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KEY WORDS: *biological treatment, biotransformation, biodegradation, intrinsic, bioremediation, engineered bioremediation, bioremoval, bioimmobilization, biomobilization, biostimulation, bioaugmentation.*

14.1 Introduction and Background

Hazardous waste is one of the most pervasive, difficult, and expensive problems confronting human society in the twentieth century. Our lack of knowledge concerning the hazards of some chemicals, lack of disposal regulations, lack of regulation enforcement, expense of treatment processes, and poor understanding of fate and effect of contaminants in the environment has led to a subsurface legacy of environmental contamination that will haunt us for hundreds of years. About 72 million U.S. citizens live within 4 mi of a USEPA Superfund National Priority List Site and 4.4 million people live within 1 mi (Bakst and Devine, 1994). The National Priority List has 1192 of the worst toxic waste sites listed and new sites are being added several times faster than sites are being removed from the list. As of December 1993, 10,624 sites were awaiting review and only 55 sites have been cleaned up and removed from the list since 1980 (Bakst and Devine, 1994). The USEPA estimated in one survey that the U.S. had more than 5 million underground storage tanks. An inspection of just 12,000 tanks revealed that 30% currently were leaking, thus as many as 300,000 to 420,000 tanks may be leaking now or will be leaking in the near future, and will require mandatory action (USEPA, 1988).

The Superfund has identified more than 36,000 toxic waste sites that will require action (Glass et al., 1995). This toxic milieu is dominated by solvents (trichloroethylene, tetrachloroethylene, vinyl chloride, carbon tetrachloride, chloroform, and chlorobenzene), petroleum products (benzene, toluene, xylene, ethyl-benzene, and total petroleum hydrocarbons), polynuclear aromatic hydrocarbons (creosote, polychlorinated biphenyls, anthracene, and benzopyrene), and metals (mercury, selenium, chromium, cadmium, lead, and radionuclides). Groundwater and soil at many waste sites, particularly landfills, contain all of these compounds above the recommended maximum contaminant levels (MCL). The health problems that these toxic waste sites represent are compounded by the increasing lack of clean surface water, thus increasing our reliance on groundwater as source water at the same time that toxic contaminant release into the terrestrial subsurface is increasing (Craun, 1986). Surveys of groundwater have revealed that 36% of more than 5000 community water sources in the U.S. had organic contaminant concentrations above the recommended MCLs for drinking water (Craun, 1986). The cost of remediating just the known contaminated sites in the U.S. is now estimated to exceed \$1.7 trillion and this number is constantly being revised upwards. Bioremediation, both above and below ground, promises to be one the premier technologies for restoring the terrestrial subsurface. Indeed, bioremediation may be the only solution to clean up many of our deepest toxic plumes.

Bioremediation is the use of biological processes to return the environment to its original state. More realistically, however, the goal of bioremediation is to make the environment less toxic. In the broadest application sense, bioremediation includes use of enzymes, growth stimulants, bacteria, fungi, or plants to degrade, transform, sequester, mobilize, or contain contaminant organics, inorganics, or metals in soil, water, or air. If we accept the "Doctrine of Infallibility", i.e., there is no compound known to humans that microorganisms cannot degrade (Alexander, 1965), then bioremediation becomes one of the great solutions for our environmental problems. Unfortunately, while the Doctrine of Infallibility may be absolutely true, the rate of biodegradation or transformation of some compounds is so slow as to be negligible for bioremediation purposes. In addition, the conditions (environmental or biological) that allow certain biological reactions to take place may not be obtainable in many environments (Fewson, 1988).

The theoretical possibilities of bioremediation (at least according to the Doctrine of Infallibility), the explosive developments of biotechnology, and our great need for better, faster, cheaper, and safer new methods of remediation have resulted in an exponential proliferation of companies offering bioremediation services. At the April 1995 3rd International Symposium of *In Situ* and On Site Bioremediation in San Diego, CA there were more than 107 companies reporting results of bioremediation field studies. Indeed, bioremediation has already become standard in the tool kits of all full-service remediation companies. Unfortunately, the hope has been largely hype by many companies with "magic bugs" and "magic potions" that will "completely" biodegrade almost anything, anywhere. Because bioremediation is "biotechnology" and has received some dramatic media attention, e.g., the Exxon Valdez spill, it is generally perceived by the public to be new technology that uses special critters that can devour any toxicant that we throw at them. Indeed, the first patent for a genetically engineered organism was granted in the U.S. in 1981 for a bacterium that degrades petroleum (Atlas, 1995).

In actuality, bioremediation as a process is not particularly new or novel. The word bioremediation, however, is fairly new. A search of paper titles, abstracts, and keywords of the major abstracting services (SciSearch™, Biosis™) indicates that the word

was not used in the peer-reviewed scientific literature before 1987. Composting, sewage treatment, and certain types of fermentation have been practiced by humans since recorded history began, and all of these are biological treatment methods that could be defined as bioremediation. We find evidence of kitchen middens and compost piles dating from 6000 B.C. (Senior, 1990). The Greeks used circular walled refuse bioreactors as early as 1900 B.C. The first public biological sewage treatment plant began operation more than 100 years ago (1891) in Sussex, U.K. (Senior, 1990). ZoBell (1946) showed that biotreatment of petroleum was possible more than 50 years ago. Since the early 1950s, petroleum land farming (bioremediation of petroleum sludge by tilling soil with fertilizer) has been used as a standard sludge disposal method. The first successful bioremediation using bioaugmentation (addition of biodegrading bacteria) was documented in 1968 for remediation of the oily bilge water in the Queen Mary when it was first brought to Long Beach Harbor for 'parking'. A patent for *in situ* bioremediation of groundwater contaminated with gasoline by stimulating indigenous bacteria via nutrient injection into the terrestrial subsurface was issued to Dick Raymond in 1974 (U.S. Patent 3,846,290). He successfully demonstrated this technology and began commercial applications in 1972 (Raymond et al., 1977). Clearly, bioremediation has been used successfully for more than 50 years and much is understood about where it is applicable, especially for petroleum contaminants. The really new bioremediation applications that have been done in the last 10 years are in the area of solvents, PAHs, PCBs, and metals. Bioremediation has been around for a long time; only its breadth of application in terms of types of contaminants and environments has increased in the last 10 years. This explosive proliferation of new applications and new environments in the last 10 years, especially by companies trying to establish themselves with a proprietary edge, has led to a large number of terms, many of which are highly redundant. Also, the bioremediation field applications that have been reported lack comprehensive field data, especially in the terrestrial subsurface. Though bioremediation has been used at a large number of sites, nearly all applications were completed by companies trying to do the study (1) for clients who usually wanted to remain anonymous, (2) at the least possible cost to the client and the vendor, and (3) protecting the vendor's proprietary edge for their product. This has led to a paucity of peer-reviewed data, misapplication of terminology, and confusion as to what some terms mean. The following is a series of terms and definitions important to bioremediation.

14.1.1 Terminology

Biological Treatment — Any treatment process that involves organisms or their products, e.g., bacteria or enzymes.

Biotransformation — A biological treatment process that involves changing the contaminant, e.g., valence states of metals, chemical structure, etc.

Biodegradation — A biological process of reducing a compound to simpler compounds. May be either complete, e.g., reduction of organic compounds to inorganic compounds, or incomplete, e.g., removal of a single atom from a compound.

Intrinsic Bioremediation — Unmanipulated, unstimulated, nonenhanced biological remediation of an environment; i.e., biologically natural attenuation of contaminants in the environment.

Engineered Bioremediation — Any type of manipulated or stimulated or enhanced biological remediation of an environment.

Biostimulation — The addition of organic or inorganic compounds to cause indigenous organisms to effect remediation of the environment, e.g., fertilizer.

Bioaugmentation — The addition of organisms to effect remediation of the environment, e.g., contaminant-degrading bacteria injected into an aquifer.

Bioventing — Originally defined as slow vapor extraction of unsaturated soils to increase flow of air into the subsurface via vents or directly from the surface, to increase aerobic biodegradation rates. Now defined more broadly to include the slow injection of air into unsaturated soils.

Biosparging — Injection of air or specific gases below ground, usually into saturated sediments (aquifer material) to increase biological rates of remediation.

Bioslurping — This treatment combines soil vapor extraction with removal of light nonaqueous-phase liquid contaminants from the surface of the groundwater table, thereby enhancing biological treatment of the unsaturated zone and the ground water, especially the capillary fringe zone.

Bioreactor — A contained vessel in which biological treatment takes place, e.g., fermentor.

Bioslurry Reactor — Biological treatment of soil by making a thin mixture with water and treating in a contained vessel.

Land Farming — A process of biologically treating uncontained surface soil, usually by aeration of the soil (tilling) and addition of fertilizer or organisms, hence farming.

Prepared Beds — A contained area (lined) above ground where soil can be tilled or variously manipulated to increase biological remediation, i.e., contained land farming.

Biopiles — Above-ground mounds of excavated soils that are biologically treated by addition of moisture, nutrients, air, and/or organisms.

Biofilters — Normally used to refer to treatment of gases by passing through a support material containing organisms, e.g., soil, compost, trickle filter. Sometimes used to refer to treatment of groundwater via passage through a biologically active area in the subsurface.

Composting — Treatment of waste material or contaminated soil by aerobic biodegradation of contaminants in an above-ground, contained, or uncontained environment.

Biocurtain — The process of creating a subsurface area of high biological activity to contain or remediate.

Bioremoval — A biological treatment involving uptake of the contaminant from the environment by an organism or its agent.

Bioimmobilization — A biological treatment process that involves sequestering the contaminant in the environment. There may not be biodegradation of the contaminant.

Biomobilization — A biological treatment process that involves making the contaminant more mobile in the environment. No biodegradation of the contaminant.

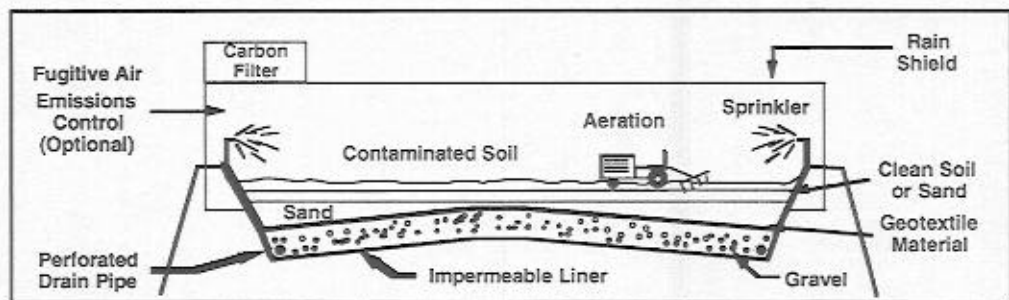


FIGURE 14.1
Prepared bed biotreatment.

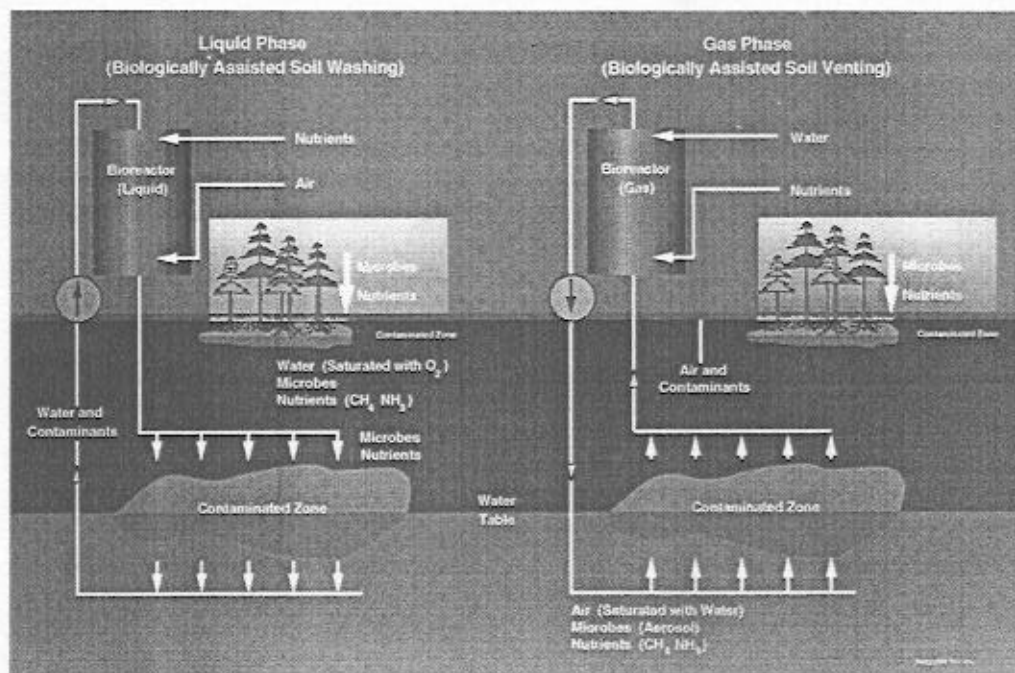


FIGURE 14.2
Bioremediation technologies.

14.1.2 Basic Schematics of Bioremediation Methods

The two schematics (Figures 14.1 and 14.2) present many of the strategies for engineered bioremediation. Figure 14.1 shows a prepared bed facility for bioremediating excavated soil. These systems consist of impermeable liner and leachate collection systems and layers of clean soil or gravel to improve drainage. A sprinkler system is often used to control moisture. Aeration, if necessary, can be as simple as rototilling or as complex as a series of vacuum blowers or compressors. Rain shields and fugitive air emissions control systems may also be necessary depending on the type of contaminants in the soil.

Figure 14.2 shows both *ex situ* and *in situ* technologies that use either liquid or gas, and treat vadose or saturated zone environments. Horizontal wells are shown, but

infiltration galleries have been widely used in the past. This figure shows strategies for biofilters, bioremediation, bioventing, biosparging, bioimmobilization, bioreactors, phytoremediation, biomobilization, biocurtain, bioaugmentation, and biostimulation.

14.2 Biostimulation and Bioaugmentation

All engineered bioremediation can be characterized as either biostimulation (i.e., the addition of nutrients), bioaugmentation (i.e., the addition of organisms), or processes that use both. The problems with adding chemical nutrients to sediment and groundwater are fundamentally different from those of adding organisms. Simple infiltration of soil, and subsequently groundwater, is physically quite different in the two processes (Alfoldi, 1988; also see Chapters 13 and 16). Even the smallest bacterium has different adsorption properties than chemicals. For example, clayey soils have very low porosity and may not physically allow bacteria to penetrate. These clays may also bind the microbes that are added by, e.g., cationic bridges involving divalent metals and the net negative charge on the surface of the bacteria and the surface of the clay. In some soils, inorganic chemicals that are injected may precipitate metals, swell clays, change redox potentials and conductivity, thereby having a profound effect on groundwater flow and biogeochemistry of the environment.

Biostimulation is dependent on the indigenous organisms and thus requires that they be present and that the environment be capable of being altered in a way that will have the desired bioremediation effect. In most terrestrial subsurface environments, the indigenous organisms have been exposed to the contaminant for extended periods of time and have adapted, or degradative organisms have even become enriched in relative community abundance through selection. This is not surprising, as many contaminants, especially organic compounds, are naturally occurring or have natural analogs that occur in the environment. Rarely can a terrestrial subsurface environment be found that does not have a number of organisms already present that can degrade or transform most contaminants that might be present. Indeed, even pristine environments have bacteria with an increasing number of plasmids with sediment depth in response to increasing recalcitrance of the organics present (Fredrickson et al., 1988).

Our ability to enhance bioremediation of any environment is directly proportional to knowledge of the biogeochemistry of the site. Finding the limiting conditions for the indigenous organisms to carry out the desired remediation is the most critical step. As with surface environments, the parameters that are usually limiting organisms are required nutrients, inorganic and organic. Of these, the most common are phosphorus, oxygen, nitrogen, and water. In the terrestrial subsurface, water can be limiting but usually is not, even in vadose zone environments. Oxygen is quite often limiting since the contaminant can be used as a carbon and energy source by the organisms and the contaminant concentration greatly exceeds the oxygen input needed by the organisms. Introduction of air, oxygen, or hydrogen peroxide via infiltration galleries, tilling, sparging, or venting have proven to be extremely effective in bioremediating petroleum contaminants and a variety of other organic compounds that are not particularly recalcitrant (Thomas and Ward, 1992). However, if the environment has been anaerobic for extended periods of time, and the contaminant has a high carbon content, it is likely that denitrification has reduced the overall nitrogen content of the environment, making this nutrient limiting. Nitrogen has been

successfully introduced into the terrestrial subsurface for biostimulation using ammonia, nitrate, urea, and nitrous oxide (USEPA, 1989). Phosphorus is naturally quite low in concentration in most environments, and in terrestrial subsurface environments even if phosphorus concentrations are high it may be in a mineral form that is biologically unavailable, e.g., apatite. Several inorganic and organic forms of phosphate have been successfully used to biostimulate contaminated environments (USEPA, 1989). In environments where the contaminant is not a good carbon or energy source and other sources of carbon or energy are absent or unavailable, it may be necessary to add an additional source of carbon (Horvath, 1972). An additional source of organic carbon will also be required if the total organic carbon concentration in the environment falls below 1 ppm and the contaminant cleanup levels have still not been met. Methane, methanol, acetate, molasses, sugars, agricultural compost, phenol, and toluene have all been added as secondary carbon supplements to the terrestrial subsurface to stimulate bioremediation (National Research Council, 1993). Even plants, e.g., poplar trees, have been used to biostimulate remediation of subsurface environments (Schnoor et al., 1995). In this latter case the plants act as solar-powered nutrient pumps stimulating rhizosphere microbes to degrade contaminants (Anderson et al., 1993).

Biostimulation strategies will be limited most by our ability to deliver the stimulus to the environment. The permeability of the formation must be sufficient to allow perfusion of the nutrients and oxygen through the formation. The minimum average hydraulic conductivity for a formation is generally considered to be 10^{-4} cm/s (Thomas and Ward, 1989). The stimulants required must be compatible with the environment. For example, hydrogen peroxide is an excellent source of oxygen, but it can cause precipitation of metals in soils and such dense microbial growth around the injection site that all soil pores are plugged. It is also toxic to bacteria at high concentrations >100 ppm (Thomas and Ward, 1989). Ammonia can also be problematic in that it adsorbs rapidly to clays, causes pH changes in poorly buffered environments, and can cause clays to swell, decreasing permeability around the injection point. Many of these problems can be handled at some sites by excavating the soil or pumping the groundwater to the surface and treating it in a bioreactor, prepared bed, land farming, bioslurry reactor, biopile, or composting. In these cases, the permeability can be controlled or manipulated to allow better stimulation of the biotreatment process. It is generally accepted that soil bacteria need a C:N:P ratio or 30:5:1 for unrestricted growth (Paul and Clark, 1989). Stimulation of soil bacteria can generally be achieved when this nutrient ratio is achieved following amendment addition. The actual injection ratio used is usually slightly higher, 100:10:2 (Litchfield, 1993), because these nutrients must be bioavailable, a condition that is much more difficult to measure and control in the terrestrial subsurface. It may also be necessary to remove light nonaqueous-phase liquid (LNAPL) contaminants that are floating on the water table or smearing the capillary fringe zone. This process has been named bioslurping (Keet, 1995). This strategy greatly increases the biostimulation response time by lowering the highest concentration of contaminant the organisms are forced to transform.

Bioaugmentation may provide significant advantages over biostimulation for (1) environments where the indigenous bacteria have not had time to adapt to the contaminant; (2) particularly recalcitrant contaminants that only a very limited number of organisms are capable of transforming or degrading; (3) environments that do not allow a critical biomass to establish and maintain itself; (4) applications where the desired goal is to plug the formation for contaminant containment, e.g., biocurtain; and (5) controlled environments where specific inocula of bacteria with high rates of degradation will greatly enhance the process, e.g., bioreactors, prepared beds,

composting, bioslurry reactors, and land farming. Like biostimulation, a major factor affecting the use of bioaugmentation in the terrestrial subsurface is hydraulic conductivity, the 10^{-4} cm/s limit for biostimulation will need to be an order of magnitude higher for bioaugmentation and may need to be higher yet, depending on the size and adherence properties of the organism being applied (Baker and Herson, 1990; also see Chapter 13). Recent studies have shown that less adherent strains of some contaminant-degraders can be produced, allowing better formation penetration (DeFlaun et al., 1994). However, the ability to rapidly clog a formation is a significant advantage of bioaugmentation in applications where containment is a primary goal. The oil industry has been using this strategy to plug fluid loss zones and enhance oil recovery for a number of years (Cusack et al., 1992). Above-ground applications allow manipulation of the permeability in order to overcome most of these problems.

A number of novel organisms have been successfully injected into the subsurface for *in situ* bioremediation of PCBs, chlorinated solvents, PAHs, and creosote (National Research Council, 1993). Surface applications of bioaugmentation for petroleum contaminants in prepared beds and land farming are routine since they help jump-start the bioremediation process.

For controlled and carefully optimized environments, e.g., bioreactors, biofilters, biopiles, and bioslurry reactors, bioaugmentation is preferred since it is easier to control and achieves higher rates of transformation or degradation. Bioaugmentation suffers the dilemma of being indistinguishable from biostimulation in many environments, since nutrients are often injected with the organisms and since dead cells are an excellent source of nutrients for most indigenous organisms. For many applications it is difficult, if not impossible, to determine if the added organisms provided a significant advantage over nutrient stimulation alone. Even some of the best-controlled bioaugmentation field studies, e.g., caisson studies of PCB biodegradation in Hudson River sediment, could not show a significant advantage for bioaugmentation over biostimulation alone (Harkness et al., 1993). Given the high cost of producing the organisms for inoculation, and the delivery problems, bioaugmentation applications will probably remain limited. However, bioaugmentation may have a very significant advantage when genetically engineered microorganisms (GEMs) are used. It is possible that a GEM could be constructed with unique combinations of enzymes to facilitate a sequential biotransformation or biodegradation of a contaminant. This would be particularly helpful for contaminants that are extremely recalcitrant (e.g., PCBs), or under limited conditions (where the contaminant can only be degraded anaerobically, e.g., tetrachloroethylene and carbon tetrachloride). In addition, GEMs could be modified with unique survival or adherence properties that would make them better suited to the environment where they are to be applied.

14.3 Treatability and Modeling

Determining and demonstrating the ability of an environment to be biostimulated or bioaugmented to effect the remediation of a contaminant is critical to the successful application of a bioremediation technique. Treatability studies need to be done to determine the (1) biodegradability or biotransformability of the contaminants, both anaerobically and aerobically; (2) effectiveness of the proposed amendments; (3) compatibility of the proposed amendment additions with the soil and groundwater matrix

at the site; (4) abiotic losses, e.g., volatilization, sorption, leaching; and (5) final toxicity of the environmental material (USEPA, 1989). All of these determinations will require collection of a large number of representative field samples (see Chapter 6). Field sample collection is critical to validate the process chosen and may require aseptic and anoxic sampling techniques for terrestrial subsurface sediments (see Chapter 3) and immediate access to laboratory facilities (see Chapter 5). A number of treatability study protocols have been published by USEPA for different types of contaminants and environmental conditions (USEPA, 1989). The reactor, flask, pan, or soil column chosen for the treatability study must mimic, in so far as possible, the environmental conditions of the site. They must also be of sufficient size to minimize "bottle effect" and subsampling errors. The systems used must also provide simultaneous testing of positive (inoculated), negative (sterile), and no-treatment (native microbiota) controls of the treatability protocol (Nelson et al., 1994). The types of systems chosen will be largely dependent on the historical data and process knowledge from the site, as well as the initial characterization. For example, when particularly volatile contaminants (e.g., VC, TCE, PCE) are considered, it may be necessary to spike samples with concentrations high enough to measure during the treatability study. This leads to inherent problems since the original contaminant source may no longer be available, forcing the use of reagent-grade chemicals which may have quite different compositions. In addition, the contaminants at the site may have been "weathered", i.e., exposed to leaching and low-level biodegradation or biotransformation and soil chemical reactions for extended periods of time, resulting in a contaminant chemical composition that is quite unique. These problems have contributed greatly to the unreliability of treatability studies to predict bioremediation in the field (Nelson et al., 1994).

Mass balance is one of the more challenging measurements that needs to be made in a treatability study. The total mass of the contaminant in the soil/ground water must first be determined. For each lab experiment, controls and amendments, one must know where all the contaminant went. This means all daughter products must be measured, as well as adsorption, volatilization, leaching, etc. Measuring only the contaminant of concern can give erroneous results. For example, tetrachloroethylene (PCE) can be reductively dechlorinated to TCE, then DCE, then vinyl chloride (VC); however, there are few situations where VC can be further reduced. If we measured only for PCE in our anaerobic treatability study, we might see its complete disappearance suggesting complete bioremediation of the sample, yet an equal quantity of VC was produced. Since VC is more toxic than PCE this would clearly be an undesirable outcome. This also illustrates the need for toxicity tests at the end of the experiment to determine if the amendments (biological or chemical) produced changes in the environment that really decreased its toxicity.

Measurements of degradation kinetics are also critical to accurately predict the amendments needed, their concentrations, rate of application, and time necessary to reach the cleanup goals for the site. These measurements can be made indirectly by modeling the mass balance through time, by direct measurement of enzyme activities or organism density changes in the reaction vessel, or by real-time measurements of terminal electron acceptor concentrations. This last technique has been increasing in popularity in recent years, especially when less volatile contaminants are considered and the desired effect is complete mineralization. These measurements are made with microrespirometers which can measure very small real-time changes in carbon dioxide, oxygen, and methane in reaction vessels (Pietro et al., 1992). This allows calculations of respiration rates for controls and amendments and, if appropriate controls can be done, a calculation of the rate of carbon dioxide or methane production from

the contaminant. Two other techniques can provide excellent kinetics and mass balance information in treatability studies. Unfortunately they are so expensive as to be prohibitive by normal bioremediation practitioners. These are radiolabeled contaminants (pulse and chase) and stable isotopic ratios of carbon. The later measurement has proven useful in both treatability studies and in field monitoring to determine mineralization rates of petroleum contaminants (Hinchee et al., 1991). Radiolabeled contaminants allow measurement of all daughter products, end products, adsorption, and even incorporation into cell components; unfortunately, these measurements are quite expensive and introduce some error from spiking of the sample with reagent-grade, unweathered contaminants. Caution must also be exercised in choosing the isotope and how the contaminant is labeled. For example, a dodecane (10 carbons) that has only 1 carbon labeled (^{14}C) will only produce 10% radiolabeled carbon dioxide molecules. Radiolabeled mineralization measurements have been used successfully both in treatability studies and in field monitoring for petroleum contaminants and chlorinated solvents (Palumbo et al., 1995).

Modeling of the bioremediation process has become increasingly important in determining the fate and effect of contaminants and predicting the outcome of different amendment scenarios. The models will only be as good as the data they receive from the characterization studies and the treatability studies. However, models can also be used to suggest treatability studies that should be performed from a minimum of characterization data. The simple kinetic models using Monod or Michaelis-Menten functions of 15 years ago are completely inadequate for current bioremediation applications in the terrestrial subsurface. One- and two-dimensional models of aerobic biodegradation of organic contaminants in ground water did not appear until quite recently (Molz et al., 1986; Widdowson et al., 1987). These models incorporated advective and dispersive transport factors coupled with an assumption of microcolonies. Widdowson et al. (1988) later added nitrate respiration as an option to their model. Perhaps the best documented and most widely used model for bioremediation has been the BIOPLUME model (Borden and Bedient, 1986). This model, now in its third version, uses a series of simultaneous equations to simulate growth, decay, and transport of microorganisms, oxygen, and hydrocarbons. The original model was used to simulate PAH biodegradation at a Texas Superfund site (Borden and Bedient, 1986). Rifai et al. (1987) later modified this model (BIOPLUME II) to incorporate the USGS two-dimensional model (Konikow and Bredehoeft, 1978). BIOPLUME II has been used to model biodegradation of aviation fuel at the U.S. Coast Guard Station at Traverse City, Michigan (Rifai et al., 1988) and to characterize benzene biodegradation over three years in another shallow aquifer (Chiang et al., 1989). Most recently, Travis and Rosenberg (1994) used a numerical simulation model to successfully predict aerobic bioremediation of chlorinated solvents in the ground water and vadose zone using methane biostimulation at the U.S. Department of Energy Savannah River Site near Aiken, South Carolina. Their model also used a series of simultaneous equations for microbial growth, nutrient limitations, and contaminant, microbe, and nutrient transport. The model predicted the amount of TCE that was biodegraded during a 14-month, full-scale demonstration, and was validated by 5 other methods (Hazen et al., 1994). As there is an increased emphasis on intrinsic bioremediation as a solution, models like these are becoming increasingly important to understand the terrestrial subsurface "black box" of bioremediation. These types of models, along with rigorous treatability studies, are required for intrinsic bioremediation to be acceptable, particularly as a solution for bioremediation of terrestrial subsurface environments.

TABLE 14.1

Bioremediation Characterization and Monitoring Methods

Measurements	Methods
Biomass	
Viable counts	Plate counts, MPN, enrichments, BIOLOG™
Direct counts	AODC, FITC, DFA
Signature compounds	PI.FA, DNA, RNA
Bioactivity and bioremediation	
Daughter products	Cl, CO ₂ , CH ₄ , stable isotopic C, reduced contaminants
Intermediary metabolites	Epoxides, reduced contaminants
Signature compounds	PI.FA, ribosome probes, BIOLOG™, phosphatase, dehydrogenase, INT, acetylene reductions, recalcitrant contaminants
Electron acceptors	O ₂ , NO ₃ , SO ₄ (microrespirometer)
Conservative tracers	He, CH ₄ , Cl, Br
Radiolabeled mineralization	¹⁴ C, ³ H-labeled contaminants, acetate, thymidine
Sediment	
Nutrients	PO ₄ , NO ₃ , NH ₄ , O ₂ , total organics, SO ₄
Physical/chemical	Porosity, lithology, cationic exchange, redox potential, pH, temperature, moisture, heavy metals
Groundwater	
Nutrients	PO ₄ , NO ₃ , NH ₄ , O ₂ , SO ₄
Physical/chemical	pH, redox potential, conductivity, temperature, heavy metals, flow (vertical and horizontal vectors)
Toxicity	Microtox™, Mutatox™

14.4 Characterization and Monitoring

Characterization and monitoring of bioremediation can be as simple as maintaining a fermentor for above-ground processes like prepared beds, land farming, bioslurry reactors, composting, and bioreactors. Monitoring the terrestrial subsurface is much more difficult, however, due to its sampling problems, poorly defined interfaces, and spatial heterogeneity. For any type of bioremediation, careful consideration and planning must be given to the remediation objectives, sampling, the types of samples, frequency, cost, priority, and background literature for method verification. The microbiology and chemistry may be of less overall importance to the remediation of the site than the hydrology, geology, meteorology, toxicology, and engineering requirements. All of these things must be integrated into the plan for characterization and monitoring of any site. Some methods that have been used for the measurement of various parameters are listed in Table 14.1. For examples of bioremediation test plans see Hazen (1991), Lombard and Hazen (1994), and Nelson et al. (1994).

The type of sample used for monitoring and characterization of sediment or groundwater can have a significant impact on a bioremediation project. Hazen et al. (1991) demonstrated that deep oligotrophic aquifers have dense, attached communities of bacteria that are not reflected in the groundwater from that aquifer. This has serious implications for the *in situ* bioremediation of deep contaminated aquifers, since monitoring of groundwater is the principal method used to characterize and control biodegradation by indigenous bacteria stimulated by nutrient infiltration. Groundwater monitoring may not indicate community or population numbers, or physiological activity of the microbes attached to sediment, and these microbiota may

be the most biologically active component of these aquifers. Harvey et al. (1984) and Harvey and George (1987) have shown that shallow, eutrophic, rapidly moving aquifers behave quite differently in that there are no significant differences between groundwater and attached sediment communities. This is reasonable because attachment in such an environment would have no significant advantage, unlike the oligotrophic deep aquifers. Fortunately, most bioremediation applications are shallow and eutrophic due to the nature of the waste mix usually deposited. Enzien et al. (1994) further underscored the need for careful sampling when they showed significant anaerobic reductive dechlorination processes occurring in an aquifer whose bulk groundwater was aerobic ($>2 \text{ mg/l O}_2$).

Determining the rate and amount of contaminant that is bioremediated in any environment is one of the most difficult measurements. Many of the problems and measurements discussed above (for mass balance in treatability studies) also apply here. In recent years, bioremediation studies have focused on measurement of biodegradation products, rather than on the organisms, because of the difficulty in measuring organisms. Soil and ground water measurements of microorganisms often require long incubations or long preparation times and the measurements are usually not specific to contaminant-degrading bacteria. Several methods have been used to determine the rate and amount of biodegradation: monitoring of conservative tracers, measurement of by-products of anaerobic activity, intermediary metabolite formation, electron acceptor concentration, stable isotopic ratios of carbon, and ratio of nondegradable to degradable substances. Helium has been used at a number of sites as a conservative tracer since it is nonreactive, nonbiodegradable, and moves like oxygen (National Research Council, 1993). By simultaneously injecting He with O_2 at known concentrations and comparing the subsurface ratios over time, the rates of respiration can be calculated. This technique has also been used to measure rates of injected methane consumption (Hazen, 1991). Bromide has been successfully used as a conservative tracer for liquid injection comparisons with nitrate, sulfate, and dissolved oxygen (National Research Council, 1993). By-products of anaerobic biotransformation in the environment have been used to estimate the amount of biodegradation that has occurred in anaerobic environments (e.g., PCB-containing sediments). These by-products include methane, sulfides, nitrogen gas, and reduced forms of iron and manganese (Harkness et al., 1993).

Measurements of chloride changes have also proven useful in indicating the amount of chlorinated solvents that have been oxidized or reduced (Hazen et al., 1994). Consumption of electron acceptors (O_2 , NO_3^- , or SO_4^{2-}) has been used for measuring rates of biodegradation and bioactivity at some bioremediation sites (National Research Council, 1993; Smith et al., 1991). Bioventing remediations of petroleum-contaminated sites rely on stable isotopic ratios of carbon, carbon dioxide production, and oxygen consumption to quantify biodegradation rates in the field (Hinchee et al., 1991; Hoeppe et al., 1991). Mixtures of contaminants, e.g., petroleum hydrocarbons, can have their own internal standard for biodegradation. By comparing concentrations of nonbiodegradable components of the contaminant source with concentrations of degradable components from both virgin and weathered sources, the amount of contaminant degraded can be calculated. These measurements have been used on the Exxon Valdez spill cleanup (Glasser, 1994) and at a number of other petroleum-contaminated sites (Breedveld et al., 1995).

Microbial ecologists have continually struggled with methods for measuring the diversity, number, and activity of organisms in the environment. For bioremediation, this information is needed regarding the contaminant-degrading microbiota. It is also critical to know if there are other organisms present that are important in terms of biogeochemistry and what proportion of the total community the degrading bacteria or other important groups represent.

Plate counts can only provide a measurement of the microbiota that will grow on the media used and under the specific conditions of incubation. Given the large number of possible media and the large number of possible incubations, this leads to an infinite number of possible interpretations. Generally, heterotrophic plate counts have been used to show that bacterial densities in the sediment or groundwater increase in response to biostimulation (Litchfield, 1993). Using contaminant enrichment media and either plates or most probable number (MPN) extinction dilution techniques, the number of contaminant-degrading bacteria can be estimated (National Research Council, 1993). However, there are serious fallacies in the underlying assumptions of many of these assays, e.g., diesel-degrading bacteria may be determined using a minimal medium with a diesel-soaked piece of cotton taped to the top of the petri dish. Are the colonies that are observed utilizing the diesel, or are they merely tolerant to the volatile components of the diesel fuel? In contrast, MPN assays have been used to conservatively measure methanotroph densities in soil and groundwater at chlorinated solvent-contaminated sites by sealing each tube under an air/methane headspace and then scoring as positive only those tubes that are turbid and have produced carbon dioxide and used methane (Fogel et al., 1986). The main drawback with these techniques is that the incubation time for plate count and MPN contaminant-degrading assays is 1 to 8 weeks, thus negating their use for real-time monitoring and control.

A number of direct count assays have been tried on contaminant-degrading bacteria, including direct fluorescent antibody staining (DFA), acridine orange direct counts (AODC), and fluorescein isothiocyanate (FITC) direct counts. The fluorochrome stains only indicate the total numbers of organisms present in the sample, they do not indicate the type of organism or its activity. However, these techniques have been used in bioremediation studies to determine changes in the total numbers of organisms (Litchfield, 1993). Increases in total counts have been found when contaminated environments are biostimulated. DFA shows promise but requires an antibody that is specific to the contaminant-degrading bacteria that are in that environment. The environment must be checked for organisms that may cross-react with the antibody and for contaminant-degrading bacteria that do not react with the antibody. DFA will be most useful in monitoring specific organisms added for bioaugmentation, though it has been used in biostimulation applications (Fliermans et al., 1994). Since the assay time is reduced to hours with these direct techniques, they have significant advantages for real-time monitoring and rapid characterization.

Biological activity at bioremediation sites has been determined in a number of ways: INT activity/dehydrogenase, fatty acid analyses, acetate incorporation into lipids, ^3H -thymidine incorporation into DNA, BIOLOGTM, phosphatase, and acetylene reduction. The INT test has been used in combination with direct counts because INT-formazan crystals can be detected in the cells. (Cells with INT-formazan crystals are assumed to be actively respiring; the reaction occurs within the electron transport system and is associated with activity.) The assay requires only a 30-min incubation; however, it can only be used in groundwater samples since particles in sediment samples cause too much interference with interpretation of the intracellular crystals. Barbaro et al. (1994) used this technique to measure microbial biostimulation of the Borden Aquifer in Canada.

Phospholipid fatty acid analyses (PLFA) have been used for characterization and monitoring at a number of bioremediation sites (for more information on this topic see Chapter 8). The PLFAs (signature compounds) that an organism has may be unique to that species or even to that strain, or they may be conserved across physiological groups, families, or even a kingdom. Certain groups of fatty acids (cis and trans isomers) may also change in response to the physiological status of the

organism. PLFAs have been used at bioremediation sites to provide direct assays for physiological status (cis/trans ratio), total biomass estimates, presence and abundance of particular contaminant-degraders and groups of organisms, e.g., methanotrophs, actinomycetes, and anaerobes (Phelps et al., 1989; Heipieper et al., 1995; Ringelberg et al., 1994). PLFAs would seem to be a panacea for characterization and monitoring of bioremediation; unfortunately, the assays require -70°C sample storage, long extraction times, have a fairly high detection limit (10,000 cells), and require expensive instrumentation. This technique merits careful consideration since it is so versatile and is a direct assay technique.

Radiolabeled acetate and thymidine incorporation into lipids and DNA, respectively, have been used at bioremediation sites to provide measurements of total community metabolic and growth responsiveness (Fliermans et al., 1988; Palumbo et al., 1995). These techniques require incubation, extraction, purification, and radiolabeled substrates, making interpretation of results difficult.

The BIOLOGTM assay has also been adapted to determine the activity of bacteria in groundwater and soil samples to contaminants. The assay consists of a 96-well microtiter plate with carbon sources and an electron transport system (ETS) indicator. It can be used to identify isolates and to examine the overall activity of a soil or water sample to a particular substrate. Gorden et al. (1993) adapted the assay to determine activity to different contaminants by using both contaminants and ETS indicator alone and adding contaminants to the plates with substrates to determine co-metabolic activity. The assay provides more rapid screening than other viable count techniques but it suffers from some of the same problems, e.g., incubation conditions and repeatability. It is also difficult to determine if the contaminants are being transformed or tolerated.

Phosphatase and dehydrogenase enzyme assays have also been used to access bioactivity in soil and groundwater during bioremediation of terrestrial subsurface sites. Acid and alkaline phosphatase have been linked to changes in ambient phosphate concentrations and bioactivity at contaminated sites caused by biostimulation (Lanza and Dougherty, 1991). The incubation, extraction, and interference caused by pH differences in samples make results difficult to interpret. Acetylene reduction has been used to indicate nitrogenase activity in a few bioremediation studies; however, the importance of nitrogen fixation at most bioremediations is probably insignificant, unless the site is oligotrophic (Hazen et al., 1994).

Nucleic acid probes provide, at least theoretically, one of the best ways to characterize and monitor organisms in the environment (Amy et al., 1990; Brockman, 1995; Hazen and Jiménez, 1988). Since many contaminants, especially the more recalcitrant ones, are degraded by only a few enzymes, it is possible to produce DNA or even RNA probes that will indicate the amount of that gene in the environment. This tells us if the functional group that can degrade or transform the contaminant is present, and its relative abundance. Since probes have also been developed for species, families, and even kingdoms, this allows soil and groundwater communities to be monitored. Recently, conserved regions in ribosomes have also been found, allowing samples to be probed for the relative abundance of ribosomes and, hence, the bioactivity of the total community (Ruminy et al., 1994). Bowman et al. (1993) demonstrated that probes for methanotrophs indicated their presence in soil at TCE-contaminated sites in South Carolina and Tennessee. Brockman et al. (1995) also showed that methane/air injection at the South Carolina site increased the methanotroph probe signal in sediment near the injection point in the aquifer. The probe signal increase for methanotrophs coincided with increases in the MPN counts for methanotrophs. Thus, sediment can be directly extracted and probed with DNA and RNA for bioremediation characterization and monitoring. As more nucleic acid sequences are described and mapped, it will be possible for us to construct

complementary sequences that can be used as probes. These probes can be used to determine the abundance of organisms in an environment that carry a degradative gene of interest, or the number genes themselves. This clearly will allow bioremediation injection strategies to have better control of the process, in terms of effecting the desired changes in the functional group responsible for the bioremediation process.

Unfortunately, nucleic acid probe technology has some serious obstacles to overcome before it becomes practical: (1) for many applications, the direct detection of nucleic acids in soil and ground water requires lysis, extraction, and purification; (2) these processes may not be equally efficient for all microbial and sediment types; (3) soil humics and groundwater pH may interfere with nucleic acid signals; and (4) the number of cells needed for these techniques may exceed those found in natural environments. The extraction and purification steps also add greatly to the cost and analysis time. These problems are not insurmountable but will impede realistic use of nucleic acid probes for bioremediation. Certainly, research in this area needs to be encouraged, especially given the sound theoretical advantages that these techniques provide for bioremediation.

14.5 Intrinsic Bioremediation

Intrinsic bioremediation is developing rapidly as an important alternative for many contaminated environments. This strategy of natural attenuation by thorough characterization, treatability studies, risk assessment, modeling, and verification monitoring of contaminated environments was first proposed by John Wilson of EPA's Kerr Lab in the early 1990s. Wilson organized the first "Symposium on Intrinsic Bioremediation" in August 1994; development and regulatory acceptance has been exponential ever since. Certainly, much of the rapid deployment of intrinsic bioremediation has been due to the crushing financial burden that environmental cleanup represents and our need to use more risk-based cleanup goals for the thousands of new contaminated sites identified every year. Intrinsic bioremediation as a strategy carries with it a burden of proof of: (1) risk to health and the environment and (2) a model that will accurately predict the unengineered bioremediation of the environment. Thus, applications of intrinsic bioremediation have been confined to environments with few risk receptors and containing contaminants with relatively low toxicity, such as petroleum in fairly homogeneous and confined, i.e., predictable, subsurface environments. The EPA reported for 1995 that intrinsic bioremediation was already in use at 29,038 leaking underground petroleum storage tank (LUST) sites in 33 states (Tremblay et al., 1995). This represents 28% of the 103,479 LUST being remediated in 1995 and an increase of more than 100% since 1993. Intrinsic bioremediation has also been implemented at a creosote-contaminated methanogenic aquifer in Florida (Bekins et al., 1993) and in three TCE-contaminated, reducing aquifers (Martin and Imbrigotta, 1994; Wilson et al., 1994; Major et al., 1994).

The coupling of intrinsic bioremediation to engineered bioremediation has been proposed but not yet tried. Nearly all engineered bioremediation projects could substantially reduce costs by stopping the biostimulation or bioaugmentation process early and allowing intrinsic bioremediation to finish the cleanup process. The only projects that would not benefit from such a strategy would be those where immediate risk to health and the environment demanded an emergency response. Intrinsic bioremediation has the same requirements for treatability, modeling, and characterization as engineered bioremediation discussed above. The only difference is that a

greater emphasis is put on risk assessment, predictive modeling, and verification monitoring. Once an intrinsic bioremediation project has been started, verification monitoring of the predictive model is initially quite rigorous. Afterwards, if the model holds true, monitoring frequency and numbers of parameters gradually decline until the site is cleaned up.

14.6 Conclusions: Bioremediation — The Hope and the Hoax

The public perception that bioremediation uses the natural cleansing capacity of the environment and can clean up any toxicant, anywhere, is such a gross oversimplification that it really represents: a *Hoax*. The popular press (newspapers and magazines) have added to this hoax in recent years by portraying smiling, hungry little blobs happily eating piles of garbage and drinking toxic chemical cocktails (see Snyder, 1993). This trivializes bioremediation in an effort to provide the public with the "cutest" understanding. The "black box" philosophy of some of the first bioremediation demonstrations may also have helped to manifest this perception, since basically all that was known was that if fertilizer or air was injected the contaminants in the aquifer and soil would go away. This has led to a proliferation of companies selling "magic bugs" or "magic potions". These enterprises are usually based on a patent for a single organism they have discovered that will degrade some toxicant, or some nutrient mix that will stimulate indigenous organisms to degrade toxicants in a jar of dirt or water. These companies are doomed to failure because bioremediation is a strategy that involves a thorough understanding of the entire environment and all the synergistic interactions therein, including a thorough study of risk. There have been and will continue to be bioremediation failures; unfortunately, those are almost never documented.

Bioremediation, both engineered and intrinsic, offers reduced cost, reduced time, improved safety, reduced risk, and better, or at least more environmentally friendly, cleanup of many of our contaminated environments, and herein lies the *Hope*. The advances in the field of bioremediation in the last 10 years have been startling. Contaminants, that 10 years ago were considered to be completely recalcitrant, can now be remediated by proven bioremediation techniques. Indeed, new methods of gaseous nutrient injection, fatty acid analyses, nucleic acid probes, 3D subsurface models, and genetically engineered microbes promise even broader and more efficient applications of bioremediation, especially in terrestrial subsurface environments (Miller and Poindexter, 1994). Regulatory acceptance and rapid implementation of intrinsic bioremediation has made it a primary driver for research and study of contaminated and pristine environments. Only through a better understanding of these environments will we be able to build successful predictive models of the environment for both engineered and intrinsic bioremediation.

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