Dominant Bacteria and Archaea in Landfills Several studies of landfill microbial demographics have been performed using 16S rRNA gene clone libraries (Pourcher et al., 2001; Huang et al., 2002, 2003, 2004; Chen et al., 2003; Uz et al., 2003; Calli et al., 2006; Laloui-Carpentier et al., 2006). The most dominant bacteria found include low G + C content gram-positive bacteria (e.g., *Bacillus, Clostridia*), *Cellulomonas, Microbacterium, Lactobacillus, Pseudomonas*-like γ–proteobacteria, and members

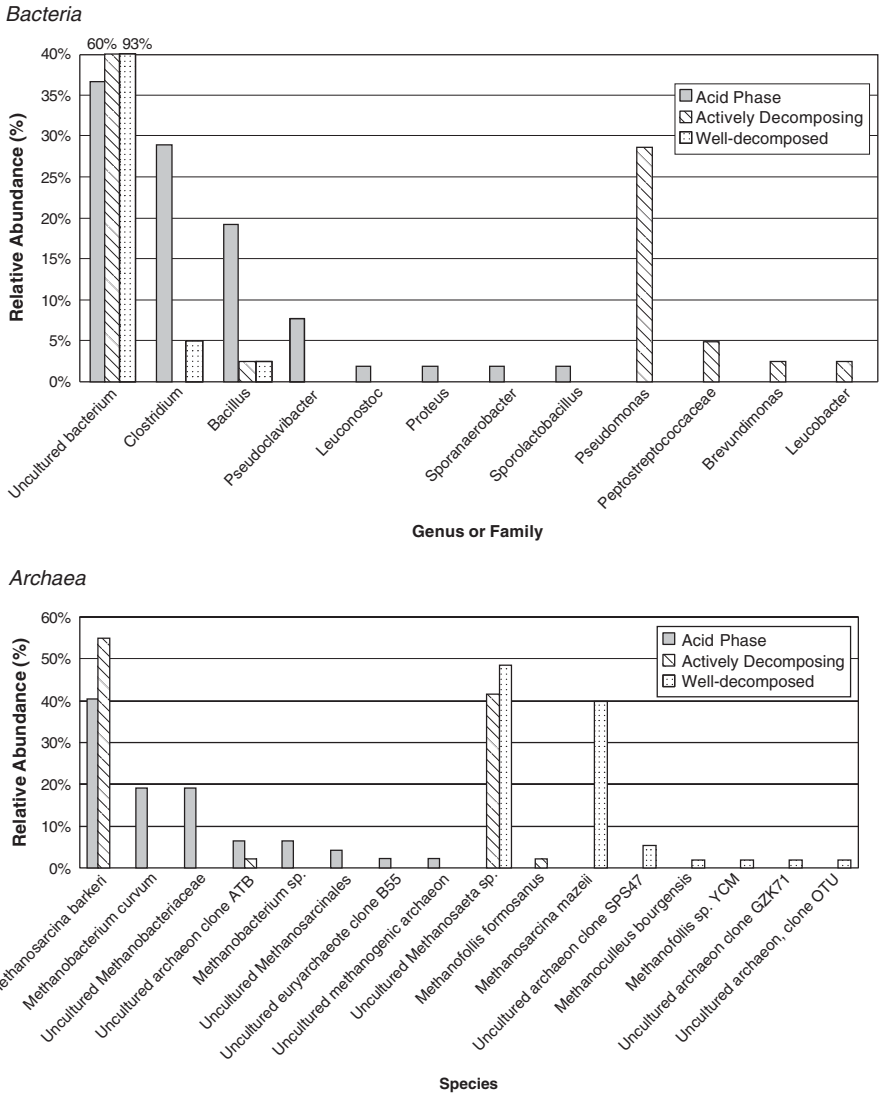

Figure 12.6 Microbial ecological succession during refuse decomposition in acid, accelerated methane (actively decomposing), and decelerated methane (well decomposed) phases for bacteria and archaea. Results are from 16S rRNA gene clone library samples from the same reactor at each phase of degradation.

of the Chlamydiae/Verrucomicrobia and Cytophaga–Flexibacter–Bacteriodes groups. Dominant Archaea found under mesophilic conditions include *Methanobacterium, Methanoculleus, Methanosarcina, Methanosaeta*, and uncultured *Euryarchaeota. Methanothermobacter* and uncultured *Methanobacteriaceae* were also dominant under thermophilic conditions (Chen et al., 2003). A number of less abundant genera were also detected. In terms of abundance, the majority of methanogens found tend to be hydrogenotrophic or use both hydrogen and acetate for energy.

Anaerobic fungi are known to biodegrade lingocellulosic substrates under anaerobic conditions in the rumen. The anaerobic gut fungi *Neocallimastigales* has been detected in two landfills in the United Kingdom using PCR with specific oligonucleotide primers (Lockhart et al., 2006).

Microbial Community Demographics During Refuse Decomposition Many chemical parameters change substantially during refuse decomposition, including pH, carboxylic acids concentrations, and solids composition (Figure 12.5). Changes to the physical structure within the refuse also occur as degradable solids are converted to gas. This affects refuse density, porosity, sorption characteristics, composition, and moisture content. Many chemical compounds are utilized or generated microbially, and solid materials are consumed as a substrate and used for cellular attachment. Thus, microbial community demographics, chemical parameters, and physical structure are interdependent.

As described above, the pH and carboxylic acid concentrations change as refuse is decomposed and this results in variable rates of CO_2 and CH_4 generation and also affects microbial population development (Figure 12.5). Bacterial and archaeal clone libraries targeting the 16S RNA gene show that substantial microbial ecological succession occurs as refuse decomposes (Figure 12.6). Ecological succession is the gradual replacement of species with new microorganisms as a result of changing environmental conditions. For example, the bacterial genus *Clostridia* contains both fermenting and cellulose-degrading species and is dominant in the acid phase, where substrate for fermenting organisms is plentiful, and the well-decomposed phase, where primarily cellulose remains. No *Clostridia* were detected during the actively decomposing CH_4 phase because fatty acid–oxidizing bacteria probably outcompete fermenting microorganisms during this stage of decomposition. For methanogenic archaea, *Methanosarcina barkeri* is dominant in the acid and methane phases but is replaced by *Methanosarcina mazeii* in the well-decomposed phase. This is because *M. barkeri* appears to function over a broader pH range than *M. mazeii* (Ferry, 1993).

12.6 ALTERNATIVE BIOLOGICAL PROCESSES

Portions of the solid waste stream are biodegradable and are amenable to other forms of biological treatment, including aerobic composting and anaerobic digestion. There are two aspects to each of these processes: (1) the fraction of the waste stream that can be collected for treatment, and (2) the actual treatment process. Although there has been work on the aerobic and anaerobic treatment of the entire MSW stream, more recently the emphasis has been on the organic fraction of MSW (OFMSW). The OFMSW includes yard waste, food waste, and soiled paper (e.g., paper towels,

napkins, tissue). Other biodegradable organics in MSW (e.g., office paper) are typically collected separately and recycled. In addition to MSW, some commercial facilities generate relatively large quantities of wastes that are amenable to biological treatment such as the food-processing industry and grocery stores.

In the United States, the disposal of yard waste is banned in many states. Yard waste is often collected separately and treated by aerobic composting. In a few cities, residents are permitted to add food waste and soiled paper to yard waste and the mixture is treated in aerobic compost systems. Composting systems are beyond the scope of this chapter, but the treatment process has been presented by Krogmann and Korner (2000), and compost microbiology has been described by Kutzner (2000). Conceptually, the waste to be composted is aerated by either turning a compost pile regularly, or by blowing or drawing air through the compost pile. After a period of time that is dependent in part on the intensity of aeration, a finished product is available for use as a soil amendment.

The OFMSW can also be treated anaerobically, which has the benefit of methane production. The microbiology of anaerobic treatment in an engineered reactor is the same as that described for a landfill. The major difference is the sophistication of the process and the retention time. At the end of an anaerobic process, the waste can be cured aerobically and used as a soil amendment as for aerobic composting.

Whether anaerobic or aerobic, good separation of the OFMSW from other waste components at the point of generation is essential for production of a soil amendment. Mechanical systems can be used for the removal metals, glass, and plastics, but separation at the point of generation is best. In some systems, the feedstock is manually sorted prior to biological treatment. Shredding and milling to reduce particle size are common in both aerobic and anaerobic systems.

A variety of engineered anaerobic reactors to treat OFMSW have been developed and are in full-scale use. These systems can be classified according to (1) the solids content of the feed, (2) the number of stages (one-stage versus multiple stages), (3) the temperature of operation (mesophilic versus thermophilic), and (4) the manner by which feed is introduced into the reactor (continuous versus batch).

When the feed is less than 20% solids, the process is called *wet digestion*. To create a slurry, low-solids waste streams such as wastewater sludge are added to OFMSW, and the resulting process is called *co-digestion*. Recycled process water is also used to lower the solids content. Processes that can treat solids concentrations as high as 35 to 40% are known as *dry digestion* systems.

Some processes use a single phase for the digestion; hydrolysis, fermentation, and methanogenesis all occur in a single reactor. In a two-phase process, hydrolysis and acidogenesis occur in the first phase, while methanogenesis occurs in the second phase, in a separate reactor. The phasing allows optimization of pH (lower in the first reactor and closer to neutral in the second reactor), mixing, retention times, and loading rates. The additional reactor makes it possible to minimize inhibitory conditions to the methanogens in the second phase.

Anaerobic reactors are operated under either mesophilic conditions, at a temperature of about 35°C, or under thermophilic conditions, at a temperature of about 55°C. These temperatures correspond to methanogen optimal temperatures, and intermediate temperatures are not typically used. The elevated temperatures are usually achieved by using some of the energy in the biogas produced. Whereas mesophilic reactors have the advantage of not requiring as much energy as thermophilic reactors, thermophilic

systems have the advantage of achieving pathogen reduction. In addition, research in the last 10 years has shown that thermophilic systems are not as unstable as previously thought, while still allowing higher rates of digestion. Temperatures can also be different for different stages, so that it is possible, for example, to have a mesophilic first stage followed by a thermophilic second stage. Combining phasing and staging has resulted in systems such as the temperature-phased anaerobic digester, where there is a thermophilic reactor for hydrolysis and acidogenesis, followed by a mesophilic methanogenic phase.

Feed can be introduced continuously (and discharged continuously) or in batch mode. Continuous-feed systems can be operated as completely mixed or as plug-flow reactors. In batch systems, OFMSW is mixed with inoculum (e.g., digested solids from a previous run), allowed to react for a certain detention time, and then discharged as a batch. Several full-scale systems (mostly in Europe) have been in operation for the past two decades. These include the DRANCO and Kompogas processes, continuous-flow, high solids, thermophilic, single-stage systems; the VALORGA process, a mesophilic semicontinuous system; the BIOCEL process, a batch high-solids mesophilic system; and the BTA process, a low-solids system that uses an upflow anaerobic sludge blanket reactor and a completely mixed reactor. Other systems that have been used at a demonstration scale include the RefCOM, SOLCOM, and SEBAC processes. The latter is a three-stage process that uses leachate recycle to achieve higher anaerobic digestion rates.

12.7 SUMMARY

Solid waste contains high concentrations of cellulose and hemicellulose that are biodegradable under both aerobic and anaerobic conditions. At this time, the majority of solid waste generated in the United States is disposed in landfills, where it decomposes to CH_4 and CO_2. The resulting landfill gas represents a source of energy. The anaerobic biodegradation of solid waste, whether it occurs in a landfill or in an engineered treatment process, requires the coordinated activity of several groups of microorganisms that are sensitive to pH. The microbial ecology of landfills is a relatively young area of study. Studies to date show a diverse population of bacteria and archaea that evolve over time as conditions in the landfill evolve from freshly buried to well-decomposed waste.

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CHAPTER 13

Low-Energy Wastewater Treatment: Strategies and Technologies

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13.1 THE LONG-TERM ENERGY PICTURE

Environmental engineering is, in essence, the implementation of strategies to break the link between economic growth and pollution. The provision of potable water and the safe disposal of wastewater is one of the most important and, in many societies, successful exemplars of this challenge. However, the suite of wastewater treatment technologies upon which we currently depend is energy intensive. In virtually all developed economies, the supply and treatment of a cubic meter of potable water uses about 0.5 kWh of electricity. Its subsequent collection and treatment require a further ~0.5 kWh (GWRC, 2008). These figures are remarkably constant across countries, climates, and regulatory systems. The UK figures are slightly higher (0.56 and 0.76 kWh/m^3, respectively). There is some national variation that presumably reflects local geography and treatment choices. For example, the most energy-intensive wastewater treatment company uses more than three times (1.4 kWh/m^3) more than the least intensive company (0.44 kWh/m^3) (Water UK, 2008). As a consequence, the water industry in developed economies is thought to use between 3 and 5% of the electricity. Moreover, this fraction is apparently a "fixed cost" (GWRC, 2008). Thus, a poorer society uncritically adopting the technology could find itself using 10 or 20% of its electricity in this manner. By the same token, as developed economies become more energy efficient, these "fixed costs" will become proportionally more important. This poses a long-term threat to the water industry and, by implication, the existence of sustainable urban societies. The threat is twofold.

First, the core assumption, that we are breaking the link between economic growth and pollution, is threatened and potentially destroyed by the carbon dioxide emissions inherent in the process. There is, to be polemical, no point in saving a river if you have to destroy a planet to do so. The need to break the link between carbon emissions and economic growth is widely recognised and will increasingly be embodied in legislation.

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Thus, in the UK the government has announced the carbon reduction commitment (CRC), a mandatory cap and trade system for those using more than 6000 MWh/yr of electricity when measured on half-hourly meters (the equivalent of an annual electricity bill of about £ 500, 000). From 2010 the water industry will have to purchase permission to emit from a diminishing pool of permits.

Second, the cost of energy is rising and, more important, is likely to remain high as global supply exceeds global demand. A temporary "blip" in the price of energy might not merit long-term investment or a fundamental technological change. However, if energy is expected to be expensive for the design life of the infrastructure, change is warranted.

The challenge for the water industry and its partners in research, consultancy, and manufacturing is to break the link between energy and carbon dioxide production in wastewater treatment. The benefits of doing so will not be cheaper water bills and compliance with the forthcoming regulations but, potentially at least, a whole new suite of wastewater treatment technologies, technologies that will cost less to build and run and are thus are within the grasp of middle- and even low-income urban populations the world over.

13.1.1 Guiding Principles

The challenge that we have described is an ambitious aim, but there are grounds for optimism. For example, since the calorific value of wastewater is 14.7 kJ/g COD (Logan, 2008), thus, at least in principle, we have within the waste itself some of the energy required to treat the waste. Moreover, the technologies we use at present do not represent the zenith of environmental engineering. Rather, they are technologies that are typically discovered accidentally and developed empirically, largely conceived and refined with only passing reference to the governing laws of thermodynamics and microbial ecology.

There is also good reason to be idealistic. In particular, we should strive to meet this goal without seeking to diminish or dilute our present and future obligations under national and regional environmental laws. This is both a principled stand and pragmatic science and science policy. As a matter of principle, we do not wish to reverse the decades of investment that have gone before us. For even if there was no climate crisis, there would still be a need to the protect the environment and public health in this and many other societies. Pragmatically, environmental legislation takes years, often decades to evolve and pass into law, especially at the European level. We cannot wait for the evolution of a more nuanced or considered environmental policy that will let us balance the cost of treatment and carbon emissions. Moreover, if we are to achieve this ambition, we must attract and retain engineers and scientists of ambition, talent, rigor, and originality. Such people will be more drawn to ambitious and principled research projects.

13.1.2 Technical Philosophy

Bruce Ritmann described the current generation of wastewater treatment systems as "falling off a log[1] technology." They cannot help but work. They were largely

[1]From the English idiom "as easy as falling off a log," meaning "to be very easy" (*Cambridge International Dictionary of Idioms*, Cambridge University Press, 1998).

developed and applied with, at best, modest understanding of the underlying science. Developments have been largely empirical, with little feel for the true limits of the technology and a tendency to take materials "off the shelf." The fundamental work that has been undertaken in relation to treatment is often poorly communicated to, and understood by, practitioners with only sincere but modest attempts at the translational research required to allow these ideas to be applied. Senior executives who sometimes have taken a sanguine view of the need for technology development have rested on the assumption that the industry can "buy in" any new technology that is required. But this *modus operandi* may not work in the future. If there were a "falling off a log" technology for low-energy wastewater treatment, we would have found it by now. The technologies required cannot be bought off the shelf, and there is an unprecedented degree of urgency. We should be aiming to have a significant effect by 2020, which in the UK is just two of the government-dictated investment cycles. Unfortunately, the long design lives of wastewater treatment infrastructure mean that each new plant potentially commits the undertaking to 25 to 50 years of emissions and enhanced energy costs.

We need a new approach more akin to the *Apollo* space program than to traditional wastewater research. We know where we want to get to, and we know that it is, in principle (i.e., thermodynamically), possible. We do not yet know how to take each step on the way. We need to identify those steps, believe that each step can be fulfilled, and fulfill them in parallel, not in series. We must be prepared to generate new technologies, techniques, and if necessary, basic science, to fulfill these steps. In doing so we must anticipate and plan for the failure of some options to fulfill their potential.

We can conceive of multiple and parallel strategies for achieving our goal, but they must be pursued rationally. This may require that we:

- Consider past investments as sunk costs (sunk costs are irrecoverable) in terms of carbon and energy and evaluate our research in terms of future savings only.
- Pursue parallel strategies.
  - Small investments: to lower costs on a short time scale by adapting existing technologies.
  - Wholly novel technologies: to eliminate energy use in the long term. These novel technologies may exploit existing sites and facilities or may require a "new build."
- Design and invest for much shorter time periods to enable the industry to be quick on its feet, adopting technologies quickly and cost-effectively dropping them when something better comes along.

Decision-making tools will be essential; we must evaluate technologies rationally using appropriate discounting rates. Energy and carbon saved now may be worth a great deal more than a larger saving made in 20 years' time (Hellweg et al., 2003). Fortunately, evaluating designs and stratagems in terms of cumulative fossil-fuel demand will often give us the lowest impact with respect to many other impact measures (Hellweg et al., 2003).

13.2 KNOWN OPTIONS

Energy (and thus CO_2) in wastewater is used for four main functions:

- Aeration for:
  - Carbon removal
  - Nitrification
  - Phosphorus removal
- Disinfection
- Micropollutant removal
- Pumping

Carbon dioxide is currently captured by the water industry (albeit accidentally) in nitrification and in certain extensive technologies such as ponds and reed beds.

The relative importance of each mechanism is site and process specific. The workhorse of the industry is the activated sludge process, which as a rule of thumb uses about 50 to 60% of the electricity on aeration. In principle, at least, no energy is required for carbon removal, and the process could be a source of energy for other treatment processes.

Nitrification absolutely requires molecular oxygen; however, some of that oxygen can be "used twice" if denitrification is required. Unfortunately, the best practice is still not informed by the best research (Rosso and Stenstrom, 2005; Park and Noguera, 2007) and there is no reason to believe that these systems are operating at their limits. Furthermore, nontraditional systems may remove ammonia by volatilization. Biological phosphorus removal is still very poorly understood, with only two biological technologies (luxury uptake and acid shock) being available at present (Mullan et al., 2006). However, it is worth noting that the biochemistry of phosphorus in microbes in treatment communities is still very poorly understood. Both micropollutant and pathogen removal can be achieved in nontraditional "extensive systems," but lower-energy oxidation technologies are still largely unexploited.

Pumping wastewater in any situation implies an assumption or calculation that the cost of pumping is less than the cost of building a treatment plant. This may not be true in the coming years. But this calculation reflects the technologies available to the engineer, and thus technologies that use less energy, are more compact, and are more reliable will help eliminate the need for pumping.

The prospects for small-scale carbon capture by the water industry are largely unrealized (Rosso and Stenstrom, 2008). However, such a technology will probably be based wholly or partly on biological processes, and the industry has an excellent track record of managing large-scale biological processes cheaply and ready access to the nutrients required to make such a process function. Carbon capture and storage will be a £5 billion market (Caffoor, 2008). Even a small fraction of this business would be well worth capturing. Moreover, an unfulfilled business opportunity of this size will draw in private capital and innovation. The most important single task is to eliminate or reduce the need for aeration in the removal of organic carbon removal, for organic carbon represents both the largest single energy sink and energy source and a major source of greenhouse gases in the water industry.

13.3 METHANOGENIC SYSTEMS

Methanogenic wastewater treatment systems are those in which the carbon in the wastewater is hydrolyzed, fermented, and broken down into acetate or hydrogen, which then donates electrons to carbon dioxide to produce methane gas. They are a well-established technology for sludge treatment, a process we do not discuss here. This is an established wastewater treatment technology that is very widely used in South America and to a lesser extent in tropical Asia. There are, for example, several hundred such systems in Brazil and Colombia, serving populations ranging from 200 to 600,000. The formats range from simple anaerobic ponds to more complicated and expensive upward-flow anaerobic sludge blanket and many variations upon that theme. In addition to using little or no energy, anaerobic systems have low yields and thus very low sludge production. The reactor volumes in South America are on the order of 0.1 to 0.5 m^3 per person and cost $25 to 100 per capita, not including posttreatment.

There is a voluminous empirical literature dealing with different methods of retaining biomass, capturing the gas, odor control, and so on. Much (though by no mean all) has been summarized by Van Haandel and Lettinga (1994). There are, however, two fundamental problems with the technology: loss of performance at low temperature, and poor effluent quality.

13.3.1 Temperature

Although there are many reports of full and pilot-scale treatment of domestic wastewater at ambient temperatures, there are no reports of effective anaerobic treatment of domestic wastewater at temperatures below 10°C. However, it is possible that reactors work at these temperatures, albeit briefly, in southern Brazil. In an experimental setting partial removal was observed at temperatures as low as 6°C, but performance was erratic.

The mechanisms governing the lower limit on temperature are poorly understood. The limit has been inferred empirically and the true rate-limiting step is unknown. Methanogenesis is thermodynamically favorable and, in nature, well documented at temperatures as low as −5°C (Hoj et al., 2005). However, psychrophillic methanogens may be naturally rare or nonexistent in temperate climates (Curtis et al., unpublished data). It is widely and probably correctly assumed that the performance observed at low temperatures in the published work represent modest acclimation by mesophillic organisms, not colonization by psychrophillic methanogens (Lettinga et al., 2001). However, methanogenesis may not be the limiting step. The hydrolysis of the wastes is reported (Elmitwalli et al., 2003) to be severely affected by lower temperatures. The temperature sensitivity of this mechanism has not been studied rigorously. A further very important point is the loss of dissolved methane in the effluent at low temperatures.

Low growth rates would exacerbate the impact of the loss of solids in the effluent, and a number of researchers are experimenting with the use of anaerobic membrane reactors to treat domestic wastewater in the UK (Hu et al., 2006; Soares et al., 2007). Performance is promising at modest temperatures (∼91% COD removal of domestic wastewater; effluent 90 mg COD per liter) and better than nonmembrane reactors at ambient temperatures, even in the tropics. This may be due in part to the physical rejection of low-molecular-weight substrate by the membrane. This technology has yet

to be challenged at temperatures characteristics of a continental winter. Moreover, the energy costs of sparging the filter have not been established.

13.3.2 Effluent Quality

The effluent quality of anaerobic systems is typically poor, Royal Commission standards (30 mg/L suspended solids and 20 mg/L BOD) for organic matter are not achieved, ammonia-nitrogen and phosphorus removal is negligible, and the removal of pathogens is only modest. This very poor removal of nutrients is probably an inevitable consequence of the biological conditions in anaerobic systems. Molecular oxygen is required for the oxidation of ammonia and phosphorus. The fundamental reason for the modest removal of organic matter is uncertain: It may represent the inherently low affinity (half saturation coefficient) of the anaerobic biomass for organic matter. However, it is possible that it is attributable to poor solids retention or hydraulic issues. If they proved cost-effective, membranes may go some way toward improving the removal of organic matter and possibly pathogens, and thus may find applications in a coastal plants. The fate of micropollutants is undocumented, although it is widely assumed to be poor, but this may not be the case.

However, the removal of nitrogen and phosphorus in anerobic systems will always be problematic. Indeed, in those countries where anaerobic domestic wastewater treatment systems are well established, the major research focus appears to be post-treatment of anaerobic effluents rather than further improvements in anaerobic processes per se. Post-treatment can take the form of any conventional aerated system, although presumably operating without the burden of organic matter removal, but with possible complicating factors such as sulfide in the influent.

13.3.3 Other Issues

Although nutrients and temperature are key issues in the context of the technology, many potentially "minor issues" such as odor and the efficient recovery of gas are likely to be very important in the near future. Moreover, anaerobic treatment systems have a reputation for instability and recovery from process failure can be a lengthy process. Therefore, failure must be avoided.

13.3.4 Applications

Incremental The incremental improvements in this approach will presumably be confined to sludge treatment. Most water companies are already committed to this line of action.

Adaptive Even if the current technology cannot be improved upon, anaerobic processes could be incorporated into current flow sheets. Possibly in lieu of primary sedimentation tanks, they may reduce energy costs and generate modest quantities of methane in the warmer months of the year.

Innovative If the temperature issue can be resolved, anaerobic treatment systems can wholly replace any wastewater treatment plant where only organic removal is required or where post-treatment nutrient removal is possible. Very compact (3- to 6-hour retention time) systems with adequate disinfection (possibly by virtue of the membrane) could replace pumping stations or for some or all of the year.

13.3.5 Research Questions

There are a cascading number of questions that can be answered to determine the potential application of this technology in domestic wastewater treatment in temperate climates such as the UK. One of the simplest and cheapest actions would be to document the nature of existing experience in anaerobic digestion in southern South America (i.e., don't reinvent the wheel).

It is crucial to determine and, if possible, lower the temperature limits of anaerobic digestion. Broadly speaking, there will be two strategies for improving the performance: biological and physical. The biological stratagems involve the acclimation and/or seeding of the reactor to operate at lower temperatures. This, in turn, will require a new generation of models and monitoring tools to ensure that the acclimation and seeding is reliable and successful. Even if successful, this approach will involve larger reactors (per capita) than are used at present. Physical strategies involve modest heating of the waste, and the use of membrane separators to minimize the loss of solids may offer quicker but more expensive solutions (Hu et al., 2006; Soares et al., 2007).

Attainable effluent standards will be critical, and the relationship between temperature and effluent quality will dictate if anaerobic reactors can be used as a stand-alone technology. Assuming that Royal Commission standards can be attained, micropollutant removal would be a considerable advantage.

High-performance stand-alone systems may require membrane separation. Both empirical and fundamental research about membranes are warranted. Empirical studies to improve existing membranes will be inevitable. However, it may be possible to develop novel membranes designed to minimize clogging in anaerobic systems using a polymer physics approach (Shannon et al., 2008).

The removal of pathogens, especially noroviruses, in membrane reactors would be highly beneficial and go some way to offsetting the cost of membranes in coastal plants. Reliable operation is critical. Rapid online and off-line monitoring of anaerobic systems will be desirable—but hopefully unnecessary. If inherently robust designs can be developed, there is evidence that the resistance to shock loads can be engineered (Hashsham et al., 2000). Assuming that the basic technology can be made to work, strategies to cope with dissolved methane and odor would need to be developed, and compact and reliable post-treatment nutrient removal technologies retrofitted in existing aerobic plants will perhaps be desirable.

13.4 MICROBIAL FUEL CELLS

Microbial fuel cells (MFCs) are also anaerobic systems in which the organic carbon in the wastewater is hydrolyzed, fermented, and broken down into simpler compounds, whose electrons are then donated to an electrode. Hydrogen ions formed at the anode travel to the cathode, where they are reunited with oxygen, in the presence of a catalyst, to form water. The electrons completing the circuit generate a modest amount of electricity. The anode potential is around -0.3 V and the cathode potential with oxygen is approximately 0.2 V, giving an overall potential [0.2 V $-$ ($-$0.3 V)] of 0.5 V. Thus, given the presence of suitable catalysts, the process occurs spontaneously: Waste is treated and electricity is harvested from the waste. Although the concept has been around for some time, the idea of using this phenomenon to treat waste has gained prominence only relatively recently. The state of the art has been well summarized

by Logan (2008), and there has recently been an excellent and well-informed critique (Rozendal et al., 2008a) of the technology.

An interesting and potentially important variation on this theme is the microbial electrochemical cell (MEC), which produces hydrogen. The cathode potential for hydrogen is approximately −0.414 V and that of the anode approximately 0.3 V. Thus, the overall potential (−0.114 V) is negative, so spontaneous hydrogen production does not occur. However, by "topping up" the potential with an external power supply with a value greater than 0.414 V (in practice, 0.25 V), hydrogen production can be initiated. The energy garnered from the hydrogen released at the anode far exceeds the energy required for its generation. In laboratory systems in carefully controlled conditions, between 150 and 500% of the energy in the electricity and up to 80% of the energy in the waste may be recovered. This remarkable result is possible because the hydrogen is garnered from the organic matter, not from water.

Broadly speaking, MFC and MEC comprise an anode, a cathode, and a means to separate the two, typically an ion-selective membrane. In MFC the cathode must either be aerated (which rather defeats the object!) or exposed to the air on one side and a catholyte (air cathode) at the other. In an MEC, air is not required and the system architecture is simpler.

On the laboratory scale, this technology looks encouraging. Current densities of about 10 A/m^2 have been achieved, which translates to about 1000 A/m^3 if a 1-cm anode spacing is permissible. An ampere is a coulomb of electrons per second, and a gram of COD represents 640 C of electrons. Thus, 1000 A/m^3 is equivalent to about 7.1 kg COD/m^3 per day, which is comparable to activated sludge (0.2 to 2.0 kg COD/m^3 per day) and anaerobic domestic wastewater treatment at 10°C (0.2 to 0.8 kg COD/m^3 per day). Thus, the systems will apparently fit within the current wastewater treatment architecture. It should be noted that the energy generated, even under optimum conditions, is modest, just enough to light a light bulb per family. The process can achieve Royal Commission standards for organic matter (> 90% removal; suspended solids have not been measured), and the anode in theory can compete with sulfate-reducing bacteria and is stable and easy to manage (at the laboratory scale).

Furthermore, the process is biologically more malleable than classical wastewater treatment processes. The oxidative processes at the anode can be complemented by reductive processes at the cathode, enhancing the range of chemical that can be removed. The cathode biology may be "fine-tuned" in MEC by varying the applied voltage. Furthermore, MFC can achieve "Royal Commission standard effluents" for organic matter when treating wastewater attaining very low yields and may be less sensitive to temperature than other anaerobic processes. There is the possibility that MEC and MFC may degrade chemicals, normally considered inert in anaerobic systems.

However, nearly all the work had been undertaken on a small scale and often with unrealistically pure wastes and almost invariably at warm temperatures. Very substantial problems remain with the application of the technology. To give just one example, power and coulombic efficiencies (the proportion of the electrons in the wastewater converted to current) have been observed to drop by a factor of 4 when wastewater is substituted for acetate in model-scale reactors (Ditzig et al., 2007). Yet the "headline data" in the literature are invariably based around acetate, while the implicit assumption is that the systems will be run on wastewater.

There are, broadly speaking, three interrelated areas in need of improvement: materials, biology, and electrochemical issues.

13.4.1 Materials

The laboratory reactors are made with graphite or carbon electrodes separated by proprietary ion-exchange membranes. The anode is invariably biological, but the cathode in most studies is platinum based. The platinum cathode has been replaced by non-noble metals and biological cathodes; although nearly all of this work has taken place in MFCs, it should also apply to MECs. Biological anodes and cathodes (e.g., Rozendal et al., 2008b) seem to be the way forward and may be superior to noble and non-noble catalysts because they have lower overpotentials (i.e., additional resistance to the flow of electrons) than those of platinum cathodes. At present, the mechanisms and basic electrochemical properties in biologically catalyzed electrodes are still very poorly characterized and poorly understood and may be contingent on both the waste deployed and the reactor format (e.g., batch reactors may favor exogenous mediators, and flow-through reactors, direct conductance by the biomass). Fortunately, many effective biological treatments have been developed in relative ignorance of the underlying biology! The membranes typically used at present (selective ion-exchange membranes) are too expensive and not very effective. Simple cheap alternatives appear to give superior results (di Lorenzo, personal communication). By contrast, the materials used for anodes and cathodes, carbon and graphite, are cheap. However, although these materials are conductive; they have a resistance more than 100 times greater than those of metals (1375 $\mu\Omega$/cm for graphite versus 9.7 $\mu\Omega$/cm for iron). This difference is negligible in small reactors but could become important at a larger scale, necessitating the use of more expensive materials. However, if these materials are reusable, they may not drastically affect the economics of the technology.

13.4.2 Biology

Detailed knowledge of the biology of the electrogens may not be a prerequisite for development of the technology. However, biology is likely to be an important factor. The effect of temperature on the process is still poorly understood, and we still do not have firm evidence that the technology will work at meaningful ambient temperatures, even in highly idealized situations. Temperature may become all the more significant with real wastewaters, where the hydrolysis of complex wastes will be required, and we still do not properly understand the effect of temperature on hydrolysis in any system, although we know that low coulombic efficiencies are not inevitable (ca. 75% recovery being achieved in Newcastle with domestic wastewater). We are aware that switching from artificial wastes to real wastes leads to a significant drop in power and coulombic efficiency in real wastewaters, but we do not know why. Low coulombic efficiencies are also seen at very high and very low loads and may reflect competition from aerobic heterotrophs and methanogens. Consequently, low coulombic efficiencies do not always presage low treatment efficiencies. Although typically viewed as regrettable, it is possible that hybrid electrogenic and methanogenic reactors could offer certain advantages. However, for MECs there will be a threshold level of coulombic efficiency below which the process is not viable, as the energy generated will be less than the energy required to drive the generation of hydrogen.

Modeling competition at the anode will help us understand competition at the anode, and biofilm and coupled anaerobic electrogenic models have been developed (Marcus et al., 2007; Picioreanu et al., 2007). Modeling has been used to highlight the fact that this is a biofilm process and, as such, is affected by mass transport considerations.

Transport of organic material into the biofilm may not always be the limiting factor; mass transport of hydrogen ions out of the biofilm must also occur, especially in weakly buffered (i.e., of modest alkalinity) wastewaters (the buffers transport the hydrogen ions). However, the modeling is weakened by conceptual uncertainties. For example, the models cited above make fundamentally different assumptions about the manner in which the electrons are transferred to the anode.

13.4.3 Electrochemistry

Even under ideal conditions the potential attainable in MFCs and MECs is a modest 0.6 V. Less than ideal electrochemical processes can reduce this to a negligible amount, so extremely efficient cell design is required. Potential can be lost in three places: at the electrode surface, in the conduction of hydrogen ions or electrons (in the liquid or the electrode), and due to pH gradients over the electrode. Losses at the electrode may be attenuated by using large surface areas and biological rather than noble catalysts (the potential losses of non-noble nonbiological catalysts are not well documented). However, neither surface area nor biological catalysts are optimized. The natural resistance of the wastewater is, in most circumstances, inescapable, it can be minimized by reducing the distance from the anode to the cathode. However, if marine disposal was anticipated, the inclusion of seawater might reduce internal resistance and improve performance. The membrane separates two compartments: the anode, in which hydrogen ions are produced, and the cathode, in which hydrogen ions are consumed. Consequently, a pH gradient is set up, which causes a potential loss of 0.06 V per pH unit. This may be overcome simply by eliminating the membrane, especially in MECs, where oxygen is not present. Alternatively, if the anolyte is passed from the anode chamber to the cathode chamber, the hydrogen ions would be carried in the liquid by mass transfer. The resistance of the materials has not yet been studied systematically; it may be overcome using standard materials and very short distances (many minicells) or by using more conductive materials. The choice of strategy will come down to the cost of the materials versus the cost of a more complex design.

13.4.4 Nutrient Removal

Although in principle, nutrient removal should not occur in MFCs, it has been observed. Air seeping in through the cathode has permitted nitrification to take place in reactors treating swine waste, and considerable nitrogen losses have been observed and attributed to volatilization. Denitrification at the cathode has also been exploited.

13.4.5 Tentative Economics

MFCs are not a working technology. Although there has been a great deal of research, much of it is served primarily to set the scene for a large-scale application. Nevertheless, it seems likely that a full-scale MFC or MEC producing electricity or hydrogen is technically feasible (real reactors may be hybrid methane–fuel cell devices). However, at present, the costs of construction, temperature sensitivity, likely effluent standards, and coulombic efficiency remain uncertain. It seems very likely that the capital costs will exceed those of activated sludge or anaerobic digestion; however, the revenues may be higher (see Table 13.1). Very tentative capital and cost estimates have been

Table 13.1 Comparison of Capital and Product Costs for Wastewater Treatment Using Aerobic, Anaerobic, and Bioelectrochemical Costs[a]

System	Product	Capital Costs (euros/kg COD)	Product Revenue (euros/kg COD)	Net Revenue (euros/kg COD)
Activated sludge	N/A	0.1	−0.3	−0.3
Anaerobic digestion	CH_4	0.01	0.1	0.1
MFC	Electricity	0.4	0.2	−0.2
MEC	H_2	0.4	0.6	0.2

Source: Rozendal et al. (2008a).

[a] Assumes a 25-year reactor design life and a 5-year design life for electrodes and membranes. The capital costs of electrochemical systems are assumed to be 5% of laboratory-scale systems.

made (Rozendal et al., 2008a). They should be viewed critically; for example, the costs of post-treatment for anaerobic digestion and the reuse of materials are ignored. It seems likely that the economics will rest crucially on the ability of the energy cost to offset the increased capital cost of the MFC/MEC format. Thus, the overall balance depends on the coulombic efficiency, the need for post-treatment, and the capital costs. Ultimately, the finite amount of energy in a unit of COD will set an upper limit on the capital costs.

13.4.6 Applications

Incremental MFC may find early application as an online BOD sensor (off-line sensors are well developed) (Gil et al., 2003). An online sensor requires small units working at high coulombic efficiency with real wastewaters on site. It may thus allow one aspect of the technology (coulombic efficiency) to be developed and yield a commercial return before other aspects of the technology are optimized.

Adaptive It is difficult to see the technology being applied to current flow sheets unless except perhaps as an experimental measure to investigate the efficacy of certain designs. There may be other "niche" applications, including treating sediments in lagoons, rivers, and especially estuaries (where salinity will reduce losses due to the low conductivity of water), where an electrogenic approach may permit the degradation of pollutants that are not normally degraded in anaerobic conditions.

Innovative MFC or MEC could replace conventional aerobic treatment, although they are much further from application than anaerobic wastewater treatment. Even if they are more expensive than anaerobic systems, they may be able to attain higher effluent standards at lower temperatures. With MECs (which produce hydrogen) the cathode design is simple and the membrane is, in all probability, dispensable. Consequently, although less well researched and somewhat counterintuitive, the MECs may be easier to scale up and exploit, especially where nutrient removal is not required. Hydrogen can probably be more easily exploited on a small scale (by virtue of interest from the auto industry) to generate energy to use on site and may be easier to sell than methane. MFC could be placed in the flow sheet after, or instead of, primary sedimentation.

13.4.7 Research Options

At present, MECs and MFCs are backstop technologies that will find application if they are able to outcompete or outperform the existing approaches, most notably anaerobic systems or trickling filters. Much of the information we have at present is based on unrealistic reactor formats and unrealistic wastes. We have to ask and answer a logical series of design questions using realistic wastes in MFC and MEC formats. In particular, we need to know if they are a credible and flexible wastewater treatment technology. To do this we must establish what is the best performance that we can expect with respect to the removal of carbon, micropollutants, and perhaps nitrogen, as well as the temperature sensitivity and the stability of the process. Moreover, we need to know this for reactors treating real wastewaters, not acetate and buffer.

To succeed as a wastewater treatment technology, MFCs and MECs must be able to exceed the performance of conventional anaerobic systems in these respects. If this is the case, the technology may be appropriate provided that running costs are low and the system is sufficiently compact to allow them to compete with conventional attached growth systems. If the performance criteria do not exceed those of anaerobic digestion (for effluent quality) and attached growth systems (for space), the cost and complexity of MFCs can probably only be justified if the energy produced will offset the cost of post-treatment. Assuming that satisfactory treatment can be achieved, there are perhaps two really important questions: What is the best coulombic efficiency that we can expect, and how much will a reactor cost?

High coulombic efficiency may be linked to process stability and temperature insensitivity as well as energy recovery. The relationship between areal loading and coulombic efficiency should be modeled and investigated empirically with particular emphasis on competition at the anode (including the effect of temperature). Strategies to minimize hydrogen-ion gradients (eliminating the membrane, flow from anode to cathode) need to be evaluated. We need to be able to predict the internal resistance caused by materials in full-scale cells, and we need to know the potential losses in noncathodes and biocathodes.

The cost of a reactor will be contingent on the materials used and the design, and will take into account the areal loading rate, the cost of a membrane (if used), and the choice and spacing of electrodes. Some of the more conductive material are assumed to be too expensive. However, if the materials are reusable, this may be less important, especially if design lives are relatively short. In addition to the cost of the reactor, one would also need ancillary equipment for energy capture and some power source for the MECs, which could be an MFC or some other renewable resource.

13.5 EXTENSIVE SYSTEMS

Extensive systems such as reed beds and lagoons are inherently low energy, can be low maintenance, and can give high effluent quality. The technology is probably under-optimized but will, in predominantly urban societies, still be confined largely to rural areas, where a relatively small fraction of wastewater is treated and a relatively small fraction of the energy is used. Consequently, although most aspects of these systems can be improved, this may not make a significant difference to the energy demand across a water company. However, treatment may become preferable to pumping, and smaller, more effective, low-maintenance low-energy wastewater treatment plants may

be important in this context. The methane footprint of these technologies is poorly understood and should be monitored on a precautionary basis.

Thus, *incremental* or *adaptive* approaches to such systems may be of limited value. However, they may find application in the polishing of slightly substandard effluents from fixed film systems. *Innovative* interventions can only be realized by solving the land issue, and this might be achieved by placing an extensive treatment facility offshore. This is not an entirely novel idea, but suitable lightweight robust offshore marine structures may now be becoming available for the first time (see below).

13.5.1 Research Questions

Simple improvements could probably be made rapidly in extensive systems by attending to the basic hydraulics of the systems. Gross reductions in size may be possible to attain in photosynthetic systems by investigating the relationship between organic load and the size of the system: for example, by understanding the effect of sulfide and iron on algae. Very large offshore systems may be possible if sufficienty large and cheap offshore marine structures could be built: These structures are being considered for carbon-capture projects (Patel, personal communication).

13.6 CARBON CAPTURE

The UK water industry will be carbon-capped under the coming carbon reduction commitment. The energy savings associated with anaerobic technologies will go some way toward meeting this target. However, carbon capture and storage is predicted to become a £5 billion per year business. This will create new technical and business opportunities. Small-scale carbon capture could very well be a biological process. Algae-based treatment systems fix carbon. Each 100 metric tons of algae produced 183 metric tons of carbon dioxide.

The nutrients in wastewater could make a valuable contribution to algal growth. We know from the stoichiometry of growth that a liter of wastewater containing 30 mg of nitrogen per liter could capture 549 mg of carbon dioxide. Even with 50% efficiency, the waste of 1 million people could be used to capture 15 kilotons of carbon a year, which currently trades at 30 euros/ton. Water companies are well placed to exploit this technology. Nitrogen and, to a lesser extent, phosphorus are limiting factors in the growth of algae and are found in sewage. Perhaps more important, water companies have extensive experience in cost-effective management of large-scale biological processes and in harvesting the biomass from them. Land and sunlight are important limiting factors in the UK. However, the technology need not work all year to be commercially effective; there is considerable scope for the intensification of algal production (e.g., bubbling carbon dioxide into algal bioreactors and placing some of the treatment just offshore, if sufficiently cheap, could help in building robust marine structures). The same technology could be used to support offshore lagoons that removed carbon, nitrogen and phosphorus. The true capacity of algal systems in temperate regions has not yet been established. The concept of harvesting algae from wastewater is not new, and an average of 28 g of CO_2 can be captured per square meter per day in Mediterranean climates using existing technology (Oswald, 2003). However, in the past, ancillary issues, particularly algae harvesting technologies and poor prices

for product, have undermined attempts to develop the technology. Those working on algae-based carbon capture believe that in addition to the almost certain market for carbon, additional revenue may be generated from algae-derived products. However, it is worth noting that analogous claims about wastewater have been made since at least 1842, when Chadwick (in the Poor Law Commissioners Inquiry into the Sanitary Conditions of the Labouring Population of Great Britain) advocated selling wastewater to farmers to help fund improvements in sanitation.

13.6.1 Research

Exploratory onshore pilot plant research will establish the credibility of this stratagem.

13.7 CONVENTIONAL AND NUTRIENT REMOVAL

The need for conventional wastewater treatment systems will remain for a number of reasons. Biofilm-based systems, potentially at least, use relatively little energy. There seems little need to improve on them. Their principal disadvantage is a lack of process flexibility. Thus, simple measures to pre- or post-treat such systems may be beneficial. Activated sludge plants may be with us for some time to come, not only because of the large number of such plants, but also because of the lack of alternative compact technologies for nutrient removal. The challenge may be both to minimize energy use of the technology in the short term and to adapt the technology to become a minimal energy post-treatment for anaerobic treatment systems. It is difficult to see how membrane bioreactors will prosper in the UK energy climate.

Practitioners are probably better placed than academics to evolve day-to-day strategies for minimizing energy demand for the removal of carbonaceous oxygen demand. However, it is worth repeating that denitrification makes it possible to save energy, as the oxygen is essentially used twice, once for nitrification and again in denitrification. There will be many proposals to adapt current aerated technologies. For example, innovative variations on the deep shaft process (Whipp and Son, personal communication) have been proposed with a view to exploiting the enhanced mass transfer that occurs under pressure. It remains to be seen if the new processes suffer from the poor settling characteristics of the original deep shaft process. Many of these innovations may emerge from industrializing giants such as Brazil, China, and India, where there are many excellent engineers and a pressing need for more and cheaper wastewater treatment technologies.

Nitrification has an absolute requirement for molecular oxygen. Where this oxygen cannot be provided passively (in biofilm systems), activated sludge may be required. There are reasons to believe that the current state of the art can be improved upon significantly. Relatively recent work from the United States suggests that nitrification, or at least the oxidation of ammonia, can be sustained at much lower oxygen requirement concentrations and flow rates than has been widely assumed if suitable bacteria can be selected for (Park and Noguera, 2007). This work was done at full scale, but the physiological basis for the observation is not known. However, there is evidence that ammonia-oxidizing bacteria in wastewaster treatment plants can grow chemoautotrophically (Milner et al., 2008), which halves the amount of oxygen required for growth. It may very well be worthwhile not only to establish the minimum oxygen requirements

for nitrification in wastewater treatment plants but also to establish the true in situ physiology of this process. Where nitrogen removal is required as a post-treatment for anaerobic or fuel cell processes, the annamox process may be required, due to the lack of carbon for conventional denitrification.

A number of monitoring tools can or could help run plants less conservatively. It appears that the probability of failure of nitrification can simply be linked to the number of ammonia-oxidizing bacteria, which in principle could be measured routinely on site. This could, in turn, lead to a more informed processes control strategy in which the number of ammonia-oxidizing bacteria was monitored and manipulated to maintain a predetermined probability of failure. More routinely, measurement of nitrous oxide offgases and ammonia in the effluent is already helping to improve process control and thus energy costs in real time.

Low-energy phosphorus removal remains an enigma. The best known strategy for biological phosphorus removal was discovered by accident and applied to activated sludge almost exclusively. The only alternative strategy, acid shock, has also been applied only in aerobic conditions (Mullan et al., 2006). In the face of such a lacuna, the best policy may be simply to explore the physiology of phosphorus removal at a very fundamental level or to have many challenge-driven research projects in the hope that something will be developed. Very similar considerations apply to endocrine-disrupter compounds in wastewater.

13.7.1 Applications

Incremental and adaptive improvements may be made in a large number of ways, some of which are outlined above. *Innovation* of the radical type is probably not possible; in the long term, we must hope that activated sludge is dead.

13.7.2 Research Questions

Nitrification should be revisited from the bottom up and the organisms and their physiology revisited with a view to introducing processes in which resource use is minimized and explicitly balanced against the probability of failure. Biological phosphorus removal is a conundrum. No obvious low- or reduced-energy technology or biology has been discovered. Blue skies research into the biology of phosphorous is warranted. As with carbon capture, there remains, the albeit distant prospect, harvesting this important nutrient from wastewater.

13.8 OXIDATION AND DISINFECTION

The state of the art for oxidation and disinfection is still, for wastewater, ultraviolet (UV) light, or perhaps ozone. Chlorine is relatively cheap but is increasingly unacceptable to environmental regulators. However, ozone and UV light represent high-energy technologies. The alternatives appear to be an alternative oxidation method. Lower-cost ozone is possible, with fuel cell–based ozone production claiming to have half the running cost of classical ozonation (Christensen et al., 2006). Titanium dioxide–based oxidation technologies have been well documented but have not been commercialized successfully, possibly because of the problem of separating the TiO_2 from the water.

Immobilized TiO_2 reactors have shown promise as excellent disinfectants but scale-up remains a problem, as mass transfer limitations appear to prevent a process dealing with very large quantities of water. Although an effective photocatalyst, TiO_2 requires very modest quantities of UV to work.

However, it appears that very low energy techniques will revolve around light. The Holy Grail is either a light-emitting diode–based system that works in the UV or a photocatalyst that works in the visible range or some combination of the two. The new generation of diode-based lights at relatively short wavelengths is a cause for optimism. There have been improvements in photocatalysts as well with the first reports of photooxdiation in the visible (Shannon et al., 2008). However, we already know that there are photocatalysts that absorb in the visible naturally present in wastewater (Kohn et al., 2007). The potential of combining short-wavelength LEDs with these naturally occurring photocatalysts is exciting.

13.8.1 Research Questions

Low-cost ozone may well be worth investigating and is a workable technology, but a rigorous accounting of inputs and costs is necessary, as well as an awareness of the impact of disinfection by-products. Immobilized titanium dioxide technology should be revisited and the fundamental limits to this technology understood. In particular, the true disinfection rates and nature of the putative mass transfer limitation need to be characterized. This would permit a decision to be made as to how, or if, to pursue the technology.

In the medium to long term, LED-based disinfection may be the most promising approach. In the first instance, this may work with available LED technology and naturally occurring sensitizers. If this stratagem does not work, either shorter-wavelength LED technology (if this is physically possible) must be found or new sensitizers need to be developed.

13.9 HOW TO MOVE FORWARD

Although there is no off-the-shelf technology to reduce energy use massively in wastewater treatment, there is no reason that such a technology cannot be developed. We have not yet exhausted either the ingenuity of environmental engineers or the natural limits of science or technology. Moreover, there is much unexploited "off-the-shelf science" that we can use. However, technological optimism must be tempered with a degree of steely realism. Time is pressing and the answers are not yet obvious. To find the multiple solutions required, we need to understand, and manage, the risk of failure in the context of probable rewards.

However, because there are no obvious solutions to the need for low-energy wastewater, multiple high-risk high-payoff strategies need to be pursued in parallel. High payoff in this context means anything that can at *least* halve the energy use of the available technology. Thus, although the risk of a single approach working may be low, the overall probability of finding a solution or solutions in a reasonable time period is higher.

We also need to "raise our game" intellectually in order to maximize the possibility of success in all our endeavors. All too often the modus operandi of problem-solving

research has been to conduct relatively short term empirical research delivering success or failure on a relatively short time frame. This approach runs two risks: Success may be difficult to replicate and judgments of failure may be premature. We minimize this risk by deepening the mechanistic basis of our understanding through a synthesis of a much better theoretical understanding of the systems we seek to engineer, coupled with improving mathematical descriptions and a far more detailed chemical and biological characterization of the systems we study. Together these innovations will lead to greater insights about the causes of failure and the predictive understanding of success required for design.

This is neither cheap nor easy. Although much relevant science merely needs to be exploited, some of the fundamental science required and ancillary technology required to make progress does not yet exist. We cannot necessarily wait for the scientific community to oblige us: We need to seek the answers ourselves. Although we can look to the national governments to support some of this work, it is difficult to imagine a coordinated approach to this challenge arising from responsive mode funding.

On the other hand, expensive precompetitive research is difficult for private companies to undertake. Nevertheless, at present the water industry as a whole has more money than time. It would make sense for like-minded companies to cooperate and move the agenda forward: with or without government support. Investment now will help ensure that the industry's costs, and thus the consumers' bills, are less sensitive to energy cost and the carbon economy. Thus, companies and countries that master the putative new generation of low-energy wastewater treatment technologies will surely be selling goods and services to those that do not.

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CHAPTER 14

Biomediated Geomechanical Processes

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14.1 INTRODUCTION

Soil is one of the most commonly used construction materials, and as a result, geotechnical engineers play a role in many types of engineering projects, including those involving earth structures, requiring a soil or rock foundation, or those constructed below the ground surface (Dunn et al., 1980). However, unlike other materials used by engineers, soil has extremely variable physical properties. To address these challenges, a significant effort has been made over the past 300 years to improve our understanding of soil properties and behavior (Mitchell and Santamarina, 2005). Most of that effort has been focused on improving our understanding of the relevant mechanical principles, as well as the roles of geology, chemistry, and mineralogy. Accordingly, when engineers have difficulties with on-site construction onto or within soils, the conventional solutions for improving the engineering properties of soils involve using chemical additives (e.g., portland cement, lime, fly ash, bitumen) and/or mechanical stabilization (e.g., mixing or blending of soils and compaction) (Army Corps of Engineers, 1994).

Geoengineers have only relatively recently become aware of the potential of biological applications for improving the mechanical properties of soils (NRC, 2006). This is surprising given that microbial activities are ubiquitous in surface and subsurface soils and have a tremendous effect on the composition, properties, and behavior of soil and rock near the Earth's surface. These soil organisms are diverse and numerous (Wollum, 1998). Bacteria are the most numerous, followed by the actinomycetes and fungi (Table 14.1); however, fungi typically have a larger total biomass in the soil. As demonstrated in Table 14.1, most of the relevant microorganisms are in a size range typical of soil particles smaller than sand, whereas most of the biological activity takes place in silt-size or coarser particles and rock fractures (NRC, 2006).

A typical soil aggregate is composed of mineral and organic components and may have many different microenvironments (Madigan et al., 1997) (Figure 14.1). For example, the outer zones of a soil particle could be fully oxic, while the center of the particle could be completely anoxic. As a result, several different types of microorganisms may also be present. Most of the growth of soil microorganisms occurs on the surfaces of soil particles, where they can exploit the nutrients that tend to absorb

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Table 14.1 Common Groups of Microorganisms Found in Soil

Soil Type Based on Particle Size[a] (μm)	Microbial Group	Example Organism	Size (μm)	Number per Gram of Soil	Biomass (kg wet mass/ha of soil)
Clay <2.0	Viruses	Tobacco mosaic	0.02×0.3	10^{10}–10^{11}	
	Bacteria	*Pseudomonas*	0.5×1.5	10^{10}–10^{11}	300–3000
	Actinomycetes	*Streptomyces*	0.5–2.0[b]	10^{8}–10^{9}	300–3000
Silt 2.0–75	Fungi	*Mucor*	8.0[b]	10^{7}–10^{8}	500–5000
	Algae	*Chlorella*	5×13	10^{5}–10^{6}	10–1500
	Protozoans	*Euglena*	15×50	10^{3}–10^{6}	5–200
Sand 75–2000	Nematodes	*Pratylenchus*	1000[c]	10^{3}–10^{5}	1–100
Gravel 2000–150,000	Earthworms	*Lumbricus*	100,000[c]	10^{1}–10^{2}	10–1000

Source: Adapted from Dunn et al. (1980) and Wollum (1998) [a] Provided for comparison. [b] Diameter of hyphae. [c] Length.

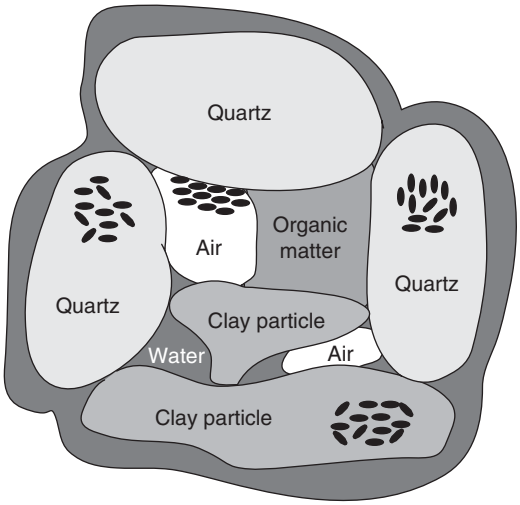

Figure 14.1 Soil aggregate consisting of mineral and organic components, with localized microcolonies of microorganisms attached to the soil particles. (Adapted from Madigan et al., 1997.)

there. The bacteria attached to the surfaces form biofilms [i.e., layerlike aggregates of bacteria and extracellular polymeric substances (EPSs)]. Soil water or gas flows past the biofilms, which extract their substrates from the fluid flow.

The physicochemical (e.g., temperature, pH, water availability, light, oxygen) and biological properties of the soil habitat have a significant impact on the growth and activity of the microorganisms (Madigan et al., 1997; Wollum, 1998). In turn, the microorganisms greatly affect the soil via the tremendous diversity of functions that they exhibit. Such geomicrobiological processes have been studied extensively by microbiologists and geologists (Ehrlich, 2002).

The National Research Council report "Geological and Geotechnical Engineering in the New Millennium" (NRC, 2006) recognized the great potential of microorganisms for modifying soil properties in field applications and improving the practice of geo-engineering in the twenty-first century through two key applications: (1) bioremediation of contaminated soil and groundwater (reviewed in Chapter 8), and (2) biomediated geomechanical processes. The focus of this chapter is on biomediated geomechanical processes. Biomediated processes can have a significant impact on the geomechanical behavior of earth materials (NRC, 2006). Examples of microbially mediated processes that have been investigated for their influences on geomechanical properties include mineral precipitation, biofilm formation and use of biopolymers, mineral transformation, and biogenic gas production (Kavazanjian and Karatas, 2008). These activities and others can alter the physical properties of soils directly or indirectly, either permanently or temporarily. Potential impacts of these processes on soil behavior include reductions in hydraulic conductivity and increases in the strength and stiffness of soils (Mitchell and Santamarina, 2005).

If biomediated geomechanical processes are going to be applied successfully in engineering practice, two questions need to be answered: (1) Do the processes occur in a time scale of interest for engineering projects, and (2) if so, how can the desired process be "engineered" so as to occur when and where it is required? The microbially mediated processes of interest (e.g., mineral precipitation) occur naturally within the geologic time frame, but there is less known about the potential for such processes to alter soil properties within the time frame of interest in engineering projects (Kavazanjian and Karatas, 2008). Nevertheless, there is clear evidence that these processes can indeed occur in the engineering time frame, based on the common observations of what are generally considered negative impacts of microorganisms in geological settings, such as clogged landfill leachate collection systems (Fleming et al., 1999).

In any engineered approach designed to exploit a naturally occurring microbiological processes, process controls are used to select for the "right" microorganisms, present in sufficient quantities to achieve the necessary biochemical reactions, thereby creating an ecological system that accomplishes the desired goal (Rittmann, 1992). The key process controls used to achieve these goals are (1) supplying the proper nutritional factors to the microbes, (2) retaining the microorganisms in the system, and (3) process "loading," that is, providing sufficient contact time between the microorganisms and their substrates. In the soil systems of interest here, cell retention is accomplished primarily via attachment of the microbes to the high amount of surface area available on the porous media, as noted above. Therefore, in the examples presented below, we focus on how nutritional factors and process loading can be used to create the necessary ecological systems and select for the desired types and amounts of microbes needed to biomediate the soil properties.

In this chapter we provide a review of what is currently known with respect to the biomediation of geomechanical processes in soils and other construction materials. First, some of the key microbially mediated processes in soils and rocks that affect the physical properties of soils directly or indirectly are reviewed, along with their effects on the soil mechanical properties. Then the potential engineering applications of those processes, their design, and potential strategies for their implementation in the field are discussed.

14.2 MINERAL PRECIPITATION

14.2.1 Potential Microbiological Improvement Mechanisms

A variety of minerals may be formed in situ (i.e., authigenic minerals) as a result of microbial activity, including iron sulfides, iron oxides, manganese oxides, silica, and calcium carbonates (e.g., calcite and aragonite, $CaCO_3$) (Ehrlich, 2002). Of the microbially induced precipitation processes, carbonate precipitation has been studied extensively (Ehrlich, 2002) and has been of the most interest for biomediation of geomechanical processes; therefore, it is the focus here.

A variety of microorganisms are involved in carbonate formation, including bacteria, fungi, and algae, with deposition occurring intra- and extracellularly (Ehrlich, 2002). Some algae and protozoans form calcium carbonate intracellularly, then export it to the cell surface to become surface structures of their cells. On the other hand, most bacteria, including cyanobacteria, and some fungi and algae induce calcium carbonate precipitation extracellularly, primarily in the bulk phase close to the cell surface, or at the cell surface. This occurs as a result of microbially mediated metabolic processes that alter the geochemistry of the pore fluid (e.g., by increasing the total carbonate content and/or pH), as reviewed below.

Carbonate System A brief review of the carbonate system is helpful for understanding how microbial processes are involved in carbonate precipitation. Four equilibrium relationships are potentially needed to describe this system, which are presented here (ignoring activity corrections) (Sawyer et al., 2003). In *open* (i.e., open to the atmosphere) *systems*, there is an interchange between atmospheric CO_2 and $H_2CO_3^*$ in solution, with the gas–water equilibrium described by Henry's law as follows:

$$H_2CO_3^*(aq) \leftrightarrow CO_2(g) + H_2O \qquad K_H = \frac{[P_{CO_2}]}{[H_2CO_3^*]} = 31.6\frac{\text{atm}}{\text{M}} \text{at } 25^\circ\text{C} \qquad (14.1)$$

where, by convention, $[H_2CO_3^*]$ equals the sum of the carbonic acid concentration, $[H_2CO_3]$, plus the dissolved carbon dioxide concentration, $[CO_2(aq)]$, and K_H is the Henry's law constant.

In water, carbonic acid is diprotic (i.e., it dissociates in two steps). First, it dissociates to bicarbonate, HCO_3^-, and a proton,

$$H_2CO_3^* \leftrightarrow H^+ + HCO_3^- \qquad K_{a,1} = \frac{[H^+][HCO_3^-]}{[H_2CO_3^*]} = 4.3 \times 10^{-7}\text{mol/L at } 25^\circ\text{C} \qquad (14.2)$$

where $K_{a,1}$ is the equilibrium constant. Second, bicarbonate ion dissociates to carbonate ion, CO_3^{2-}, and a proton,

$$HCO_3^- \leftrightarrow H^+ + CO_3^{2-} \qquad K_{a,2} = \frac{[H^+][CO_3^{2-}]}{[HCO_3^-]} = 4.7 \times 10^{-11}\text{mol/L at } 25^\circ\text{C} \qquad (14.3)$$

where $K_{a,2}$ is the equilibrium constant.

Based on these equilibria, a mass balance on the total concentration of dissolved inorganic carbon (DIC) species gives

$$C_T = [H_2CO_3^*] + [HCO_3^-] + [CO_3^{2-}] \qquad (14.4)$$

In open systems, $[H_2CO_3^*]$ is constant with pH (Eq. 1), while C_T changes with the pH. For example, as the pH increases, $[HCO_3^-]$ and $[CO_3^{2-}]$ increase [equations (14.2) and (14.3)], causing C_T to increase. However, in *closed systems* (i.e., closed to the atmosphere), C_T is constant with pH if no precipitation occurs.

If a carbonate solid is also present or forms, an additional reaction describing its solubility is required. For example, for calcium carbonate, $CaCO_3(s)$,

$$CaCO_3(s) \leftrightarrow Ca^{2+} + CO_3^{2-} \qquad K_{sp} = [Ca^{2+}][CO_3^{2-}] = 5 \times 10^{-9} mol^2/L^2 \text{ at } 25^\circ C \tag{14.5}$$

where K_{sp} is the solubility product. The product of the calcium ion and carbonate concentrations must exceed the solubility product for precipitation to occur (i.e., the solution must be oversaturated). This can be quantified using the saturation state, S, of the solution:

$$S = \frac{[Ca^{2+}][CO_3^{2-}]}{K_{sp}} \tag{14.6}$$

When $S > 1$, the reacting solution is over- or supersaturated.

A key process control for promoting biocementation via carbonate precipitation is to supply substrates whose transformation shifts the carbonate system to increase the carbonate concentration while ensuring adequate concentrations of appropriate cations, such as calcium, to promote precipitation [equation (14.5)]. As reviewed below, precipitation of $CaCO_3$ and other insoluble carbonates can be induced via a variety of heterotrophic and autotrophic microbial processes.

Heterotrophic Microbial Processes Contributing to Carbonate Precipitation

Aerobic and anaerobic oxidation of organic compounds resulting in the addition of dissolved $H_2CO_3^*$ will increase the total concentration of the dissolved carbonic species [equation (14.4)]. If such oxidations occur in a well-buffered neutral or alkaline environment, at least some of the $H_2CO_3^*$ produced will be transformed into carbonate [equations (14.2) and (14.3)], which will precipitate if sufficient Ca^{2+} or other appropriate cations are present [equation (14.5)] (Ehrlich, 2002). For example, Krumbein (1974) demonstrated the formation of aragonite and other calcium carbonates under these conditions by heterotrophic bacteria and fungi in media containing organic matter.

If an environment is not already well buffered and neutral or alkaline, an increase in alkalinity and pH is necessary to ensure that the CO_2 produced is being transformed into carbonate [equations (14.2) and (14.3)] for forming carbonate precipitation. Alkalinity is a measure of the capacity of a water to neutralize strong acid. In natural waters, this is mostly attributable to the bases of the carbonate system (HCO_3^-, CO_3^{2-}) and OH^-; therefore, total alkalinity (TA) is often defined as

$$[TA] = [HCO_3^-] + 2[CO_3^-] + [OH^-] - [H^+] \tag{14.7}$$

However, in some natural and contaminated waters, other salts of weak acids such as phosphates and weak organic acids may contribute to the TA. Note that precipitation of calcium carbonate [equation (14.5)] will decrease the pH and TA [equations (14.2), (14.3), (14.5), and (14.7)] (e.g., Abd-El-Malek and Rizk, 1963b).

An increase in alkalinity and/or pH can be accomplished directly via several microbially mediated processes. One of these processes is the aerobic or anaerobic biotransformation of organic nitrogen compounds, either via oxidation (e.g., of amines, amino

acids, purines, pyrimidines), or hydrolysis (e.g., of urea) (Ehrlich, 2002). In particular, much of the interest in promoting biocalcification in soils and other construction materials has focused on urea hydrolysis (or ureolysis) (Gollapudi et al., 1995; Stocks-Fischer et al., 1999; Urzi et al., 1999; Fujita et al., 2000; Bang et al., 2001; Ramachandran et al., 2001; Ross et al., 2001; DeJong et al., 2006; Chou, 2008; Whiffin et al., 2007):

$$NH_2CONH_2 + H^+ + 2H_2O \rightarrow 2\ NH_4{}^+ + HCO_3{}^- \tag{14.8}$$

Biotransformation of organic nitrogen compounds has the advantage of releasing NH_4 as well as CO_2, resulting in an increase in pH and alkalinity [equation (14.7)]. This creates an environment where at least some of the CO_2 produced will be transformed into carbonate. Thus, in unbuffered environments containing adequate amounts of $Ca_2{}^+$ and other cations, $CaCO_3$ precipitation can result if the solubility product is exceeded [equation (14.6)]. For example, Stocks-Fischer et al. (1999) observed urea degradation in batch systems to increase ammonia and pH levels, with microbiological $CaCO_3$ precipitation beginning at pH 8.3, completed at pH 9, and correlated to growth of *Bacillis pasteurii* (now *Sporosarcina pasteurii*). Similar trends were observed by others (Fujita et al., 2000; Bang et al., 2001; Chou, 2008).

Oxidation of organic electron donors under denitrifying, sulfate-reducing, and methanogenic conditions can also result in CO_2 production and an increase in pH, thereby promoting calcium carbonate precipitation in the presence of calcium and other counter ions. These processes have the advantage of not requiring the addition of oxygen to the soil or subsurface, although they still require the presence of a suitable carbon and energy source, and the appropriate electron acceptor. Heterotrophic denitrifying bacteria oxidize an organic electron donor while reducing $NO_3{}^-$, producing N_2 gas, CO_2, and alkalinity:

$$4NO_3{}^- + 5CH_2O + 4H^+ \rightarrow 5CO_2 + 2N_2 + 7H_2O \tag{14.9}$$

where organic carbon is represented by the generic formula for a carbohydrate, CH_2O. This creates a favorable environment for carbonate precipitation in the presence of calcium and other counter ions (Abdelouas et al., 1998; Karatas et al., 2008). Similarly, oxidation of organic matter by sulfate-reducing bacteria (SRB) results in a release of sulfide and carbon dioxide, in addition to increasing the alkalinity and pH (Abd-El-Malek and Rizk, 1963a):

$$SO_4{}^{2-} + 2CH_2O + 2H^+ \rightarrow 2CO_2 + H_2S + 2H_2O \tag{14.10}$$

Precipitation of calcium carbonate induced by SRB has been observed under a variety of conditions relevant to biomediation of soil engineering properties (Abd-El-Malek and Rizk, 1963b; Van Lith et al., 2003; Aloisi et al., 2006).

Laboratory and field studies indicate that the primary cause of clogging in landfill leachate collections systems is calcium carbonate precipitation (Fleming et al., 1999). Based on theoretical and laboratory studies, the main process driving this precipitation is aceticlastic methanogenesis,

$$CH_3COOH \rightarrow CO_2 + CH_4 \tag{14.11}$$

which increases the total carbonate concentration and shifts the acid–base control from acetate to the weaker carbonic acid system, thereby increasing the pH (Rittmann et al., 2003; VanGulck et al., 2003). The pH increase is enhanced by the loss of CO_2 to the gas phase in the open system [equations (14.1) to (14.3)], as discussed further below.

Autotrophic Microbial Processes Contributing to Carbonate Precipitation Chemo- and photosynthetic autotrophs can also induce the precipitation of carbonates when they remove CO_2 from bicarbonate-containing solutions in the presence of an adequate supply of Ca^{2+} or other appropriate cations (Ehrlich, 2002). For example, calcium carbonate precipitation has been observed during hydrogenotrophic methanogenesis (Castanier et al., 1999) and autotrophic denitrification (Lee and Rittmann, 2003).

One of the most important mechanisms for CO_2 removal and formation of biogenic $CaCO_3$ in the open, aerobic environment is photosynthesis (Ehrlich, 2002). In surface waters where cyanobacteria and algae are growing rapidly, pH values as high as 10 have been observed due to the removal of CO_2 to support photosynthetic activity (Sawyer et al., 2003). Algae can cause such an increase in pH by reducing the free carbon dioxide level to below its equilibrium with air [equations (14.1) to (14.3)]. As the pH increases, the forms of alkalinity change, and CO_2 is extracted from bicarbonates and carbonates, according to the following equilibrium reactions:

$$2HCO_3^- \leftrightarrow CO_3^{2-} + H_2O + CO_2 \tag{14.12}$$

$$CO_3^{2-} + H_2O \leftrightarrow 2OH^- + CO_2 \tag{14.13}$$

This results in a shift in the forms of alkalinity present from bicarbonate to carbonate, and from carbonate to hydroxide. When the carbonate-ion concentration, coupled with the amounts of Ca^{2+} present, exceeds the $CaCO_3$ solubility product sufficiently, calcium carbonate precipitation occurs [equation (14.6)]. Such processes result in the production of marl deposits in lakes (Thompson and Ferris, 1990). In terrestrial systems, physiologically and ecologically significant amounts of light generally do not appear to penetrate more than 4 to 5 mm through the soil (Tester and Morris, 1987); therefore, application of photosynthetic processes for biomediation of soil properties is probably limited to surface applications.

Nucleation Whatever the metabolic processes involved, formation of the solid phase (e.g., $CaCO_3$) from an oversaturated solution typically involves three key steps (Stumm and Morgan, 1981): (1) homogeneous or heterogeneous nucleation, as ions or molecules interact, leading to formation of clusters and then nuclei that allow spontaneous growth to occur; (2) crystal growth, as material is deposited on nuclei forming crystallites; and (3) ripening, as large crystals form from fine crystallites. Biocalcification probably occurs via a combination of homogeneous and heterogeneous nucleation (Mitchell and Ferris, 2005, 2006). Homogeneous carbonate nucleation results when the heterotrophic and autotrophic metabolic processes discussed above cause changes (e.g., increases in pH and DIC concentration) that lead to oversaturation in the bulk solution chemistry and in microenvironments surrounding cells, thereby catalyzing precipitation (Schultze-Lam et al., 1996). Heterogeneous nucleation occurs when bacterial cells (living or dead) function as nucleation sites (Schultze-Lam et al., 1996), via the binding of cations to the cell wall (Greenfield, 1963; Stocks-Fischer et al., 1999;

Van Lith et al., 2003; Aloisi et al., 2006) and EPS (Braissant et al., 2007; Ercole et al., 2007), both of which carry a net negative electric charge. For example, if free Ca^{2+} is available, the conditions are favorable for it to attach on cells due to the attractive force between the cations and the negatively charged cell (Stocks-Fischer et al., 1999):

$$Ca^{2+} + \text{cell} \rightarrow \text{cell} - Ca^{2+} \tag{14.14}$$

In the presence of carbonate, $CaCO_3$ precipitates form on the cells according to

$$\text{cell} - Ca^{2+} + {CO_3}^{2-} \rightarrow \text{cell} - CaCO_3 \downarrow \tag{14.15}$$

Under in situ soil and aquifer conditions, the porous medium provides additional sites for nucleation on the matrix mineral surfaces (Ferris et al., 2004).

14.2.2 Effects on the Mechanical Properties of Soils

Microbially induced carbonate precipitation has been observed to have direct effects on two important soil properties for geotechnical engineering design: hydraulic conductivity and strength. Laboratory studies of bio-induced calcite precipitation in sands have demonstrated reductions in the porosity and hydraulic conductivity of the biomediated sand as a result of mineralogical plugging (Stocks-Fischer et al., 1999; Chou, 2008). Scanning electron micrographs (SEMs) of the porous media from these studies typically show the accumulation of $CaCO_3$ precipitates and microbial cells between sand grains and in pore throats (Figure 14.2), while x-ray diffraction analysis in one study (Stocks-Fischer et al., 1999) indicated that the precipitation was primarily calcite.

Some studies have demonstrated an increase in strength as well as a decrease in hydraulic conductivity. For example, Chou (2008) examined the effects of biocalcification on sand by using columns that could function as completely mixed biofilm reactors (CMBRs), and provide specimens for measuring bearing strength via the California bearing ratio [CBR = (test unit stress/standard unit stress) $\times 100$]. Poorly graded (uniform) sand in the CMBRs was subjected to microbial $CaCO_3$ precipitation via ureolysis catalyzed by *S. pasteurii* (ATCC 11859) under different conditions [soil relative density, cell types (i.e., dead, resting, and live cells), and cell concentration]. SEMs of the column porous media after treatment are shown in Figure 14.2. The addition of cells, particularly at higher concentrations, generally increased the CBR values, with the greatest improvements in soil strength (110 to 580%) observed following addition of live cells, particularly to dense sand. These changes in bearing strength were accompanied by a greater than 80% reduction in hydraulic conductivity.

Although a reduction in hydraulic conductivity has often been observed and may be the goal of the process, it is not always desirable in terms of soil improvement (Whiffin et al., 2007). For example, if the hydraulic conductivity is reduced in the target region, groundwater flow will bypass the treatment area to some extent, which can result in an increase in the soil pore pressure and increased risk of soil failure. Whiffin et al. (2007) describe a one-dimensional flow-through system designed to achieve a strength increase via ureolysis without excessive clogging of the soil under low-flow conditions. Under these conditions, $CaCO_3$ was precipitated along the column length, but a nonuniform strength improvement was observed, with a greater increase in the confined compressive strength at the column inlet due to the greater cementation,

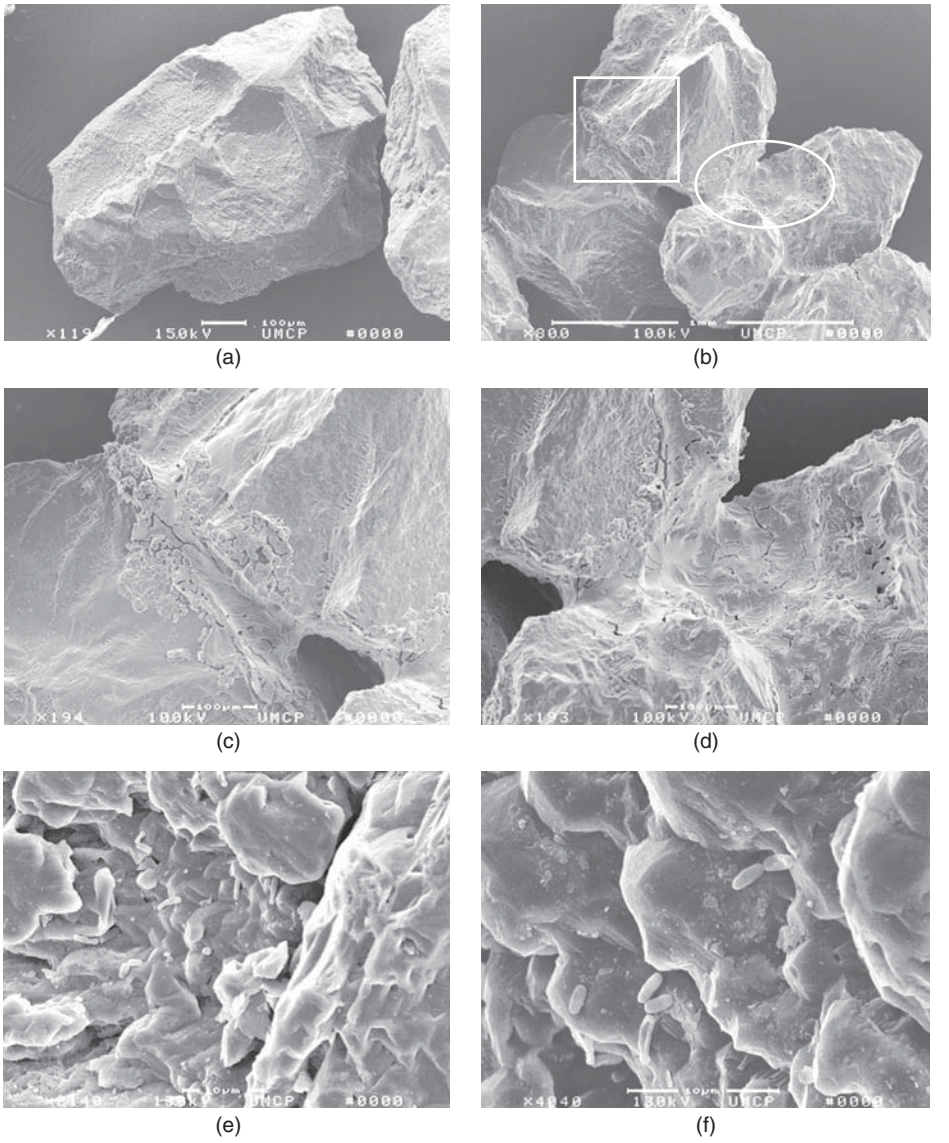

Figure 14.2 Scanning electron micrographs: (a) of untreated sand with washing before analysis; (b) of sand treated with live *S. pasteurii*, $CaCl_2$, and urea, without washing before analysis; (c) focused on the area within the square boundary in (b); (d) focused on the area within the oval boundary of (b); and of the surface of biotreated sand without (e) and with (f) washing before analysis. (From Chou, 2008.)

and no significant effect on compressive strength for $CaCO_3$ concentrations below 60 kg/m^3. Porosity losses were proportional to the amount of $CaCO_3$, but changes in hydraulic conductivity were small and not correlated with $CaCO_3$.

A critical question regarding application of the microbial cementation method is how the mechanical properties of microbially cemented soils compare with those of soils

subjected to conventional treatment using calcium-rich admixtures (e.g., lime, portland cement, gypsum cement). DeJong et al. (2006) compared specimens of initially loose, collapsible sands that were microbially cemented via ureolysis catalyzed by *S. pasteurii* with specimens that were treated with gypsum cement, and observed similar behavior for both treatments. For example, the microbially cemented sands had shear strengths comparable to those treated with 5% (by weight) gypsum cement when subjected to drained triaxial shear tests. Compared to untreated loose sands, the biotreated sands had a high initial stiffness and peak strength (Figure 14.3). In addition, biotreated and untreated sands were compared based on the shear wave (S-wave) velocity, V_s,

$$V_s = \sqrt{\frac{G_{\text{soil}}}{\rho_{\text{soil}}}} \tag{14.16}$$

where G_{soil} represents the shear modulus of the soil and ρ_{soil} the soil density. The shear wave velocity is preferred for the characterization of near-surface deposits when the soil mass is saturated, because the shear modulus of the soil depends only on the skeleton shear stiffness and is not affected by the bulk stiffness of the pore fluid. Measurements by DeJong et al. (2006) showed that V_s of the microbially modified sand increased from 180 m/s to 540 m/s upon treatment (Figure 14.3); thus, the treated soil exhibited the characteristics of very dense soil and soft rock.

The biocalcification process has also been used in recent years for remediation of fractures in geomedia and damaged structural materials. In geomedia, microbial processes have been used for plugging cracks in permeable rock formations (Urzi et al., 1999; Ross et al., 2001). For example, Gollapudi et al. (1995) used a silica fume/sand slurry amended with *S. pasteurii* to remediate cracks in granite, resulting in increased compressive strengths and reduced hydraulic conductivity.

Microbially induced calcite precipitation was also studied by Ramachandran (2001) and Bang et al. (2001) for remediation of cracks in concrete by filling the cracks with mixtures of *S. pasteurii* and sand, or polyurethane (PU)-immobilized *S. pasteurii* cells, respectively. In both cases, cracks filled with the biotreatment demonstrated an increase in compressive strength compared to cracks without cells. SEM analysis indicated that treatment with cells and sand resulted in calcite precipitation primarily close to the surface areas of the crack, whereas PU-immobilized cells induced calcite precipitation throughout the PU matrices, which protected the microbes from the very alkaline concrete environment and provided additional nucleation sites.

14.3 BIOFILMS AND BIOPOLYMERS

14.3.1 Potential Microbiological Improvement Mechanisms

As noted above, most microbes (80 to 90%) in porous media are found attached to solid surfaces (Figure 14.1), with the rest free-living (Maier and Pepper, 2000). For example, studies of autochthonous subsurface microbes show that the numbers of microorganisms attached to aquifer sediments usually are one to three orders of magnitude greater than the numbers of free-living microorganisms (Harvey et al., 1984). The attached microorganisms are typically found in nonuniform “patches” or “microcolonies” on the particle surfaces (Maier and Pepper, 2000). As defined above, such associations

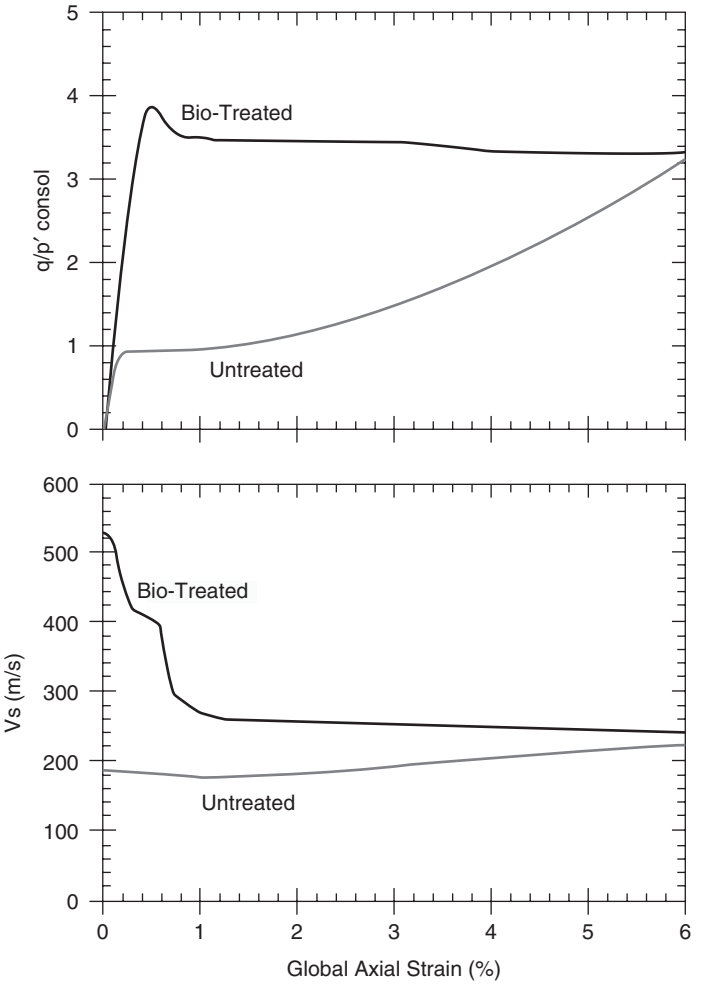

Figure 14.3 Effect of microbial cementation on strength and shear wave velocity (V_s) of Ottawa sand [p' = preconsolidation pressure and $q = (\sigma_1 - \sigma_3)/2$, where σ_1, and σ_3 are major and minor principal stresses, respectively]. (From DeJong, 2008.)

of microorganisms and extracellular products with a solid substratum are in general referred to as *biofilms*.

Increasing the biomass associated with the porous medium is another way by which the geomechanical properties of a porous medium can be biomediated. Increased microbial growth alters the pore geometry, which can cause a decrease in the hydraulic conductivity and porosity of the porous media, a phenomenon referred to as *bioclogging* (Baveye et al., 1998). Bacteria often appear to be the dominant organisms in the biomass of clogged porous media, but fungi and algae have been found as well, and protozoans may also be involved (Mattison et al., 2002).

The development of bioclogging is a result of numerous physical, chemical, and microbial mechanisms (Baveye et al., 1998). Of most interest here are the microbial mechanisms, which include the accumulation of microbial cells (e.g., at grain contacts

or pore necks), production of EPS, accumulation of precipitates, and entrapment of gaseous metabolic end products. As mineral precipitation and biogenic gas production are discussed above and below, respectively, the focus here is on the impact of accumulating biomass (i.e., bacteria plus EPS produced by the bacteria) on the porous medium. The net accumulation of biomass is controlled by four processes (Rittmann, 1993): (1) microbial growth, which is proportional to the rate of substrate utilization; (2) deposition of suspended bacteria, which also increases the attached biomass and is controlled by the free-living cell concentration and their transport and adsorption; (3) decay of biomass (e.g., for maintenance energy supply), which reduces the attached biomass proportionally to the amount of active biomass; and (4) detachment, which decreases the attached biomass and increases the amount of free-living cells, generally at rates proportional to the amount of attached biomass and the hydrodynamic shear stress. Correspondingly, the addition of growth substrates (e.g., carbon and energy sources and nutrients), which results in larger net biomass accumulation, has been observed to accelerate the bioclogging process (Baveye et al., 1998).

Different explanations have been offered as to how increases in biomass cause reductions in hydraulic conductivity (Rittmann, 1993). Some researchers (Taylor and Jaffé, 1990; Cunningham et al., 1991) have conceptualized the biomass as forming a continuous biofilm on the particle surfaces, which is assumed to be impermeable to water flow and reduces the hydraulic conductivity by decreasing the pore size and porosity. In comparison, others (Vandevivere and Baveye, 1992) have observed isolated bacterial aggregates, which reduce the hydraulic conductivity by accumulating at pore constrictions and forming plugs. Yet others have observed *biowebs*, with strands of biomass crisscrossing the pores (Paulsen et al., 1997). In some cases, the various forms of biomass growth have occurred simultaneously (Dupin and McCarty, 2000). Rittmann (1993) used a tool of biofilm kinetics, the normalized surface loading, to interpret these conflicting claims about the characteristics of attached bacteria in the subsurface. As discussed further below, analysis of a range of experiments indicated that the magnitude of the surface loading could be used to differentiate between conditions leading to continuous and discontinuous or patchy biofilm forms.

Resting bacteria known as *ultramicrobacteria* may be used to facilitate the bioclogging process (Perkins et al., 2000). These bacteria are formed when active vegetative cells are starved, resulting in a reduction in size and metabolic rates. When the cells have penetrated to the desired location in the porous media, they can be regrown by stimulation with nutrients, resulting in biomass growth and clogging. For example, MacLeod et al. (1988) conducted tests on sintered glass-bead cores using vegetative and resting cell cultures of *Klebsiella pneumoniae*. While the vegetative cells caused a greater initial decrease in the hydraulic conductivity than the starved cells (99% versus 71% reduction), the starved cells were able to penetrate deeper into the cores and could later be stimulated to produce reductions in hydraulic conductivity.

Rather than using biofilms to modify the geomechanical properties of a porous medium, a related approach is to use commercial biopolymers. For example, several EPS are now available as products of biotechnology (Sutherland, 1998). One of the most widely used of these is xanthan (also known as xanthan gum). Xanthan is produced by the plant pathogen *Xanthomonas campestris* (Sutherland, 2002). It has a cellulosic backbone, with a trisaccharide side chain attached to every second glucose residue. Xanthan has been used in enhanced oil recovery to reduce the hydraulic conductivity of the porous medium (Sandvik and Maerker, 1977). This occurs due to

retention of biopolymers like xanthan in the porous medium via two possible mechanisms that alter the pore geometry: chemical adsorption onto pore channel walls, which reduces the cross-sectional area for flow; and physical entrapment in narrow pore constrictions, which restricts flow to a portion of the interconnected pore network.

14.3.2 Effects on the Mechanical Properties of Soils

Various researchers have analyzed the effects of naturally occurring biofilms on the clogging of soil pores and the use of biopolymers for the beneficial improvement of geotechnical properties of soils. In particular, engineered growth of biofilms and biopolymer addition have usually been considered for their effects on the hydraulic characteristics of the candidate porous media, as discussed above. For example, many one-dimensional laboratory column studies of bioclogging have been performed in which nutrient solutions were pumped through porous media inoculated with bacteria, resulting in large decreases in porosity and several orders of magnitude reductions in hydraulic conductivity, especially near the column inlet (MacLeod et al., 1988; Taylor and Jaffé, 1990; Cunningham et al., 1991; Vandevivere and Baveye, 1992; Cunningham et al., 1997; Perkins et al., 2000; Mattison et al., 2002; VanGulck and Rowe, 2004). Most of these studies focused on bioclogging due to biomass accumulation, although in some cases biogenic gas may have been partially responsible (Mattison et al., 2002), as well as $CaCO_3$ precipitation and suspended solids removal (VanGulck and Rowe, 2004).

Modeling and experimental studies with more realistic two-dimensional sand tank studies have also demonstrated bioclogging downgradient of the nutrient injection point (Kildsgaard and Engesgaard, 2002; Thullner et al., 2002; Seki et al., 2006). However, although the hydraulic conductivity was reduced in the clogged region, leading to preferential flow patterns, there was minimal change in the bulk hydraulic conductivity of the sand tank. In one of the few field studies of bioclogging, Oberdorfer and Peterson (1985) injected wastewater into wells at two sites in different sedimentary formations and observed that clogging initially occurred primarily within 50 cm of the injection wells (probably due to entrapment of suspended organic matter in the wastewater), with the most severe clogging later observed 70 to 130 cm from the injection wells (probably due to N_2 gas production, which is discussed below).

Biopolymers have also been shown to cause significant decreases in the hydraulic conductivity of porous materials. Khachatoorian et al. (2003) conducted laboratory column tests to compare the hydraulic performance of five different biopolymers—xanthan gum, polyhydroxybutyrate (PHB), guar gum, polyglumatic acid (PGA), and chitosan—for their possible use in biobarrier applications. The greatest reduction in hydraulic conductivity was observed for PHB (six orders of magnitude), followed by PGA and chitosan (three orders of magnitude). Similarly, Martin et al. (1996) observed that mixing silty soils with 0.3% (by weight) xanthan gum resulted in a two-order-of-magnitude decrease in the hydraulic conductivity that lasted for six months (Kavazanjian and Karatas, 2008).

In addition to the effects of biofilms and biopolymer addition on hydraulic characteristics, there has been increasing interest in the potential effects of these processes on soil strength. In an attempt to evaluate the effects of biofilm formation on strength, Perkins at al. (2000) conducted one-dimensional compression (i.e., oedometer) tests as well as consolidated-drained and consolidated-undrained triaxial shear tests on sand

inoculated with *Klebsiella oxytaca*. A negligible increase in strength and stiffness was observed as a result of the biofilm treatment. However, results from oedometer tests indicated that the secondary creep index, defined as the slope of the one-dimensional strain versus the log-of-time curve, increased 2.5 times as a result of the biofilm treatment. Perkins at al. (2000) noted that the viscous nature of the biofilm may have reduced the friction angle of the sandy soil. The shear strength of soils has cohesive and frictional components, and it is possible that the potential beneficial effects of the biofilm bonding process for increasing cohesive strength may have been offset due to the lubricating properties of the biofilm. It might be expected that dilation of the sand may still be present, but the frictional component between the minerals may have decreased due to the viscous nature of the biofilm.

Martin et al. (1996) showed that the strength of silty soils increased by 30% with an addition of 0.3% (by weight) xanthan gum when subjected to consolidated-undrained triaxial shear tests. The aging of soil–biopolymer mixtures had a clear effect on strength, with about 20 days of aging following the hydration of the mixture required to observe the full (maximum) strength increase. Cabalar and Canakci (2005) also reported a fivefold increase in drained shear strength of sand with an increase in xanthan gum from 1% to 5% (by weight).

Once a bioclogg barrier has been established, laboratory studies suggest that periodic maintenance (i.e., feeding) will be required for the long-term maintenance of the changes in the soil properties (e.g., reductions in hydraulic conductivity) (Cunningham et al., 1997). Thus, if suitable environmental conditions are not actively maintained, the permanence of these improvement in soil properties becomes a concern (Kavazanjian and Karatas, 2008). Nevertheless, even if the effects of biofilms and biopolymers are not long term, it may still be appropriate or desirable for applications requiring only temporary soil improvement (e.g., stabilization and groundwater control for excavations and tunneling) (Perkins et al., 2000, Kavazanjian and Karatas, 2008).

14.4 MINERAL TRANSFORMATION

14.4.1 Potential Microbiological Improvement Mechanisms

In addition to the microbially induced precipitation processes described above, microorganisms may also influence other geological processes, including diagenesis and weathering (Ehrlich, 2002). For instance, reactive microbial metabolic products such as inorganic (e.g., NH_3, HNO_3, H_2SO_4, H_2CO_3) and organic (oxalic, citric, and gluconic) acids may promote rock weathering by altering or solubilizing some of the rock mineral constituents. In addition, some microbes may also perform a direct enzymatic attack on certain oxidizable or reducible rock minerals. An example of the latter, which is of interest for the biomediation of soil properties, is the microbially mediated reduction of Fe(III) in iron-rich smectite.

Fe(III) in clay minerals can be an important electron acceptor supporting the growth of bacteria (Kostka et al., 2002). Several pure and enrichment cultures have been shown to catalyze the rapid reduction of Fe(III) within smectite clay minerals (Gates et al., 1993; Kostka et al., 1996, 1999b). More recently, Kim et al. (2004) presented evidence that the bacterium *Shewanella oneidensis* strain MR-1 promoted the microbial dissolution of smectite via reduction of Fe(III), resulting in the formation of illite. The mechanism of interaction between the dissimilatory iron-reducing bacteria and

the iron-rich clays is not well understood (Stucki and Kostka, 2006). Some studies indicate that the electron transfer mechanism between the bacterial cells and clays requires direct contact (Kostka et al., 1996), while other studies report an increased rate or extent of Fe(III) reduction if an electron shuttle is added (Kostka et al., 1999a).

Engineering a soil system to promote this reaction requires the provision of several conditions. The structural Fe(III) in a swelling clay can provide the electron acceptor for iron-reducing bacteria when soils and sediments are subjected to reducing conditions in the absence of competing electron acceptors (e.g., O_2 and NO_3^-), such as during soil flooding (Kostka et al., 1996). In addition, an appropriate electron donor and environmental conditions are needed. For example, using organic acids (formate or lactate) as the electron donor was shown to promote reduction of structural Fe(III) by *Shewanella putrefaciens* strain MR-1 (Kostka et al., 1996, 1999a), with the following stoichiometry (Stucki and Kostka, 2006): for formate,

$$0.73[Fe(III)_{2.73}]_{smectite} + HCOO^- + H_2O \rightarrow 0.73[Fe(II)_{2.73}]_{smectite} + HCO_3^- + 2H^+ \quad (14.17)$$

and for lactate,

$$1.47[Fe(III)_{2.73}]_{smectite} + CH_3CHOHCOO^- + 2H_2O \rightarrow$$
$$1.47[Fe(II)_{2.73}]_{smectite} + CH_3COO^- + HCO_3^- + 5H^+ \quad (14.18)$$

Kostka et al. (1996) also demonstrated that smectite reduction by *Shewanella putrefaciens* strain MR-1 occurred at pH (optimum pH 5 to 6) and temperature (optimum 25 to 37°C) values common to soils and sediments.

14.4.2 Effects on the Mechanical Properties of Soils

Reduction of Fe(III) in crystalline clay minerals results in a collapse of the clay structure (Figure 14.4). This causes several fundamental changes to the physicochemical properties of clays, including swell capacity, surface area, cation exchange capacity, and flocculation properties (Kostka et al., 1996; Stucki and Kostka, 2006). Of these changes, perhaps of most interest to the geotechnical engineer is the observation by several researchers that bacterial reduction of structural Fe(III) in a smectite decreased the water content and swelling pressure of the clay (Gates et al., 1993, Kostka et al., 1999b). When water is added to expansive clays, such as smectite, the water molecules are pulled into gaps between the clay sheets, causing the soil volume to expand. Reduction of the structural Fe results in a greater fraction of collapsed clay layers, which do not participate in free swelling, and thus a reduced water-holding capacity (Figure 14.4) (Stucki et al., 2002). This is important because swelling of clays controls soil permeability and water drainage (Gates et al., 1993).

It has also been shown that the microbial reduction of Fe(III) to Fe(II) decreases the specific surface area, whereas the cation-exchange capacity and potential for mineral dissolution and transformation increase as a result of this reduction (Kostka et al., 1999b; Stucki and Kostka, 2006). In addition, Kostka et al. (1999b) found that microbial reduction of Fe(III) to Fe(II) enhanced the hydrophilic character of the clay mineral surface. Finally, Kim et al. (2005) showed that the microbial reduction by *Shewanella oneidensis* promotes clay flocculation, which is known to increase the hydraulic conductivity of the clay (Lambe, 1958).

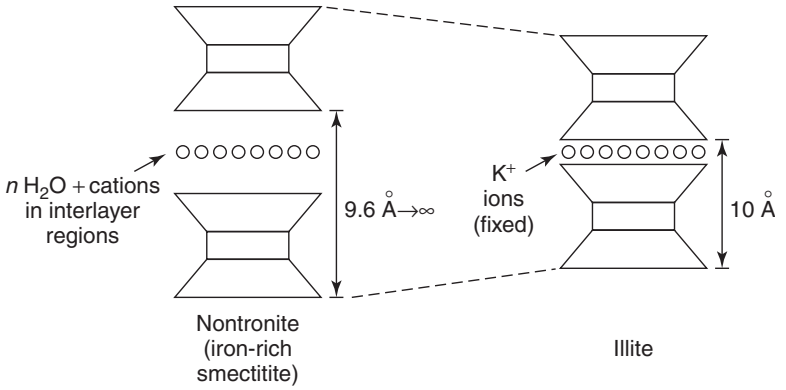

Figure 14.4 Effect of the reduction of structural Fe(III) to Fe(II) on the interlayer collapse of smectite with K^+ as the exchanged cation. The trapezoids represent tetrahedral sheets and the rectangles represent octahedral sheets. The circles are interlayer cations. (Adapted from Stucki et al., 2002.)

14.5 BIOGENIC GAS PRODUCTION

14.5.1 Potential Microbiological Improvement Mechanisms

Microorganisms produce a variety of poorly soluble gases, including CO_2, N_2, H_2, and CH_4. Correspondingly, many field and laboratory studies have reported the presence of a gaseous phase in submerged soil profiles (Baveye et al., 1998). Much of this literature has been focused on N_2 gas production during denitrification, probably due to the importance of the nitrogen cycle, and the fact that H_2 has rarely been found to accumulate in the soil gas, presumably because it is readily used by other microorganisms. In terms of the biomediation of soil properties, N_2 gas has several advantages over the other gases (Rebata-Landa, 2007): (1) unlike CH_4 and H_2, it is not combustible; (2) unlike CH_4, it is not a greenhouse gas; and (3) it has a very low water solubility, which means that less gas is required to produce bubbles and, once produced, bubbles will remain undissolved for a longer time. However, N_2 gas is formed via the reduction of nitrate, which is a water pollutant of concern (USEPA, 2008).

As biogenic gases are produced (e.g., due to denitrification), it causes changes in the partial pressure of individual dissolved gases and thus in the total dissolved gas pressure (Amos and Mayer, 2006). When the total gas pressure becomes greater than a threshold pressure, which is equal to the sum of the hydrodynamic and capillary pressures, nucleation and growth of gas bubbles may occur. Subsequently, gases partition between the aqueous and gas phases according to Henry's law [equation (14.1)], resulting in bubble growth or shrinkage, depending on whether the gases are produced or consumed. The bubble will disappear when the total dissolved gas pressure becomes less than the threshold pressure. Formation of the gas phase significantly affects the dissolved gas concentrations because of the insolubility of most dissolved gases. As a result, the dissolved concentrations of nonreactive gases (e.g., Ar and N_2) will decrease as they partition into the bubbles.

Biogenic gas production has been observed to mimic bacterial population growth patterns (Sills and Gonzalez, 2001), as expected. Thus, promoting the desired

gas-producing process to biomediate soil properties requires providing the required nutrients (electron donors and acceptors, and other nutrients), along with the appropriate environmental conditions. For example, addition of organic matter is expected to enhance biogenic gas production via heterotrophic processes (Ronen et al., 1989).

14.5.2 Effects on the Mechanical Properties of Soils

The entrapment of gaseous metabolic products has been investigated for its role in the clogging of saturated soils and aquifer materials (Baveye et al., 1998). If gas bubbles formed in a porous medium are sufficiently large to become trapped in the pore network, the hydraulic conductivity will be reduced due to the decrease in the size of the water-conducting pores (Figure 14.5). For example, when Orlob and Radhakrishna (1958) [cited by Baveye et al. (1998)] increased the air content in a uniform sand by 10%, the hydraulic conductivity was reduced by 35%, and desaturation had an even greater effect on hydraulic conductivity when fine and large grains of sand were present. Gas bubbles actually do not need to be larger than the pore diameters to accumulate in the pores, because they can adhere to surfaces (Figure 14.5) (e.g., at hydrophobic sites) (Baveye et al., 1998) or become trapped in the exopolymer matrix surrounding biofilms (Battersby et al., 1985). This gas accumulation is likely to occur simultaneously with the bioclogging, due to the biofilm formation discussed above, with the decrease in hydraulic conductivity a result of the combined effect (Seki et al., 1998).

Biogenic gas occurring in offshore and lake sediments and landfill sites is also an important factor affecting other geotechnical characteristics of soils (Sills and Gonzalez, 2001; Rowe et al., 2002). For example, Sills and Gonzales (2001) conducted a series of

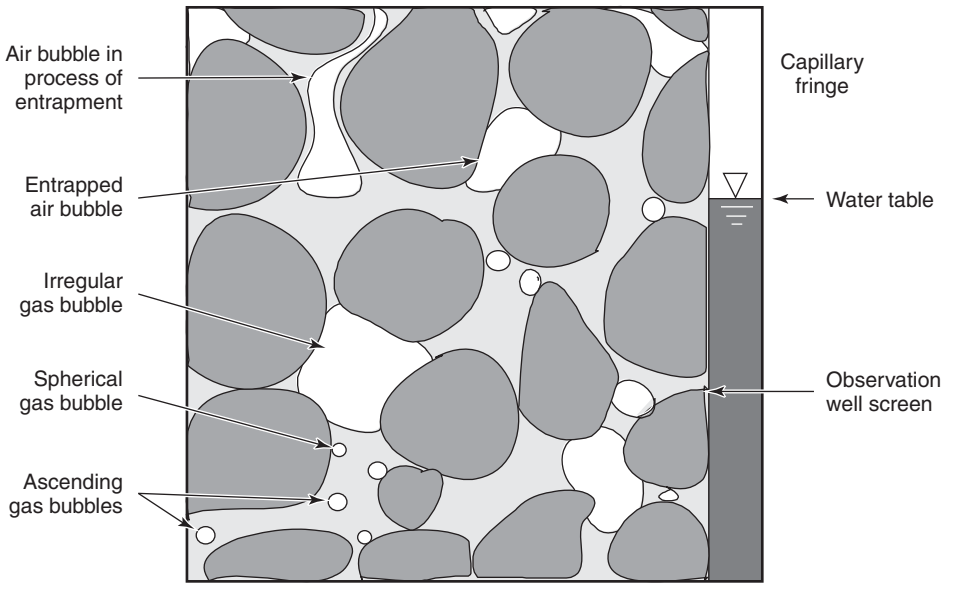

Figure 14.5 Porous media with entrapped air bubbles and biogenic gas bubbles. (Adapted from Ronen et al., 1989.)

consolidation experiments on soils that naturally contained biogenic gas. Two phases of consolidation were observed. In the first phase, gas was produced and accumulated within the soil, and the pore pressures fluctuated unpredictably. In the second phase, the settlement accelerated, as cracks and fissures provided a quick route for gas and pore water dissipation.

Biogenic gas production may be engineered for soil improvement during construction applications or against earthquake loads. Rebata-Landa (2007) conducted a laboratory study of the effects of N_2-producing bacteria on the mechanical properties of various soils and sediments. The soils and sediments were inoculated with the denitrifying bacterium *Paracoccus denitrificans*, placed in closed containers, the volume of gas produced was determined, and P-wave velocity, V_p, measurements were conducted. P-waves are a factor of the bulk stiffness of the pore fluid and are commonly used to define the effect of degree of saturation on soil stiffness (Fratta et al., 2005). The experimental results of Rebata-Landa (2007) showed that P-wave velocity was affected by the nutrient availability but not by the material property (e.g., specific surface area). Specimens inoculated with bacteria consistently had lower P-wave signatures than those of the original soil and sediment specimens ($V_p \sim 1500$ m/s versus 600 m/s). Rebata-Landa (2007) correlated the P-wave velocities to the saturation level and normalized cyclic stress ratio, a parameter indicative of the number of cycles required to attain soil liquefaction under earthquake loads. Earthquakes can induce liquefaction of saturated soils, giving them the consistency of a heavy liquid and damaging overlying structures. Lower P-wave velocities were measured when the saturation level was decreased. This is consistent with the observation of Fratta et al. (2005) that a 5 to 10% decrease in the degree of saturation of a fully saturated soil ($S = 100\%$) can decrease the P-wave velocities significantly. It is well known that the normalized cyclic stress ratio increases with decreasing soil saturation (Yang et al., 2004) and P-wave velocities (Ishihara et al., 1998; Tsukamoto et al., 2002). Thus, a decrease in the degree of soil saturation due to biogenic gas generation is a potential tool for increasing the liquefaction resistance of soils subjected to cyclic loading.

14.6 BIOSOIL INTERACTIONS AND ENGINEERING

14.6.1 Potential Engineering Applications

There is a growing need for new technologies that improve and stabilize soils over wide areas and at low cost. At the same time, there is increasing interest in engineering approaches that mimic and maximize the benefits of natural processes, such as those reviewed above, including microbial calcite precipitation, engineered biofilm development, use of biopolymers, microbial mineral transformation, and biogenic gas production. These biological processes may provide cost-effective solutions for in situ site improvement in a variety of applications.

Mineral Precipitation Microbiological $CaCO_3$ precipitation is a ubiquitous process and has a number of potential applications, including capping of high-water-content wastes. A variety of contaminated high-water-content materials, such as polychlorinated biphenyl (PCB)–containing wastewater treatment sludges, contaminated harbor dredgings, waste pickle liquor sludges, asbestos-containing sediments, mine tailings,

and contaminated river bottom sediments are present at large industrial waste storage facilities that were constructed prior to the emergence of modern environmental regulations. The materials are typically contained in surface impoundments such as lagoons, ponds, or old quarries, and capping, if practical, often offers the least expensive solution for remediation of these impoundments (Edil and Aydilek, 2001). Microbiological calcite precipitation is expected to facilitate the capping of high-water-content wastes primarily by increasing the strength of the capping material and decreasing slightly the hydraulic conductivity of the candidate capping material, which is typically a sandy soil.

Portland cement or lime are sometimes added to chemically stabilize surface soils subject to erosion. The biocalcification process could potentially be used as an alternative to cement or lime stabilization for use in erosion control of naturally occurring surfaces, such as slopes and bluffs, which have been deforested or naturally exposed. Similarly, biocalcification could be used to create a light temporary surface crust that is permeable to water and could be used for dust control on heavy haul roads (e.g., at mining operations) and at construction sites, especially when dust is an environmental concern (Bang et al., 2009), as well as at campgrounds, parks, and race tracks, and other recreational facilities. Some have even proposed that biological crusts could be used for dust control in enclosed spaces on Mars or the Moon (Liu et al., 2008).

Possible geotechnical applications of biocalcification at relatively deeper locations include increasing the bearing capacity of soils to reduce settlement of structures (e.g., due to earthquakes) (Gallagher and Mitchell, 2001). Biocalcification can also be used to increase the liquefaction resistance of soils. Current approaches for increasing the resistance of soils to liquefaction (e.g., compaction grouting) are expensive and/or cause large ground deformation. Microbially induced precipitation could offer a cost-effective alternative for treatment of soils prone to liquefaction. The technique may also be suitable for remediation of liquefiable sand deposits along seashores due to the abundance of minerals that aid in the bioprecipitation process (Kavazanjian and Karatas, 2008). Similarly, biocalcification could be used to improve the poor shear characteristics of marginal soils such as loose sands, fills, mine spoils, and collapsible soils.

Biocalcification may also play a role in the long-term control of groundwater flow. For example, biocalcification could be used to control seepage and reduce surface settlements effectively by reducing permeability in a cutoff wall of soil surrounding an excavation, a tunnel, or the foundation of a structure. The process also has great potential for improving dam and levee safety by plugging erosive piping or for retrofitting building or bridge foundations. In addition, plugging via microbiological calcite precipitation has been studied for use as a microbially enhanced oil recovery (MEOR) process by the oil industry (Ferris et al., 1996). Specifically, calcium carbonate precipitation is used to control production of excess water by plugging the layers surrounding the oil-bearing layer during pumping. Finally, biocalcification could potentially be used in carbon sequestration by transferring carbon into a more reduced, deeper environment through formation of $CaCO_3$.

Biofilms and Biopolymers The engineered development of biofilms or addition of biopolymers may provide for short-term control of groundwater flow by plugging soil pores and reducing hydraulic conductivity. Examples of applications in which temporary groundwater control is needed include excavation dewatering (Geo-Solutions, 2008) or temporary confinement of in situ contaminants during site remediation (e.g.,

through use of biosorptive barriers) (Demirkan, 2008). Another application of these techniques is the MEOR process, which often uses biofilms or biopolymers for plugging high-permeability layers surrounding the oil-bearing layer to facilitate oil production (Finnerty and Singer, 1983).

Engineered biofilms are much less effective at reliably increasing the long-term bearing capacity and strength of soils. However, a limited amount of data suggests that small additions of biopolymers may cause significant increases in the strength of the soil (Martin et al., 1996). The use of biopolymers to strengthen soils could potentially be used in the improvement of shallow foundations, slope repair and stabilization, and excavation support. However, biopolymers are generally biodegradable, and changes in strength may not be long term. Thus, biopolymer-amended soils should be considered primarily for temporary applications, and long-term research studies should be conducted if use in permanent structures is considered.

Mineral Transformation Biocalcification and biofilm formation/biopolymer addition are microbial processes that can be used to enhance the physical properties of coarse-grained soils. However, fine-grained soils, particularly expansive or swelling soils, which typically contain expansive clay minerals, may also be improved through microbial reduction of Fe(III) in smectite and other Fe(III)-rich expansive clays, thereby converting them to illite or another mineral with little swelling potential. Expansive clays exhibit cyclic shrink–swell behavior, due to seasonal moisture changes. These changes influence the suitability of soils for engineering purposes. For example, these soil movements result in uneven, rough, and cracked pavement surfaces (Puppala et al., 2007). Swelling may also lead to deformation of foundations, uplift of bridge piers, and cause damage to tunnels, resulting in millions of dollars in annual repair costs to rehabilitate the structures (Madsen and Müller-Vonmoos, 1985). The high plasticity of swelling soils also pose workability problems during construction.

Biogenic Gas Production Biogenic gas generation is another process that could potentially be used to modify the geotechnical properties of soils. The bulk stiffness of the pore fluid in soils can be softened by injecting gas into soils, but this softening may also be achieved via microbially mediated processes (Rebata-Landa, 2007). Such decreases in pore pressures, in turn, lead to increases in the bearing capacity and shear strength of the soil. Increasing the soil strength and bearing capacity can be beneficial in various geotechnical applications, including roadway construction and foundation design and repair. Biogenic gas generation can also be a potential tool for increasing the liquefaction resistance of soils subjected to cyclic loading by causing a decrease in the degree of saturation of the soil.

14.6.2 Engineering Process Control

Balanced reactions were provided for many of the microbially mediated processes reviewed above. The stoichiometry of these microbiological reactions, which relates the quantities of reactants and products, provides one of the key quantitative tools for the design of engineered systems for biomediation of soil properties. The other key quantitative tool is the reaction kinetics, which describe the rate at which the reaction takes place and, coupled with the desired treatment time frame, determine the rate at which the required stimulatory materials must be added to the treatment zone (i.e., the

process loading) (Cookson, 1995). The key concept is to provide enough contact time between the microorganisms and the substrates to meet the treatment goals. Together, these data can be used to size treatment process facilities, and to estimate the required project time and cost.

Conceptual Models Formulation of a mathematical model of the rate of microbial substrate utilization within a saturated porous medium requires development of an appropriate conceptual model. As reviewed by Baveye and Valocchi (1989), the key conceptual models of bacterial growth and transport of biodegradable substrates in saturated porous media include the strictly macroscopic, microcolony, and biofilm models. The strictly macroscopic model only assumes that the bacteria are immobilized, without regard to spatial distribution. In comparison, the microcolony model assumes that the biomass is present as small colonies growing on the solid surfaces (Molz et al., 1986), while the biofilm model assumes that the bacteria and their EPS are distributed as a continuous film (Rittmann and McCarty, 1980, 1981).

Rittmann (1993) discussed the importance of differentiating between continuous and discontinuous biofilm forms when modeling subsurface biological processes. For modeling substrate flux in low-substrate-loading environments (e.g., as commonly found in groundwater situations), distinguishing between the strictly macroscopic model, microcolony model, or full biofilm model has no impact (Odencrantz et al., 1990). However, when modeling the spatial distribution of biomass or the loss of permeability due to biomass, the distinction is more important (Rittmann, 1993), as discussed above. For example, if the biomass forms a continuous biofilm on the particle surfaces (Taylor and Jaffé, 1990; Cunningham et al., 1991), the key factor affecting the hydraulic conductivity is the biofilm thickness, which decreases the pore diameter and liquid porosity. However, if the hydraulic conductivity is being reduced by the accumulation of biomass at pore constrictions (Vandevivere and Baveye, 1992), the key factor is biomass deposition at those points. Thus, depending on the specific situation and the goals of the modeling, one or more of these approaches may be applicable or equivalent. Because the strictly macroscopic and microcolony models can be viewed as limiting cases of the biofilm model, the biofilm model kinetics and their use are reviewed briefly here.

Biofilm Kinetics The key process loading criterion for the practical design and operation of biofilm processes is the flux, J [M/L^2/T], of the substrate from the water to the microorganisms attached to the soil surfaces (Rittmann, 1987). Therefore, the rate of removal of substrate from the water phase is described as (Bouwer, 1992)

$$\text{substrate loss rate} = -JA \tag{14.19}$$

where A is the surface area of the system [L^2]. The fixed-film models of Rittmann and McCarty (1980, 1981) provide relatively simple techniques to compute the substrate flux J under steady-state and non-steady-state conditions. Another key parameter for biofilm processes is the reference substrate flux, J_R, which represents the minimum flux supporting a deep biofilm (i.e., one in which the substrate concentration approaches zero within the biofilm because of diffusion limitations) (Rittmann, 1987).

Rittmann (1993) analyzed experimental data for colonization of porous media using the normalized surface loading:

$$\overline{J} = \frac{J}{J_R} \tag{14.20}$$

This analysis demonstrated that the surface loading could be used to differentiate between conditions leading to continuous ($J/J_R > 1.0$) and discontinuous biofilm forms (possible for J/J_R less than about 0.25), with an undefined transition in continuity. With high loading conditions ($J/J_R > 1.0$), biofilm accumulation is large and substrate removal is controlled by biokinetics and mass-transport resistance. However, with low substrate loading (e.g., in the subsurface) the substrate utilization kinetics are controlled by biomass accumulation, not internal mass transport.

The biofilm modeling approach was used in a series of modeling and experimental studies of the clogging of landfill leachate collection systems, which is relevant to several processes described above. Cooke et al. (1999, 2001) used a biofilm kinetics model successfully to evaluate column experiments with synthetic leachate and linked the clogging to the formation of microbial biofilms during volatile fatty acid (VFA) (acetate and propionate) degradation and to $CaCO_3$ precipitation. A key parameter in this modeling was the experimentally determined calcium yield coefficient, Y_{ca}, which relates the amount of calcium carbonate precipitated to the chemical oxygen demand removed. This model was later improved by defining a new parameter, the carbonic acid yield coefficient, Y_H, which relates carbonic acid production via microbial fermentation of VFAs to calcium precipitation (VanGulck et al., 2003), and incorporating the biofilm kinetics model into a generalized one-dimensional transport and clogging model (Cooke et al., 2005).

14.6.3 Engineering Strategies for Biomediation of Geomechanical Properties in the Field

Because the biomediation of soil geomechanical properties is a nascent field, the strategies for engineering applications in the field are largely undeveloped. Nevertheless, some general statements and proposals can be made, drawing on experience from the fields such as bioremediation and MEOR. Certainly, the strategies for biomediation of soils will significantly differ depending on the zone of treatment, but in all cases the goal will be the same—to develop an in situ bioreactor, or biologically active zone (BAZ).

Surface soil treatments, such as for dust control and capping of contaminated sludges, should be relatively simple to implement. If the necessary microorganisms exist at the site, the key will be to adjust appropriately the field conditions that will affect the process (i.e., perform biostimulation). Such applications could potentially exploit processes driven by photosynthesis, in which case energy will be supplied from the sun. For chemoorganotrophic applications, carbon and energy sources could be applied via surface spraying of aqueous solutions, as could nutrients and other amendments (e.g., calcium, pH control) needed to stimulate phototrophic and chemotrophic processes. These applications will probably occur under aerobic conditions, with oxygen from the atmosphere serving as the electron acceptor. However, if significant quantities of organic substrates are added into soils, the oxygen dissolved in the water- and gas-filled pores will be consumed rapidly, and reaeration from the overlying air may be inadequate to support appreciable microbial activity (Alexander, 1999). Given the shallow nature of these applications, tilling of the soil using agricultural implements could be used to aid at least in the initial aeration and mixing of amendments. If the necessary microorganisms are not present, it will also be necessary to add them (i.e., perform a bioaugmentation). In some cases it may be possible to use dead or

resting cells to achieve the biomediation, as discussed above (e.g., Chou, 2008). Or, it may only be necessary to supplement the soil with the required enzymes (e.g., Bang et al., 2009). The effectiveness of these treatments for altering the soil mechanical properties could be evaluated by measuring factors such as soil stiffness, strength of the surface, and percent weight loss of soil due to wind erosion.

In the case of deeper applications (e.g., in the unsaturated or saturated zones), implementation of biomediation will be made more challenging by the intrinsically inaccessible and heterogeneous nature of the subsurface. In addition, oxygen availability for promoting aerobic processes is likely to be a major issue in all applications given the limited amount of oxygen ($\approx$ 8 to 10 mg/L) that can be delivered from the air into water, coupled with the high oxygen demand associated with the organic substrates that may be added. In the unsaturated zone, it is possible to supply oxygen for aerobic processes by forcing air though the BAZ, as in bioventing for in situ bioremediation, which is advantageous because air is a more efficient carrier than water for delivering oxygen to the BAZ (Leeson and Hinchee, 1997). Alternatively, or in addition, water added via infiltration galleries can be used as a carrier for dissolved biostimulatory amendments, and microorganisms if necessary.

If the zone to be treated is saturated, or can be converted to saturated conditions, wells or infiltration galleries can be used to inject the biostimulatory amendments and create the BAZ, analogous to biobarrier systems used in in situ bioremediation. The injection system could also be coupled with extraction wells or trenches, making it possible to control the system hydraulically, forcing the amendments through the BAZ, as in water circulation approaches to in situ bioremediation. As discussed above, supplying adequate oxygen to support aerobic processes will be challenging in the saturated subsurface if air-saturated water is used as the oxygen carrier. Therefore, promoting microorganisms such as denitrifiers or SRBs that require an anaerobic subsurface environment may be advantageous (Kavazanjian and Karatas, 2008). Another option for supplying biostimulatory amendments to the saturated zone is to use solid slow-release materials such as gypsum to supply sulfate and calcium (e.g., Roemer and Schwartz, 1965; cited by Ehrlich, 2002) and wood chips and other inexpensive organic material to supply carbon and energy sources (e.g., Kim et al., 2003). Proprietary materials are also available to provide the slow release of oxygen and hydrogen.

One issue that is likely to be a challenge in the saturated zone is clogging of the formation. Bioclogging is the goal of some of the processes of interest discussed above; however, some care will be required in designing amendment addition schemes and maintaining the injection systems so as to not to clog the injection well screen or infiltration gallery. Some options for reducing bioclogging include periodic well redevelopment, periodic addition of inhibitory materials (e.g., hydrogen peroxide) to discourage biofilm growth, and pulsing of substrates, especially when more than one substrate is required to promote downgradient microbial activity (McCarty et al., 1998).

14.7 SUMMARY AND CONCLUSIONS

Although long ignored by the geotechnical engineering community, microbiological processes such as mineral precipitation, biofilm formation, mineral transformation, and biogenic gas production have significant effects on the mechanical properties of soils. Some of the key geomechanical properties that can be biomediated include reductions in hydraulic conductivity and increases in the strength and stiffness of soils.

Potential applications of the biomediation of soil geomechanical properties are many. For example, biocementation or biofilm/biopolymer processes could be used during construction in sandy soils to prevent infiltration of groundwater, control movement of soil into the excavation, and minimizing subsidence of adjacent structures. Such processes could also be used passively to stabilize loose saturated sands susceptible to liquefaction in seismic areas, while biogenic gas generation has the potential to reduce the liquefaction potential of these soils by changing the bulk stiffness and saturation. Another example of a potential application for biomediated geomechanical processes is reduction in the swell potential of expansive soils via mineral transformation.

Building on the significant history of work by geomicrobiologists and others, much has been learned in recent years about these microbially mediated processes and their impact on soil mechanical properties. Nonetheless, we have only begun to the scratch the surface in terms of understanding how we might implement these processes in practice. The future possibilities are exciting. For example, imagine using such processes to produce self-healing infrastructure, to "grow" foundations in unconsolidated soils, or to develop "smart" materials comprised of soil and appropriate microbial systems that could respond to changing conditions such as earthquakes (NRC, 2006). Given the breadth and potential of this field, and the issues that remain to be resolved, this should be a ripe area of research for many years to come.

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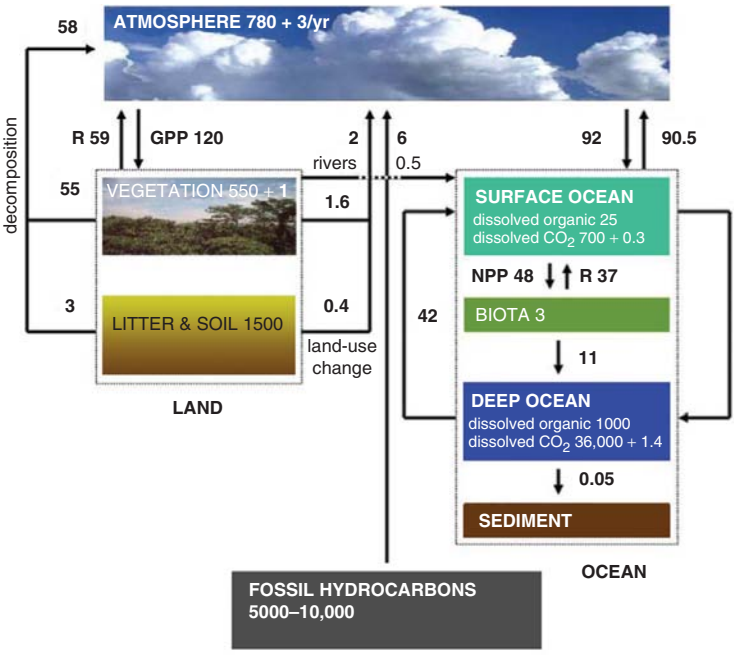

Figure 1.1 The global carbon cycle, including human perturbations in the 1990s. The quantities in the boxes are the size of the carbon reservoir in petagrams (Pg; 10^{15} g), with the annual growth, if any, due to the perturbations. Note that there is direct exchange between the atmosphere and terrestrial ecosystems, whereas exchange with the ocean is mediated by the physicochemical exchange across the air–sea interface. The downward transport of organic carbon, both particulate and dissolved, constitutes the biological pump. There is a riverine input of about 0.5 Pg from the land to ocean, balanced by outgassing and burial in sediments. Currently, the annual net land sink for atmospheric CO_2 is 1 Pg and the ocean sink is 2, leaving an annual net anthropogenic accumulation in the atmosphere of 3.2 Pg. (Modified from Houghton, 2007.)

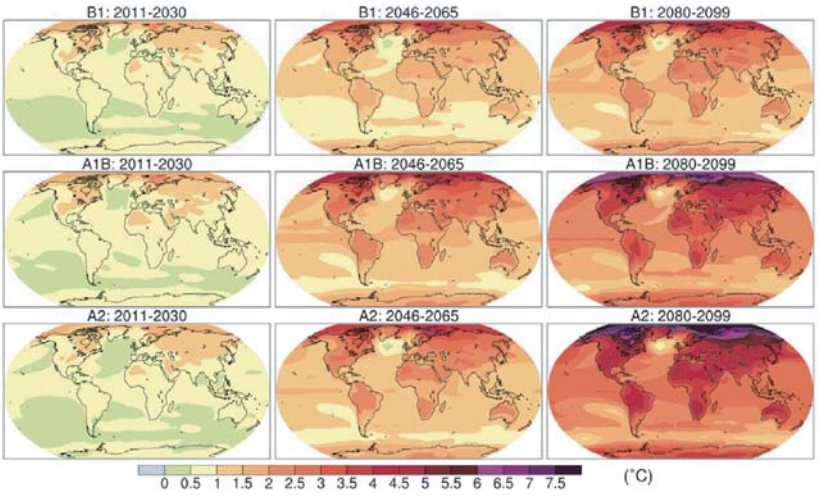

Figure 1.2 Multimodel mean of annual mean surface warming (surface air temperature change,°C) for scenarios B1, A1B, and A2, and three time periods, 2011 to 2030 (left), 2046 to 2065 (middle), and 2080 to 2099 (right). Stippling is omitted for clarity (see the text). Anomalies are relative to the average of the period 1980–1999. (From IPCC, 2007b, with permission of the IPCC, http://www.ipcc.ch/graphics/graphics.htm.)

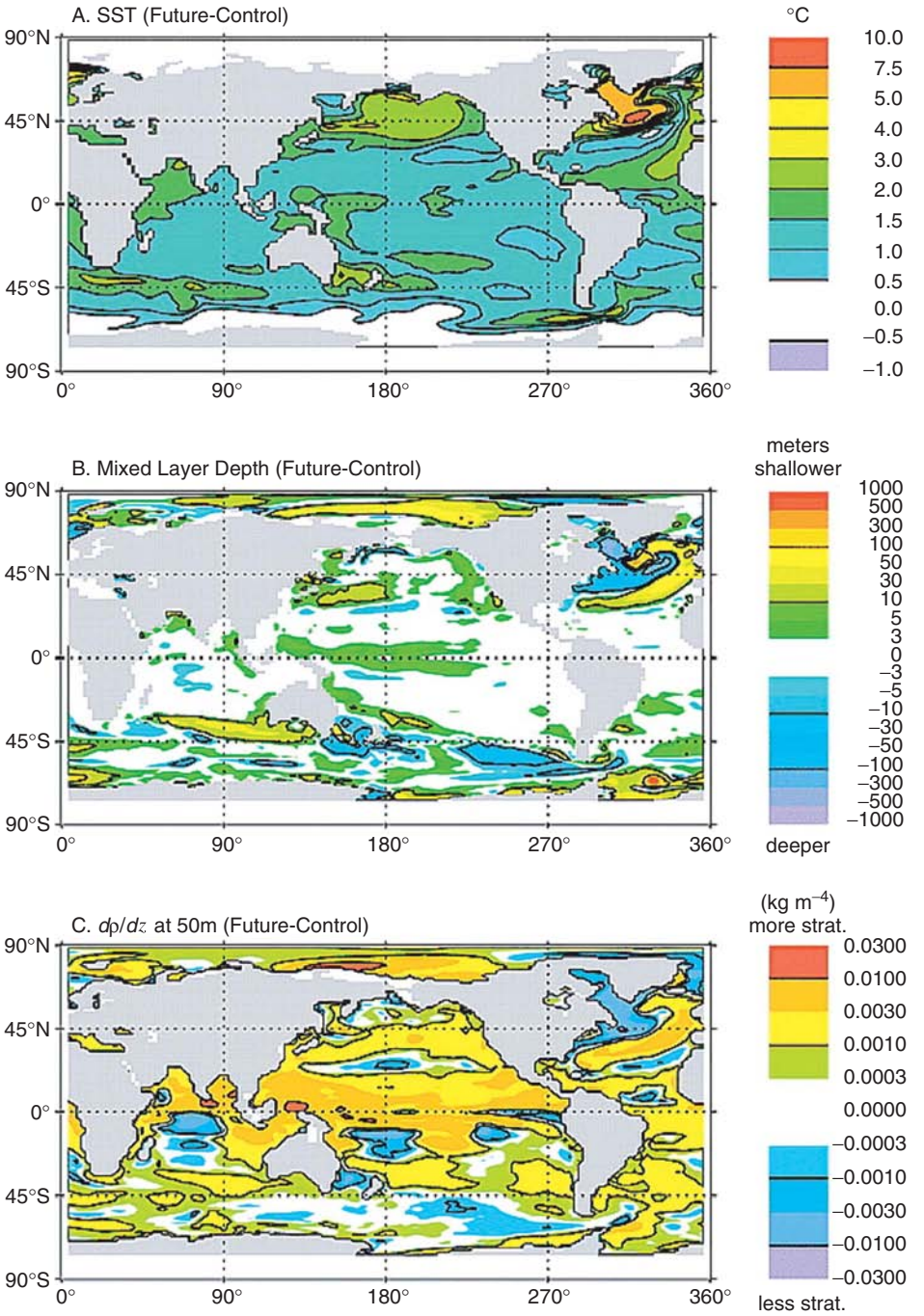

Figure 1.3 Projected climate-mediated changes in ocean physical forcing (future-control, i.e., 2060–2070 minus the present) from the NCAR Community Climate System Model for (A) sea surface temperature, (B) mixed-layer depth, and (C) upper ocean (50 m) stratification. (From Boyd and Doney, 2002, with permission of the American Geophysical Union.)

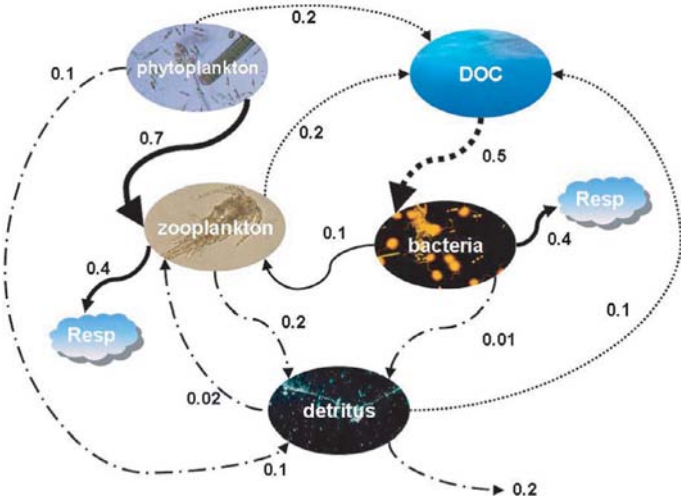

Figure 1.4 Microbial food-web diagram, showing exchanges of carbon in the oceanic surface layer. The flows are normalized to NPP = 1.0. The partitioning of flows among compartments is based on the physiological budget model given in Anderson and Ducklow (2001). Note that the carbon flows are dominated by zooplankton grazing (70% of NPP), DOC uptake by bacteria (50%), and heterotrophic respiration (80%). In this depiction the respiration is divided evenly among zooplankton and bacteria, but note that oceanic zooplankton may be dominated by protozoans smaller then 20 μm. Here the bacterial production is 12% of the particulate NPP, the fraction approximated by traditional ^{14}C assays, and a typical value for the open sea (Ducklow, 1999). Solid lines, biomass flows and respiration; dotted lines, dissolved flows; dashed–dotted lines, detrital flows and mortality.

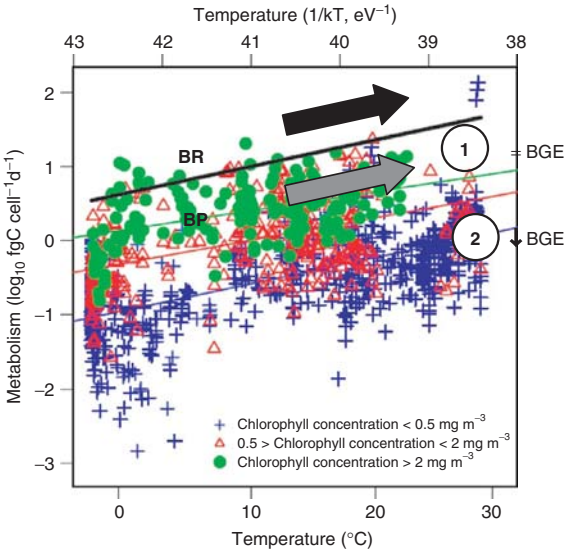

Figure 1.6 Individual rates of bacterial production (BP_i) and respiration (BR_i) versus temperature at three levels of chlorophyll concentration. The black line represents the relationship between ln BR_i and $1/kT$ ($y = 26.49 - 0.59x$) and the colored lines represent the ln BP_i–temperature relationships for each data subset (green, $y = 18.14 - 0.42x$; red, $20.54 - 0.50x$; blue, $22.58 - 0.58x$). An increase in temperature with no changes in resource availability would result in similar increases in BP_i and BR_i (i.e., the same BGE: case 1). Resource limitation would slow the rate of increase of BP_i with temperature compared to BR_i, thus lowering BGE (case 2). (Modified from López-Urrutia and Morán, 2007.)

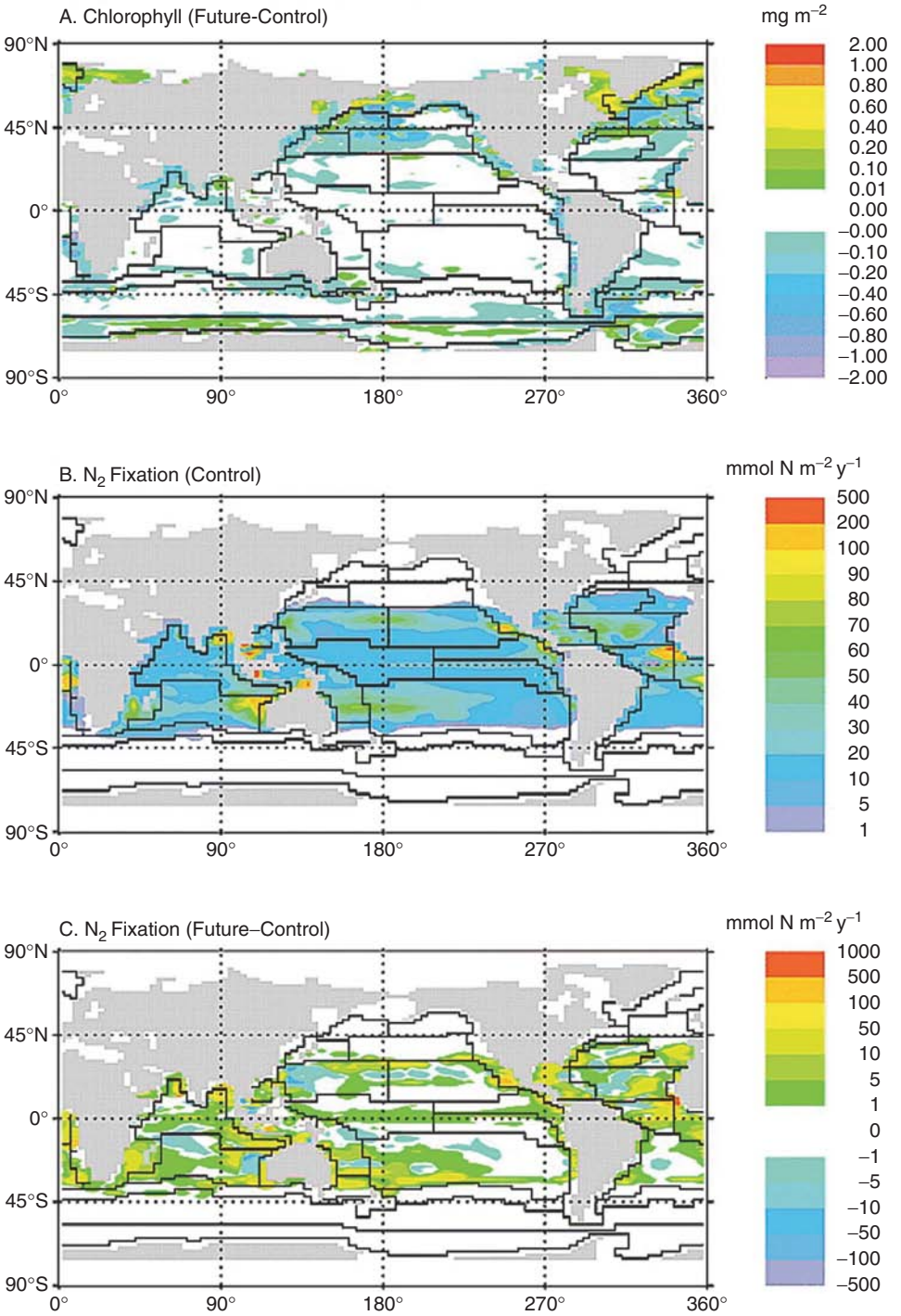

Figure 1.8 Numerical model simulations of ocean ecosystems using the CSSM (see Figure 1.3) with an off-line, multispecies pelagic ecosystem model for (A) the difference between predicted chlorophyll with warming and a control run (future-control), (B) N_2 fixation (control), and (C) N_2 fixation (future-control). (From Boyd and Doney, 2002, with permission of the American Geophysical Union.)

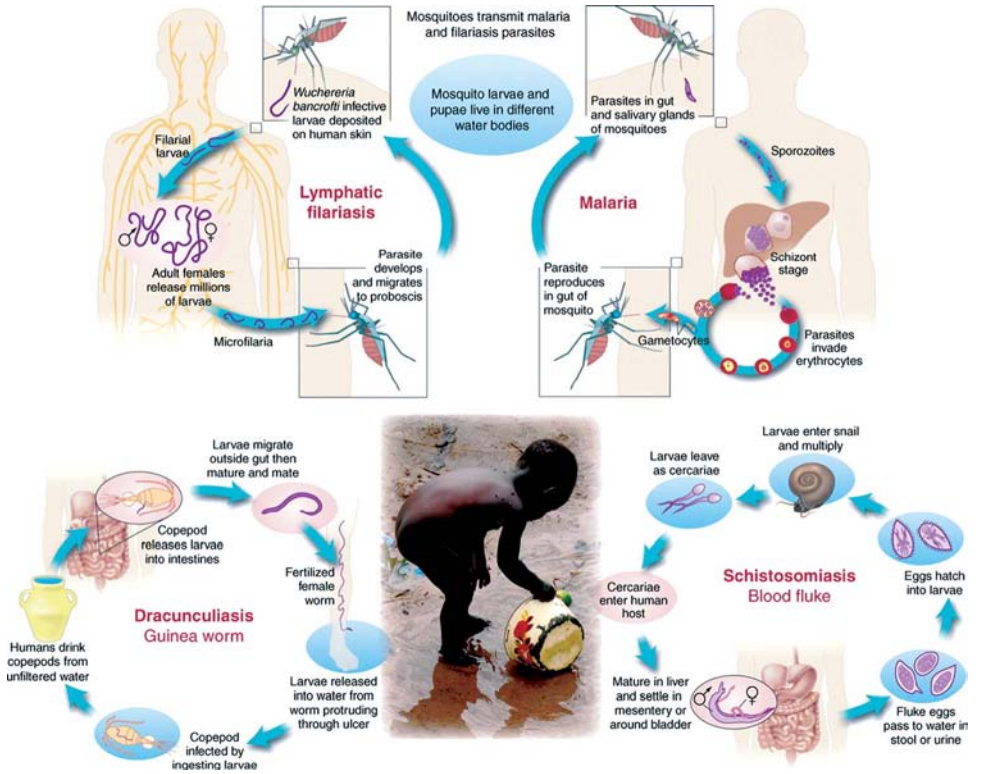

Figure 2.1 Transmission cycles of lymphatic filariasis, malaria, dracunculiasis, and schistosomiasis. (From Fenwick, 2006.)

Figure 2.2 Multiple uses of the Ganges River, Varanasi, India, in which *E. coli* O157:H7 has been detected. Clockwise, beginning from upper left: religious bathing, commercial laundry washing, washing and watering of cattle, milkmen washing pails in a "least polluted" section of the river, where the fecal coliform count exceeds 10^4 CPU/1000 mL (From Hamner et al., 2006, 2007.)

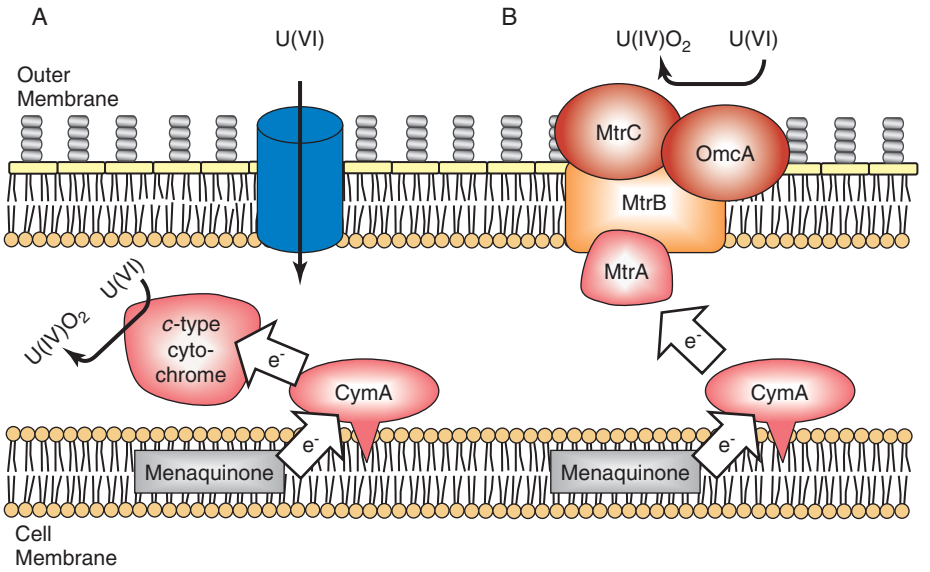

Figure 4.2 Working model for cytochrome *c*–facilitated U(VI) reduction in *Shewanella oneidensis* MR-1: (A) reduction of U(VI) by periplasmic *c*-type cytochromes; (B) reduction of U(VI) by outer membrane *c*-type cytochromes.

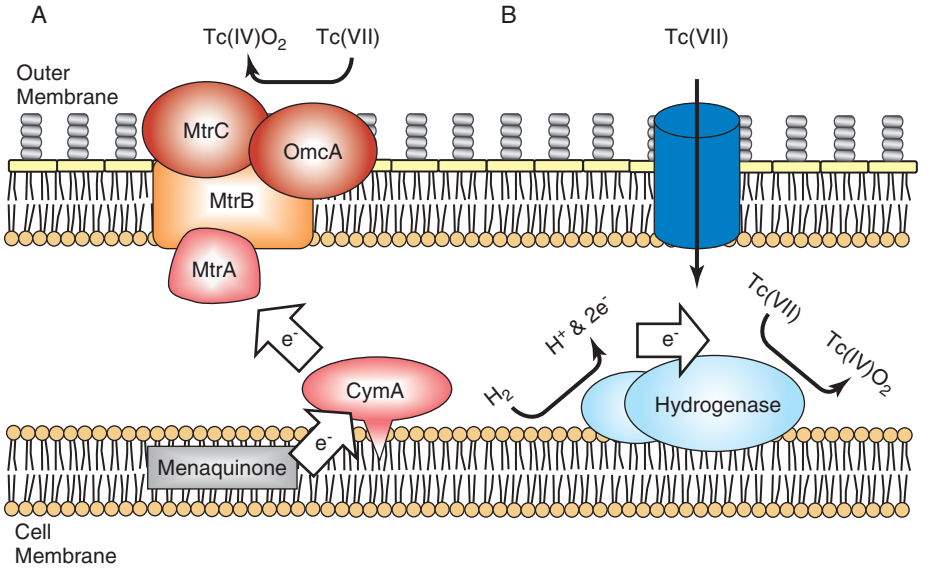

Figure 4.3 Working model for Tc(VII) reduction in *Shewanella oneidensis* MR-1: (A) outer membrane cytochrome *c*–facilitated Tc(VII) reduction; (B) periplasmic hydrogenase-facilitated Tc(VII) reduction.

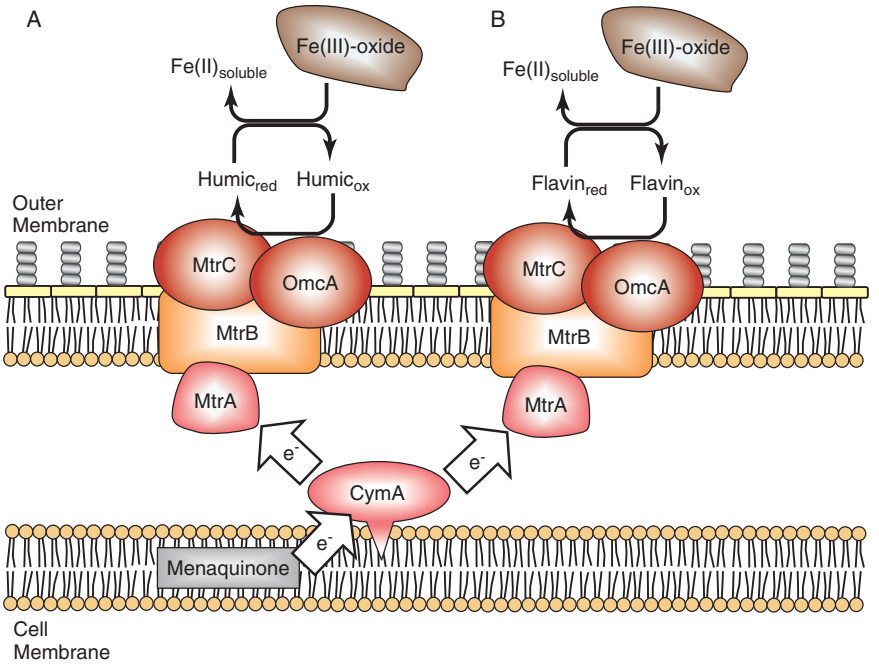

Figure 4.4 Working model for shuttle-facilitated Fe(III)-oxide reduction in *Shewanella oneidensis* MR-1: (A) reduction via an exogenous electron shuttle (humics); (B) reduction via an endogenous electron shuttle (flavins).

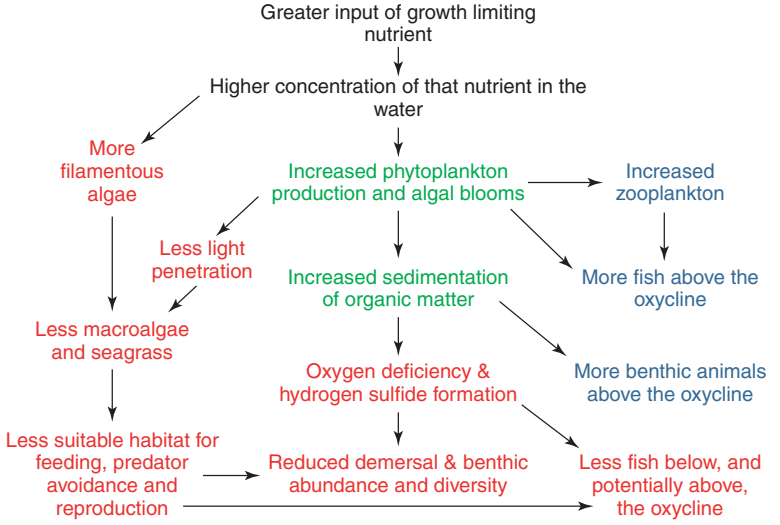

Figure 5.1 Series of responses within a coastal ecosystem to the increased input of a limiting nutrient. Green processes indicate eutrophication. Blue processes or conditions indicate increased secondary production. Red processes or conditions indicate the negative effects of eutrophication.

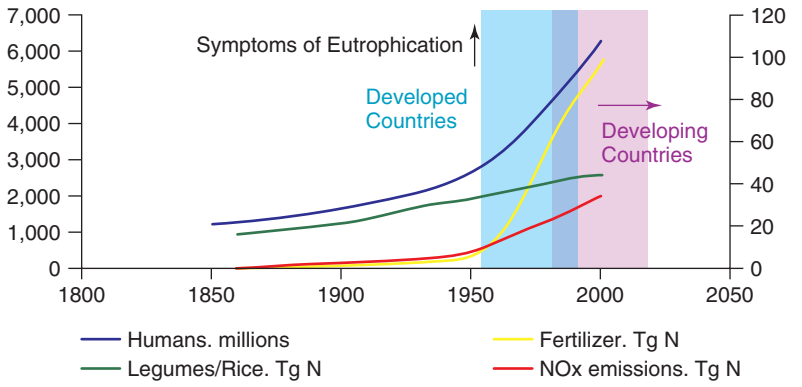

Figure 5.2 Period of the explosive increase in coastal eutrophication in relation to global additions of anthropogenically fixed nitrogen. Most of the symptoms for developed nations were manifested in the 1960s to 1980s (aqua) but are becoming more evident in developing countries with increases in fossil-fuel consumption and use of artificial fertilizers (lavender). (Modified from Boesch, 2002; Galloway and Cowling, 2002.)

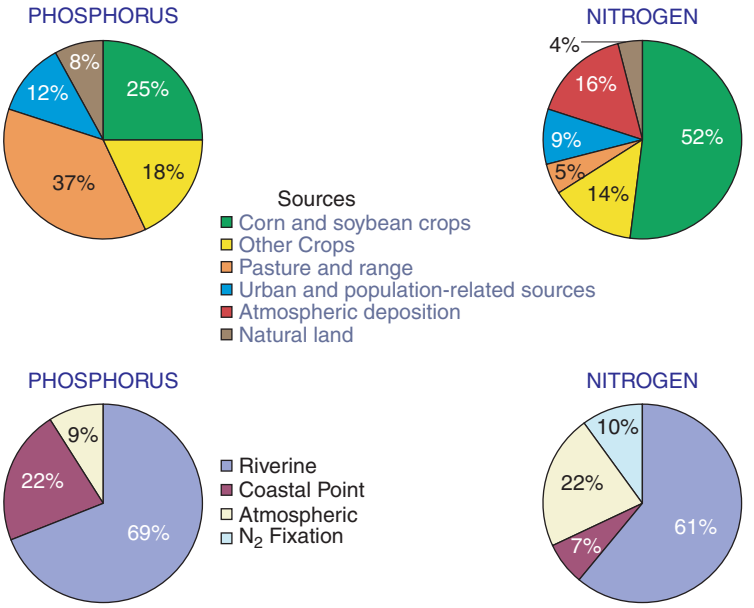

Figure 5.3 Relative proportion of sources or transport mechanisms of nitrogen and phosphorus to the northern Gulf of Mexico from the Mississippi River watershed (upper panels) (from Alexander et al., 2008, http://water.usgs.gov/nawqa/sparrow/gulf_findings/) and to the Baltic Proper (from Grimvall and Stålnacke, 2001.)

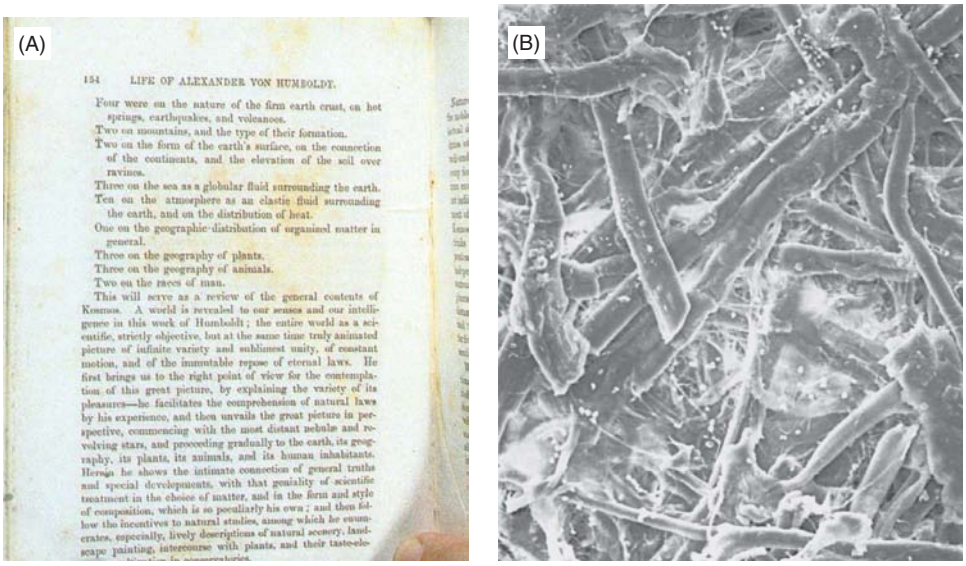

Figure 6.2 (A) Reddish-brown fox spots in the book *Lives of the Brothers Humboldt*, published in 1854. (B) Scanning electron micrograph of fungal growth occurring in a fox spot on paper. (Adapted from Florian, 1996.)

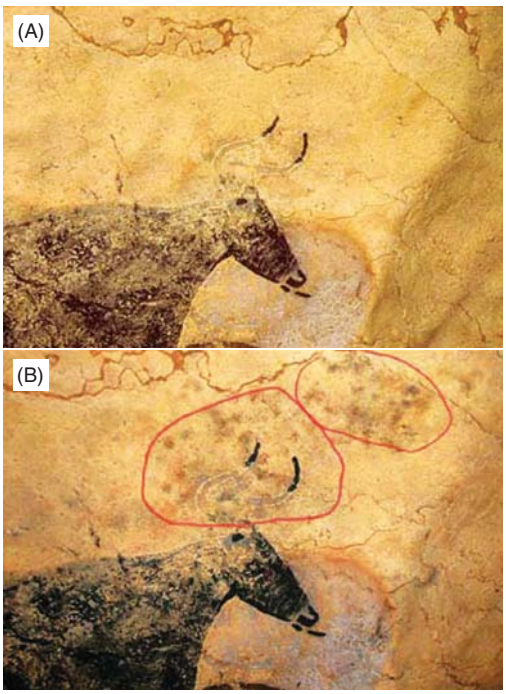

Figure 6.3 Painting of a bull on a wall in Lascaux before (A) and after (B) growth of grayish-black fungi. (From Bahn, 2008.)

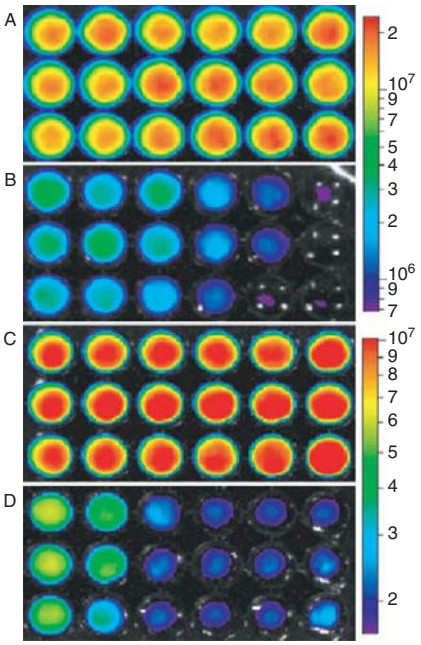

Figure 9.5 *S. cerevisiae luxCDABE* bioreporter assay in a high-throughput 96-well microtiter plate format imaged in real time with a Xenogen IVIS Lumina imaging system. Bioluminescence emission (as photon counts per second) by the bioreporters BLYRa (A) and BLYES (B) in response to a range of 17β-estradiol concentrations, and bioreporters BLYRa (C) and BLYAS (D) after exposure to a range of dihydrotestosterone concentrations. Each panel shows an 18-well exposure profile ranging from 1μM (top, left well) to 2.5 pM (bottom, right well) (1 : 10 dilutions were performed across each row and 1:2 dilutions down each column). Quantification of bioluminescence output from each well permits formulation of a standard curve and determination of estrogenic or androgenic equivalents present in the sample.

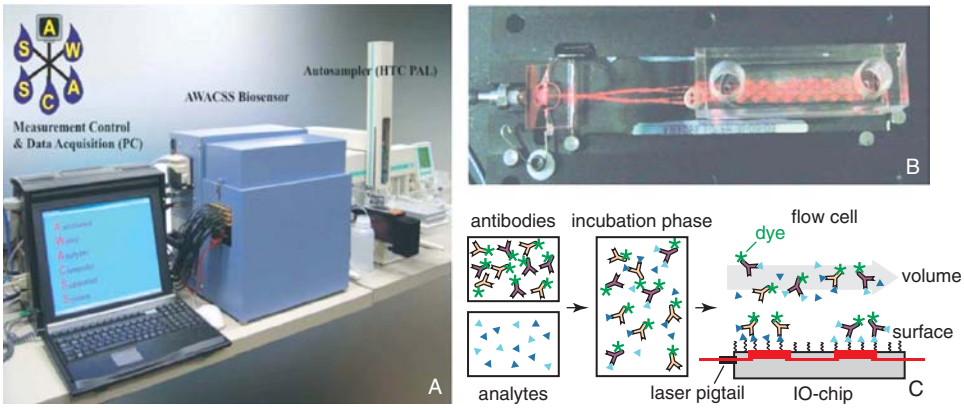

Figure 9.6 The AWACSS immunosensor (A) is capable of simultaneous multianalyte detection of up to 32 different target contaminants. Its detection methodology uses a semiconductor laser flow cell (B) to excite fluorophore-tagged antibody/target analyte complexes bound to the surface of a multisensor optical waveguide chip (C). (From Tschmelak et al., 2005, with permission.)

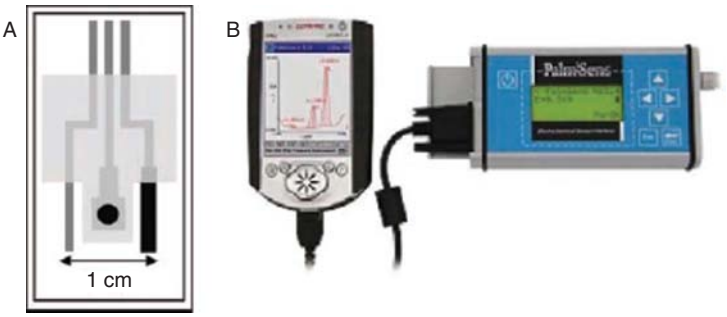

Figure 9.7 The screen-printed electrode (A) consists of three electrodes: a graphite working electrode in the center onto which the DNA is immobilized, and a silver reference electrode and graphite counter electrode on either side. The handheld potentiostat (B) on the right monitors variations in the electrochemical properties of the DNA, such as those occurring upon mutagenic chemical exposure. (From Lucarelli et al., 2003, with permission.)

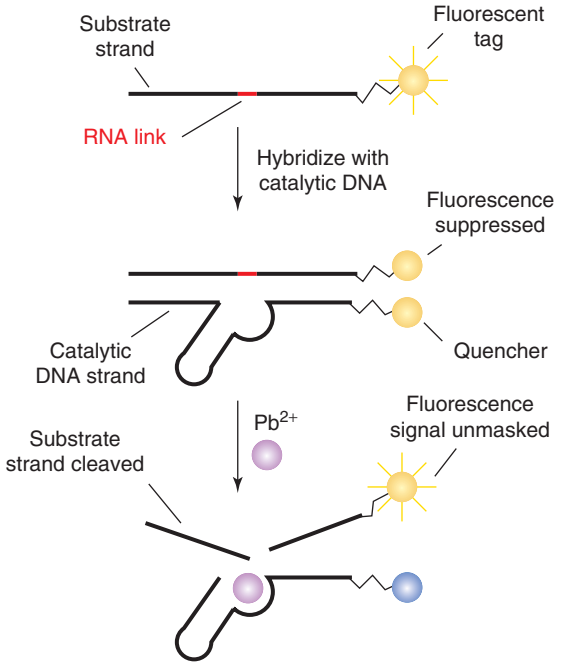

Figure 9.8 Catalytic beacon fluorosensor for lead (Pb^{2+}). In its uninduced state, fluorescence is suppressed due to close quencher proximity. Upon exposure to lead, the substrate strand is cleaved, thereby disassociating the fluorescent tag from the quencher to yield a quantifiable fluorescent signal. (From Borman et al., 2000, with permission.)