Introduction

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Most organic chemicals and many inorganic ones are subject to enzymatic attack through the activities of living organisms. Most of modern society's environmental pollutants are included among these chemicals, and the actions of enzymes on them are usually lumped under the term *biodegradation.* However, biodegradation can encompass many processes with drastically differing outcomes and consequences. For example, a xenobiotic pollutant might be mineralized, that is, converted to completely oxidized products like carbon dioxide, transformed to another compound that may be toxic or nontoxic, accumulated within an organism, or polymerized or otherwise bound to natural materials in soils, sediments, or waters. More than one of these processes may occur for a single pollutant at the same time. The chapters in this book will discuss these phenomena in relation to specific groups of xenobiotic pollutants, since these processes ultimately determine the success or failure of bioremediation technologies.

Bioremediation refers to the productive use of biodegradative processes to remove or detoxify pollutants that have found their way into the environment and threaten public health, usually as contaminants of soil, water, or sediments. Though biodegradation of wastes is a centuries-old technology, it is only in recent decades that serious attempts have been made to harness nature's biodegradative capabilities with the goal of large-scale technological applications for effective and affordable environmental restoration. This development has required a combination of basic laboratory research to identify and characterize promising biological processes, pilot-scale development and testing of new bioremediation technologies, their acceptance by regulators and the public, and, ultimately, field application of these processes to confirm that they are effective, safe, and predictable. Examples of these research and development strategies, both successful and unsuccessful, can be found in chapters dealing with specific types or classes of pollutants.

Levin and Gealt (1993, p. 4) estimated the costs of biotreatment of biodegradable contaminants in soils to range between $40 and $100 per cubic yard, as compared

with costs as high as $250–$800 per cubic yard for incineration and $150–$250 per yard for landfilling. Considering that billions of dollars in cleanup costs may be saved when these rates are projected over the whole of the industrialized world, and also that incineration and landfilling are no longer an alternative for many wastes, it is no surprise that bioremediation is receiving so much attention from the scientific and regulatory communities.

Bioremediation can be applied to an environmental problem in a variety of ways. Litchfield (1991) listed five general approaches to bioremediation: aboveground bioreactors, solid phase treatment, composting, landfarming, and *in situ* treatment. These five types of bioprocess largely cover the variations among bioremediation procedures, though there is considerable diversity in technologies within any one area.

Aboveground bioreactors are used to treat liquids (e.g., industrial process streams, pumped groundwater), vapors (e.g., solvents vented from contaminated subsurface environments, factory air), or solids in a slurry phase (e.g., excavated soils, sludges, or sediments; plant materials). They may use suspended microorganisms or adsorbed biofilms, singly or in combination; native microbial populations indigenous to the material being treated; pure microbial cultures isolated from appropriate environments; or genetically engineered microorganisms (GEMs) designed specifically for the problem at hand. They may operate with or without additions of oxygen or other electron acceptors (nitrate, carbon dioxide, sulfate, oxidized metals) and nutrient feeds (nitrogen, phosphorus, trace minerals, co-substrates). Other variables that may be controlled or that must otherwise be accounted for may include ratio of solids to water, biodegradation rates for specific pollutants or mixtures of pollutants, adsorption/desorption of pollutants to matrices such as soil or carriers used to provide surfaces for biofilms, pH, temperature, and redox potential. Bioreactors, as compared with other bioremediation techniques, can be controlled and their processes modeled mathematically with great precision. There are many physical designs of bioreactors, some of them quite novel and designed specifically for bioremediation. Bioreactors may be used in treatment trains (e.g., sequencing batch reactors), with multiple designs being used concurrently. Chapter 1 will discuss some of these designs and their application to environmental cleanup. Other bioreactor technologies will be discussed in chapters dealing with specific pollutants.

Soils are often treated by solid-phase technologies. This usually means placing excavated soils within some type of containment system, e.g., a lined pit with leachate collection and/or volatile compound entrapment equipment, and then percolating water and nutrients through the pile. Oxygen may or may not be supplied, depending on the bioremedial process being encouraged or supported. Inocula may or may not be added, depending on whether or not an indigenous microbial population can be stimulated to remove the target pollutant(s). Solid-phase treatments are particularly useful for petroleum-contaminated soils, but they are constantly finding new uses. For example, fungal mycelia carried on materials such as wood chips have been incorporated into contaminated soils to

promote biodegradation of xenobiotic contaminants, a process receiving considerable interest among bioremediation scientists (Lamar, 1990). A typical aerated static pile system for the bioremediation of petroleum-contaminated soil is shown in Figure 1.

Composting is a variation of solid-phase treatment that involves adding large amounts of readily degradable organic matter to a contaminated material, followed by incubations, usually aerobic, lasting several weeks or months. Adjustments of carbon: nitrogen ratios in composting systems require particular attention, and the

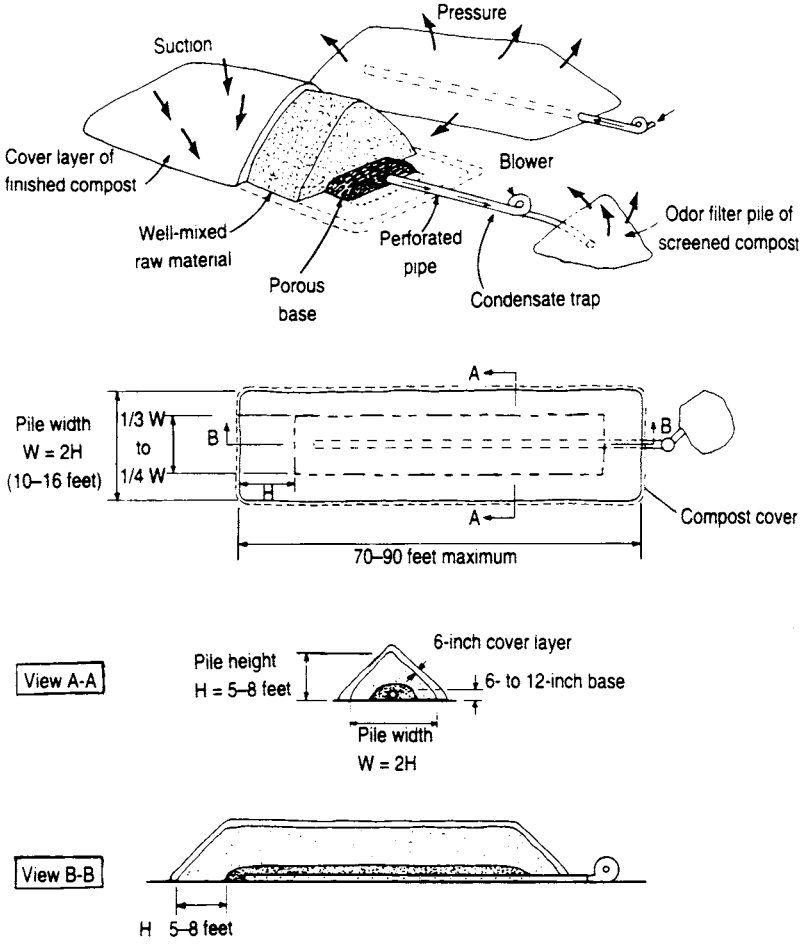

Figure 1. Aerated static pile for *ex situ* bioremediation of chemically contaminated soil. Adapted from Rynk (1992). Reprinted with permission from Northeast Regional Agricultural Engineering Service (NRAES), Cooperative Extension, Ithaca, New York.

need for frequent turning of the piles makes this a labor-intensive treatment technology. Special equipment may be needed for this task as well. Though this technology is well known as a means to convert waste organic matter (leaves, agricultural wastes, manures) to useful soil amendments, it only recently has been applied to the problem of bioremediation of hazardous compounds. Composting can be carried out at mesophilic (20–30 °C) or thermophilic (50–60 °C) temperatures. Compost piles typically contain aerobic, microaerophilic, and anaerobic microhabitats, promoting simultaneous growth of fungi, actinomycetes, and eubacteria. Thus, biodegradative processes in these systems can be very complex. For example, some xenobiotic compounds are polymerized into the organic compartments of composted soils (Williams, Ziegenfuss & Sisk, 1992). Composting is a technology whose true limits and effectiveness have not yet been established, but the technology shows considerable promise for some specific applications such as treatment of petroleum-contaminated soils.

In *landfarming*, contaminated soils, sludges, or sediments are spread on fields and cultivated in much the same manner as a farmer might plow and fertilize agricultural land. It has been used most commonly as an inexpensive and effective process for the treatment of petroleum-contaminated materials. Though simple in design, landfarming must be performed carefully to avoid creating a second, possibly larger, hazardous waste contamination problem, should the process fail. Clearly, many toxic materials should not be landfarmed. It is a technique to be used only for readily biodegradable chemicals. Even with easily degraded compounds there may be potential for leaching of contaminants to groundwater, so most regulatory agencies require that groundwater be deep below a landfarm site, or that there be some type of confining layer (natural clays) or barrier (reinforced liner) between the cultivated material and the subsurface water. A landfarm site must be managed for its moisture content, and fertilization with nitrogen and/or phosphorus may often be required. Landfarming and its variations, as applied to petroleum contaminants, will be discussed in detail in Chapter 4. A photograph of a typical landfarming operation is shown in Figure 2.

Since so many instances of environmental contamination involve soil, and since soil is a highly complex biological, chemical, and physical matrix, we have provided a full chapter (Chapter 2) on the influences of soil properties on bioremediation processes. These influences must be understood if bioremediation is ever to become a dependable technology.

In situ bioremediation is currently receiving a great deal of attention among bioremediation researchers. This attention is warranted because *in situ* processes, which would not require impossibly expensive excavations of vast amounts of contaminated vadose-zone soils, or unending pump-and-treat schemes for large, deep aquifers, might save vast amounts of money and potentially solve problems that are not approachable by off-the-shelf technologies. Most *in situ* processes involve the stimulation of indigenous microbial populations so that they become metabolically active and degrade the contaminant(s) of concern. The best examples,

Figure 2. Landfarming of petroleum-contaminated soil. A 10-acre site for treatment of about 20 000 cubic yards of excavated soil.

some from as early as the mid-1970s (Raymond, 1974), involve treatment of aquifers contaminated by petroleum hydrocarbons. Free product floating on the aquifer surface was first removed by pumping appropriately located wells. Nutrients (primarily nitrogen and phosphorus) were then injected and electron acceptors (usually oxygen, but sometimes nitrate) were supplied either by sparging wells (air) or through the addition of solutions of hydrogen peroxide or nitrate salts. Problems encountered during *in situ* stimulation of microbial populations include the plugging of wells and subsurface formations by the tremendous amounts of biomass that may be generated through microbial growth on hydrocarbons, difficulties in supplying sufficient oxygen to the subsurface, and the inability to move nutrients and electron acceptors to all regions of heterogeneous subsurface environments. Also, it is rarely possible to remove all free product, so reservoirs of slowly released contamination may be present for many years in some situations.

A process known as 'bioventing' is becoming an attractive option for promoting *in situ* biodegradation of readily biodegradable pollutants like petroleum hydrocarbons. Bioventing involves the forced movement of air (oxygen) through the vadose zone of contaminated sites (Hinchee, 1994). When oxygen is provided as a terminal electron acceptor, indigenous microorganisms multiply at the expense of the carbon present in the contaminating material. The goal is to provide sufficient oxygen to allow degradation of the pollutants to proceed to completion, without vaporizing contaminants to the surface. Air may be forced through the vadose zone

either by injection through sparging wells, or by vacuum extraction through appropriately located infiltration and withdrawal wells (Figure 3). In soils that are impermeable (e.g., clay-rich soils) bioventing may not be possible since air cannot be moved through these soils at sufficient rates to supply microbial populations the electron acceptors required. Sometimes such soils can be 'opened' by fracturing with pressurized air or water, improving their permeability for air transport. In theory, bioventing should work for both volatile and non-volatile contaminants, and petroleum or non-petroleum compounds, as long as the contaminants are inherently biodegradable and an indigenous degrader population of microbes exists in the zone of contamination. If nitrogen is also limiting microbial growth in a contaminated vadose zone, it might be supplied by gaseous ammonia vapors along with the infiltrated air. Expect to see bioventing become a major technique in future bioremediation efforts worldwide.

Since petroleum and petroleum-derived products are the single most pervasive environmental contamination problem, we have provided two chapters directly related to hydrocarbon treatment. Chapter 4 specifically addresses petroleum, while Chapter 5 discusses polycyclic aromatic hydrocarbons, a particularly problematic component of petroleum and other fossil residues.

In situ bioremediation has also been proposed for the cleanup of aquifers contaminated by solvents such as trichloroethylene (TCE) and dichloroethylene (DCE). Compounds like TCE are usually not degraded as sole sources of carbon and

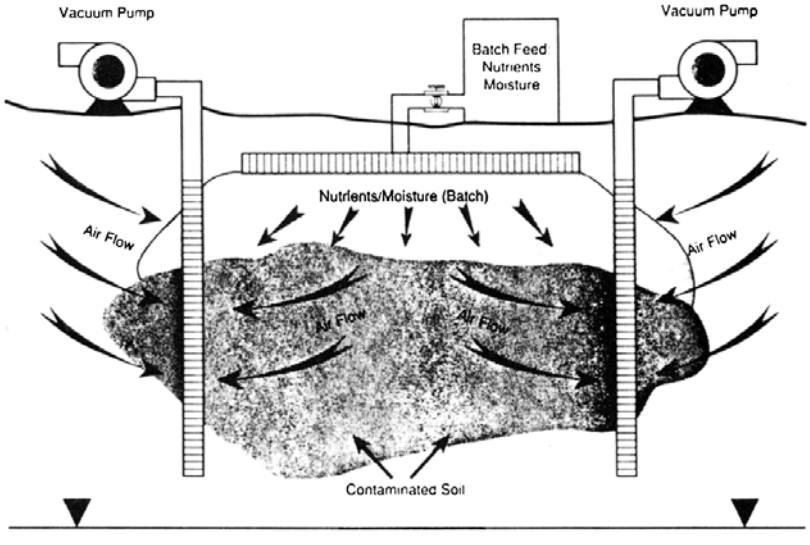

Figure 3. Common bioventing system for treatment of vadose-zone contaminants using oxygen as a terminal electron acceptor. Reprinted from *In Situ Bioremediation: When Does It Work?* Copyright 1993 by the National Academy of Sciences. Courtesy of the National Academy Press, Washington, DC.

energy for microbial growth. They are, however, degraded by processes generally called *cometabolism.* During cometabolic processes contaminants are degraded fortuitously by enzymes that microbes normally employ to degrade substrates that do provide carbon and energy. The primary example studied for its application to *in situ* bioremediation is cometabolism of halogenated solvents during microbial growth on methane. Methane-oxidizing bacteria produce a methane monooxygenase (MMO) that oxidizes methane to methanol using reducing power derived from cellular-reduced pyridine nucleotides (NADH). Certain forms of this enzyme show extraordinarily broad substrate specificities, oxidizing perhaps hundreds of substrates in the place of methane (Henry & Grbić-Galić, 1991), including compounds like TCE and DCE. Once oxidized by MMO, TCE, for example, decomposes to largely innocuous products. Through sparging a TCE-contaminated aquifer with an appropriate mixture of methane and oxygen, the growth of methane-oxidizing bacteria and the concomitant cometabolism to TCE can be stimulated. This practice has worked well in some model systems and in small, well-controlled aquifers (Semprini *et al.*, 1990) but has not yet shown great success in real-world situations. The outlook for this technology, however, is improving as more work is done to perfect the *in situ* techniques. Biodegradation of chlorinated aliphatic compounds will be discussed in depth in Chapter 9.

The phenomenon of cometabolic degradation of pollutants will undoubtedly be harnessed in the future for many compounds, including classes of chemicals beyond the chlorinated solvents. Chapter 7 will specifically discuss the polychlorinated biphenyls (PCBs), where cometabolic processes play a very important part in the biodegradation of multiple pollutant isomers. Work at the University of Idaho has shown that munitions compounds such as 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and herbicides such as 2-*sec*-butyl-4,6-dinitrophenol (dinoseb) are degraded to innocuous products, i.e., volatile organic acids, by consortia of microorganisms fermenting carbohydrates (Kaake *et al.*, 1992; Funk *et al.*, 1993; U.S. EPA Fact Sheet, March 1994). *Clostridium* spp. appear to be prime players in these processes (Regan & Crawford, 1994), in which the anaerobic bacteria get most of their energy from fermentable carbohydrates, simultaneously reducing and degrading the nitrated contaminants. It should be possible to reproduce this process *in situ* by injecting the appropriate nutrients (soluble starch or molasses) into nitro-compound-contaminated aquifers to stimulate growth and reductive activities of endogenous clostridia. If such clostridia are not present in sufficient numbers, they could be introduced in the form of spores. Because nitroaromatic compounds are one of the world's major environmental problems, Chapter 6 is provided as an entire chapter on their bioremediation.

Anaerobic processes are now known to be much more diverse in biodegradation of pollutants than was thought even a few years ago. Anaerobic bioremediation of nitro-substituted compounds, halogenated molecules, and even hydrocarbons now appears possible, employing electron acceptors such as nitrate, halogenated

compounds themselves, carbon dioxide, sulfate, and oxidized metals such as iron. This rapidly moving field is summarized in Chapter 3. An important subclass of pollutants treatable both anaerobically and aerobically, the chlorinated phenols, is covered in Chapter 8 to illustrate one of the better model systems for bioremediation.

In another approach to *in situ* bioremediation, investigators have developed methods to encapsulate pure microbial cultures in small beads (5–10 μm diameter) that might be used as transport and survival vehicles for introducing unique microorganisms into aquifers (Stormo & Crawford, 1992, 1993). Preliminary work at the University of Idaho in near-surface, heterogeneous aquifers has shown that bacteria-loaded microspheres can be introduced into and transported within the subsurface. Problems still to be overcome include the cost of preparing large quantities of beads in the smallest size ranges, and developing dependable ways to assure good distribution of the beads throughout the aquifer or in the path of a contaminant plume. The U.S. EPA has developed a method to use hydrofracturing to introduce lenses of porous sands into subsurface soils (Vesper *et al.*, 1994). The addition of encapsulated bacteria and nutrients to such sand lenses to intercept pollutant plumes is a possible variation of the encapsulated microorganism theme. A photograph of a microencapsulated *Flavobacterium* that degrades pentachlorophenol (PCP) is shown in Figure 4.

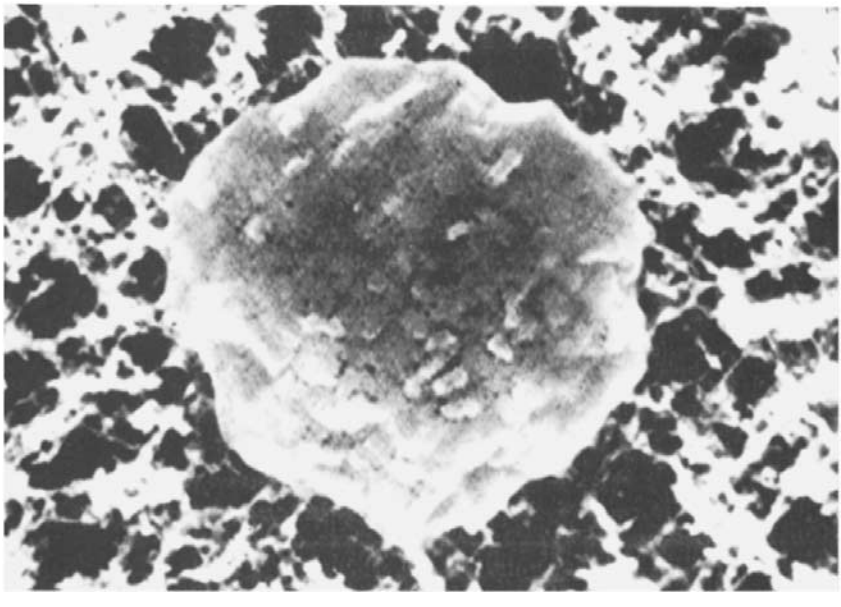

Figure 4. Microencapsulated *Flavobacterium* cells. The bead (10 μm diameter) is composed of alginate. Similar beads can be prepared for agar, polyurethane, and other polymeric materials. From Stormo & Crawford (1992). Reprinted with permission of the American Society for Microbiology.

Chapter 11 will discuss the rapidly developing potential for use of genetically engineered microorganisms (GEMs) by the bioremediation industry. Some very important environmental pollutants are not readily biodegradable by known biological processes; that is, they are not subject to attack by existing enzymes, and microorganisms apparently have never evolved the capability to degrade these structures. These recalcitrant chemicals may not provide sufficient energy for growth of microorganisms, may be too toxic to allow for growth and mutation over long periods of time, may not be inducers of appropriate enzymes, or may be so new to nature that evolution has not proceeded to the point of modifying existing pathways sufficiently to allow for enzymic attack on the novel structures. The modern tools of molecular biology allow for human intervention in this process of evolution. Genes from different organisms can now be cloned and reassembled under proper regulatory control into new pathways for biodegradation of previously nondegradable or highly recalcitrant compounds. These novel pathways can be placed in new hosts, from *Escherichia coli* and *Pseudomonas* to a variety of other microbial strains, depending on their intended uses. For example, several of the genes encoding enzymes for the complete biodegradation of pentachlorophenol (PCP) have been cloned, sequenced, and moved from a *Flavobacterium* into *Escherichia coli* and placed under the control of the *lac* operon promoter (Xun & Orser, 1991; Orser *et al.*, 1993a,b; Lange, 1994). The recombinant *E. coli* was shown to detoxify PCP faster than the original host of the pathway. In the original *Flavobacterium*, PCP is first oxidatively dechlorinated by a PCP-4-monooxygenase, encoded by the *pcpB* gene. Two subsequent dechlorinations are catalyzed by a glutathione-dependent reductive dehalogenase, encoded by the *pcpC* gene. The product of the *pcpA* gene may be a ring-fission oxygenase or an oxygenase component, but this remains to be established; the gene has been cloned and sequenced. The pathway also contains a *lysR* type regulatory gene and a gene encoding a reductase that probably functions with the hydroxylase. The recombinant *E. coli* strain that converts PCP to dichlorohydroquinone has been constructed, as shown in Figure 5 (Lange, 1994) by cloning *pcpB* and *pcpC* into the recombinant host. Efforts are underway to move the remaining PCP pathway genes into the recombinant. Such recombinant microorganisms will probably first see use in bioreactors, where it should be easier to contain their novel genotypes than it would be after direct release to soil or water. However, we should expect to see releases of GEMs directly to the environment for bioremediation of specific pollutants in the near future, as regulatory questions and concerns about environmental risks are addressed. This approach to bioremediation is one of the most exciting areas of the new discipline known as environmental biotechnology.

Toxic metals are a special class of environmental pollutants. Metals cannot be degraded, but only changed from one form (oxidation state) to another. Thus, bioremediation processes for metal-contaminated environments aim at sequestering the metals to make them unavailable to biological components of the ecosystem, or mobilizing them in a manner that allows their 'flushing' from the system for

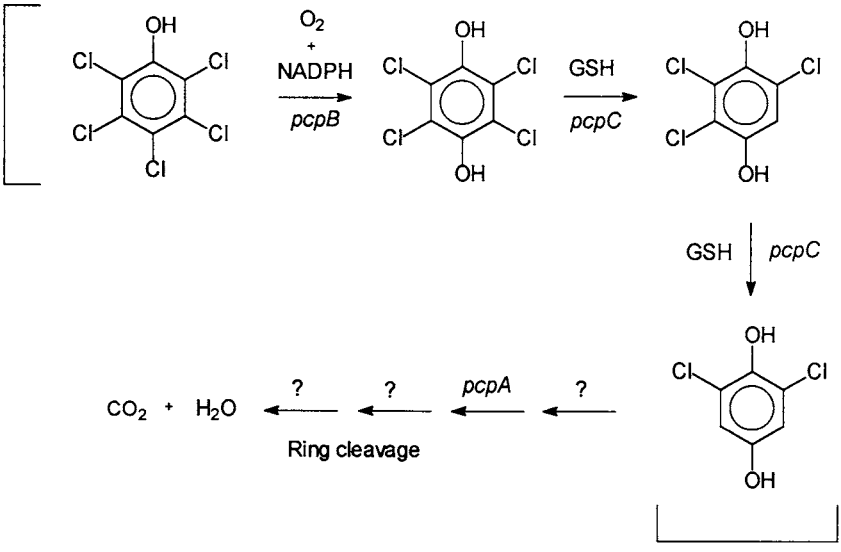

Figure 5. *Flavobacterium* ATCC 39723 pentachlorophenol pathway genes expressed in *Escherichia coli*. Brackets indicate genes cloned into and functional in a recombinant *E. coli*.

collection and disposal. Chapter 10 covers this uniquely difficult class of toxins.

Throughout the world, the problems of environmental contamination by toxic chemicals are enormous. The projected costs of cleaning up just the worst instances by means of available technologies run into the hundreds of billions of dollars. In some cases, no appropriate technologies are available at any cost. Bioremediation offers a partial solution to this dilemma. As compared with incineration or landfilling, biodegradation of pollutants can be inexpensive. It can be a permanent solution when pollutants are mineralized, and it can be combined with other procedures in treatment trains to deal with the complex problems associated with many sites. Bioremediation may be the only possible approach for cleaning some environments, such as deep aquifers. Yet, bioremediation is still an unpredictable technology that may be simple in concept, but sometimes hard to apply in practice. The bioremediation business has suffered from some overselling of the technology, which has not always worked as advertised. In some cases bioremediation simply is not the technology of choice. Research, however, is changing the status quo. Some of the world's best scientists are using their skills to design experiments that lead to a better understanding and tighter control of biodegradative processes. As those processes are being applied in well-engineered systems to the treatment of contaminated environments, the role for bioremediation in environmental restoration is steadily increasing. We hope the discussions in this book will convey to our readers our excitement about the progress being made in the field of bioremediation.