BIOREMEDIATION OF CONTAMINATED SITES*

Karl J. Rockne and Krishna R. Reddy
University of Illinois at Chicago
Department of Civil and Materials Engineering
842 West Taylor Street
Chicago, Illinois 60607, USA

ABSTRACT

Bioremediation is a process in which microorganisms metabolize contaminants either through oxidative or reductive processes. Under favorable conditions, microorganisms can oxidatively degrade organic contaminants completely into non-toxic by-products such as carbon dioxide and water or organic acids and methane. Highly electrophilic compounds such as halogenated aliphatics and explosives typically are bioremediated through reductive processes that remove the electrophilic halogens or nitro groups. Bioremediation processes may be directed towards accomplishing: (1) complete oxidation of organic contaminants (termed mineralization), (2) biotransformation of organic chemicals into smaller less toxic metabolites, or (3) reduction of highly electrophilic halo- and nitro- groups by transferring electrons from an electron donor (typically a sugar or fatty acid) to the contaminant, resulting in a less toxic compound. With increasing numbers of successfully demonstrated cleanups, biological remediation alone or in combination with other methods, has gained an established place as a soil restoration technology.

INTRODUCTION

Pollution of groundwater and soil is a worldwide problem that can result in uptake and accumulation of toxic chemicals in food chains and harm the flora and fauna of affected habitats. The contamination of groundwater resources by organic chemicals is a significant environmental problem, with an estimated 300,000 to 400,000 contaminated sites in the USA alone (Doust and Huang 1992; USEPA 2000). Contaminated sites often contain numerous pollutants, which can constitute a risk to health of humans, animals and or the environment. Although substantial progress has been made in reducing industrial releases over recent years, major releases still occur; a considerable number of known polluted sites exist and new ones are continually being discovered. Many of these sites threaten to become sources of contamination of drinking water supplies and thereby constitute a substantial health hazard for current and future generations. To remedy this situation, numerous remediation techniques have been developed. Primarily due to the cost and time consideration physical and/or chemical treatment processes are currently the most widely used remediation methods. Nevertheless, biological remediation alone or in combination with other methods, has gained an established place as a soil restoration technology. Bioremediation is a process in which microorganisms metabolize contaminants either through oxidative or reductive processes. Under favorable conditions, microorganisms can oxidatively degrade organic contaminants completely into non-toxic by-products such as carbon dioxide and water or organic acids and methane (USEPA 1991). Highly electrophilic compounds such as halogenated aliphatics and explosives typically are bioremediated through reductive processes that remove the electrophilic halogen or nitro groups.

The process of bioremediation refers to the enhancement of this natural process, either by adding microorganisms to the soil, referred to as *bioaugmentation*, or by providing the appropriate conditions and/or amendments (such as supplying oxygen, moisture and nutrients) for growth of the microorganisms to the soil, referred to as *biostimulation*. Bioremediation is also called enhanced bioremediation or

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engineered bioremediation in the published literature (USEPA 1991; USEPA 1992; USEPA 1994a). Bioremediation in the presence of air or oxygen is called *aerobic bioremediation* and typically proceeds through oxidative processes to render the contaminant either partially oxidized to less toxic by-products or fully oxidized to mineral constituents: carbon dioxide and water. Under anaerobic conditions, bioremediation processes are more complex. In anaerobic respiration, organic contaminants can be mineralized provided sufficient nitrate or sulfate is present. In methanogenic bioremediation, the contaminants are converted to methane, carbon dioxide and traces of hydrogen. Another type of anaerobic bioremediation is reductive dehalogenation where the contaminants are rendered less toxic by removal of halogens such as chlorine or nitro groups. Typically, aerobic bioremediation is quicker than anaerobic bioremediation; therefore, it is often preferred. However, many compounds can only be metabolized under reductive conditions and therefore anaerobic treatment is the only option. Bioremediation can be accomplished under in-situ conditions, called *in-situ bioremediation*, or under ex-situ conditions, called *ex-situ bioremediation* (USEPA 1988a; USEPA 1988b; Thomas and Ward 1989; Cauwenberghe and Roote 1998; Cookson 1995).

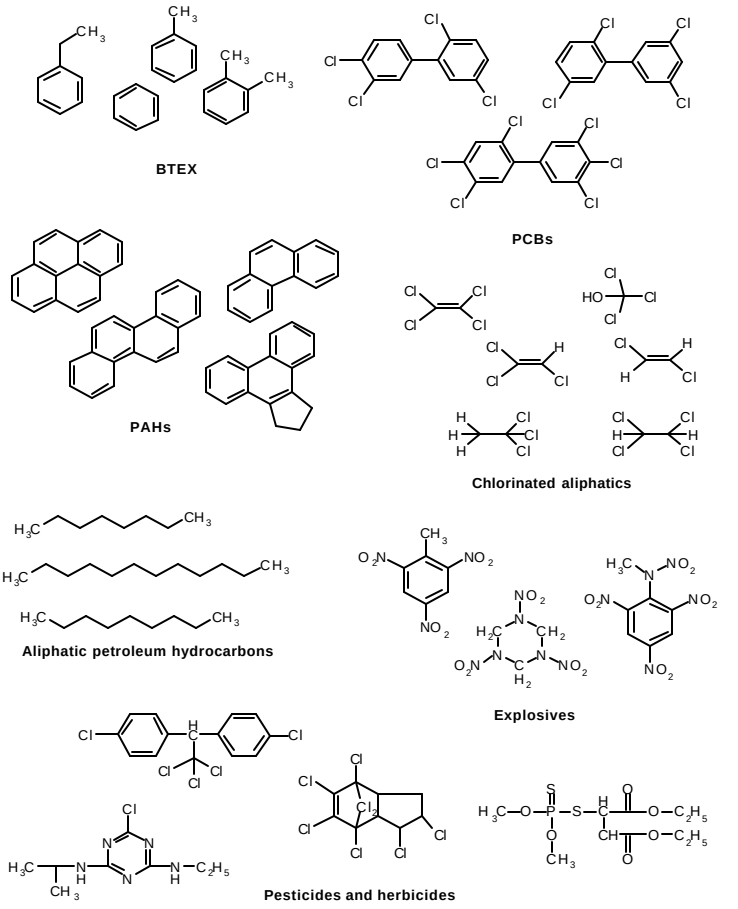

Figure 1. Representative organic pollutants in contaminated soils. Note that many classes of compounds are present as co-contaminants at the same site. For example, PAHs, BTEX, and aliphatic hydrocarbons are commonly found in petroleum-impacted sites.

At many contaminated sites, microorganisms naturally exist that have developed the capability of degrading the contaminants. However, not all sites have competent microbes and typically lack environmental conditions (such as sufficient electron acceptor levels and/or bioavailability restraints) conducive for rapid degradation of the contaminants. Engineered bioremediation, therefore, typically involves supplying oxygen (or other electron acceptor), moisture, and nutrients to the contaminated soil zone so that the naturally existing microorganisms stimulated to degrade the contaminants. For the degradation to occur it has to be ensured that electron acceptor, moisture and nutrient concentrations are maintained in sufficient amounts and at the proper rate. This requires extensive monitoring to assure that the process is proceeding satisfactorily. The monitoring can be done by maintaining monitoring wells and also by measuring the concentrations of carbon dioxide and other metabolites. The increase in biological activity will be marked by the decrease in oxygen concentration (for aerobic processes) or by the buildup of metabolites (e.g. ethene from the reductive dechlorination of tetrachloroethene).

Bioremediation is commonly used for the treatment of soils and groundwaters contaminated with organic contaminants (see Figure 1). Some inorganic pollutants such as ammonia, nitrate, and perchlorate can also be successfully transformed by microbes. Although microbes cannot degrade heavy metals, they can be used to change the valence states of these metals thus converting them into immobile or less toxic forms. For example, microbes can convert mobile hexavalent chromium into immobile and less toxic trivalent chromium.

Bioremediation can be used in any soil type with adequate moisture content, although it is difficult to supply oxygen and nutrients into low permeability soils. It should be noted that very high concentrations of the contaminants may be toxic to microorganisms and thus may not be treated by bioremediation. Therefore, a feasibility investigation is needed to determine if biodegradation is a viable option for the site-specific soil and contaminant conditions (USEPA 1985; Aggarwal et al. 1990).

Bioremediation has the following advantages:

- It may result in complete degradation of organic compounds to nontoxic byproducts.
- There are minimum mechanical equipment requirements
- It can be implemented as in-situ or ex-situ process. In-situ bioremediation is safer since it does not require excavation of contaminated soils. Also, it does not disturb the natural surroundings of the site.
- Low cost compared to other remediation technologies.

Bioremediation has the following disadvantages:

- There is a potential for partial degradation to metabolites that are still toxic and/or potentially more highly mobile in the environment.
- The process is highly sensitive to toxins and environmental conditions.
- Extensive monitoring is required to determine biodegradation rates.
- It may be difficult to control volatile organic compounds during ex-situ bioremediation process
- Generally requires longer treatment time as compared to other remediation technologies.

FUNDAMENTAL PROCESSES

Bioremediation is a common technology for the treatment of organic compounds; however, the use of this technology for the treatment of heavy metals is still new (Means and Hinchee 1994). Therefore, the fundamental processes involved in biodegradation of organic contaminants will be the focus of this section.

Bioremediation processes may be directed towards accomplishing: (1) complete oxidation of organic contaminants (termed mineralization), (2) biotransformation of organic chemicals into smaller (hopefully less toxic) constituents, or (3) reduction of highly electrophilic halo- and nitro- groups by transferring electrons from an electron donor (typically a sugar or fatty acid) to the contaminant, resulting in a less toxic compound (see Figure 2 for overview).

Microbes are known for their metabolic diversity. One consequence of this diversity is the fact that many toxic or persistent anthropogenic organic compounds are degraded by microbial activities. In simple terms, microorganisms must gain energy from the transformation of contaminants in order to survive. In addition, they must also have a source of carbon to build new cell material. Absent these, biodegradation will not proceed. In the case of biodegradation of organic pollutants, the carbon typically comes from the pollutant being degraded. Although a multitude of reactions are used by microbes to degrade and transform pollutants, *all energy-yielding reactions are oxidation-reduction reactions*. In oxidative attacks, microbes oxidize a contaminant by transferring electrons from the contaminant (termed the electron donor) to an electron acceptor to gain energy. Typical electron acceptors are oxygen, nitrate, Fe(III),

sulfate, and carbon dioxide (Figure 2, Table 1). In reductive attacks, microbes utilize some easily metabolized organic electron donor (such as sugars or short chain fatty acids) and transfer the electrons to the pollutant to gain energy (Figure 2). This process is only possible with electrophilic pollutants such as halogenated aliphatics and explosives which contain nitro groups.

Microbial energetics

In order for energy to be released from an oxidation/reduction reaction, an overall negative Gibb's free energy must exist (i.e. the reaction must be thermodynamically favorable). The oxidation of most organic contaminants (electron donating half reaction) has a slightly positive to slightly negative standard state Gibb's free energy. In order to determine the overall free energy of the system, the electron donating half reaction must be balanced with the electron accepting half reaction. A variety of inorganic compounds can be used as terminal electron acceptors by bacteria during respiration (Table 1). Anaerobic respirative bacteria have generally lower energy yielding mechanisms than aerobic bacteria.

At a particular oxidation/reduction potential (ORP), a single electron acceptor will be favored by these thermodynamic considerations. When oxygen is present it is the dominant electron acceptor because it has the highest energy reduction half reaction (so-called terminal electron accepting process, or TEAP) (Lovley 1991). If no oxygen is present, the ORP decreases and various redox reactions become more important. Under these conditions, the availability of electron acceptors is the main selective factor determining microbial diversity. Typically, electron acceptors are utilized by bacteria in order of their thermodynamic energy yield (from highest to lowest) in soil: oxygen, nitrate, iron, and sulfate. Although oxygen and nitrate are highly energetic (Table 1), their reduction zones frequently do not penetrate deeply into contaminated soil zones, particularly in heavily polluted areas because they are rapidly utilized (Devol and Christensen 1993). At a slightly lower oxidation-reduction potential (ORP), the Fe(III)/Fe(II) couple occurs, followed by sulfate reduction and, finally, methanogenesis once all the other electron acceptors are exhausted (Devol and Christensen 1993).

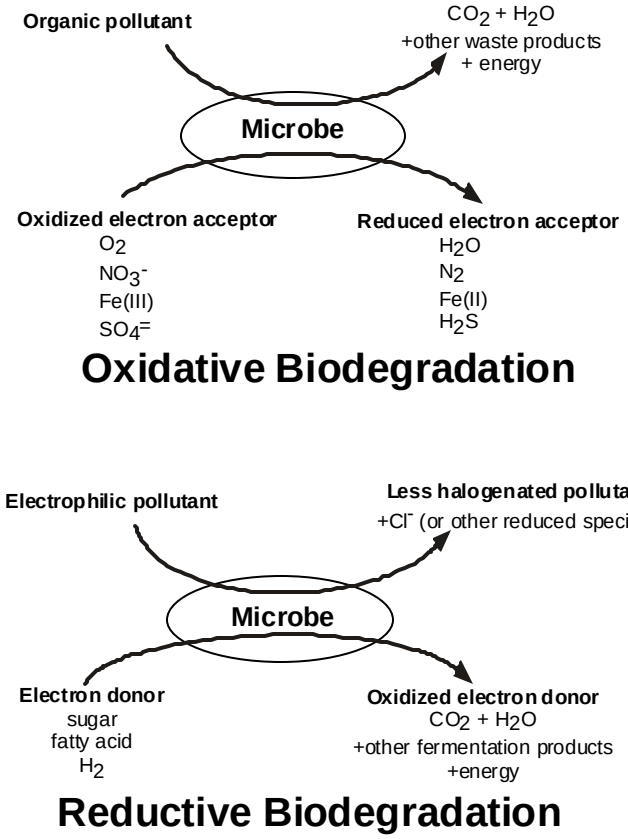

Figure 2. General schemes to biodegrade organic pollutants. In oxidative attacks (upper) pollutants are oxidized by external electron acceptors such as oxygen or sulfate. In reductive attacks (lower), electrophilic halogen or nitro groups on the pollutant are reduced by microbes consuming sugars, fatty acids, or hydrogen. The halo- or nitro- group on the pollutant serves as the external electron acceptor.

Bacteria can mediate all of these reactions. Denitrifying bacteria are commonly facultative anaerobes which preferentially use oxygen to oxidize organic matter under aerobic conditions and switch to a denitrifying pathway under anoxic conditions. Several bacteria can reduce Fe(III) coupled to the oxidation of organic matter. Some respirative bacteria, such as *S. putrafaciens* and *G. metallireducens*, are highly versatile in the types of electron acceptors they can couple to the oxidation of organic matter. For example, *G. metallireducens* can reduce many transition metal cations, including Mn(IV), U(VI), Co(III), and Cr(VI) (Lovley 1991).

At lower ORP, sulfidogenic and methanogenic bacteria mediate the oxidation of organic matter with sulfate and carbonate as the electron donor, respectively. Organic matter oxidation coupled to sulfate and carbonate reduction is characterized by low Gibbs free energy yields. Because of this low energy yield, there is the possibility that the oxidation of organic compounds such as aromatic hydrocarbons is endergonic under sulfidogenic and methanogenic conditions.

Table 1. Gibbs free energy[1] of one electron reduction for various electron acceptors found in soils.

	Reaction	ΔG_r^0 (w) kJ/reaction
Aerobic oxidation	$1/4O_2 + H^+ + e^- \rightarrow 1/2H_2O$	-83.5
Denitrification	$1/5NO_3^- + 6/5H^+ + e^- \rightarrow 1/10N_2 + 3/5H_2O$	-79.9
Fe reduction	$Fe(OH)_3(am) + 3H^+ + e^- \rightarrow Fe^{2+} + 3H_2O$	-7.8
Sulfate reduction	$1/8SO_4^{2-} + 19/16H^+ + e^- \rightarrow 1/16H_2S + 1/16HS^- + 1/2H_2O$	7.7
Methanogenesis	$1/8CO_2 + H^+ + e^- \rightarrow 1/8CH_4 + 1/4H_2O$	23.4

[1]Adapted from Rockne (1997). Free energy calculated for neutral conditions (pH=7) and typical concentrations of reactants in contaminated soils.

In summary, we can see that high energy electron acceptors such as nitrate and oxygen are preferred by microbes because they can gain much more energy from the oxidation of an electron donor. Under low energy electron accepting conditions, minimal energy may be available for microbes, and in some cases it may not be energetically possible for microbes to oxidize the contaminant. In these conditions, either a reductive attack is necessary, or the compound may not be biodegradable.

Biochemistry of biodegradation

All reactions in cells are controlled by enzymes. Enzymes catalyze both the oxidation and reduction of organic compounds for energy (called catabolic reactions) as well the production of new cell components during growth (called anabolic reactions). The degradation of any organic molecule, thus, requires the production and efficient utilization of enzymes. As discussed above, transfer of electrons from the electron donor to the electron acceptor requires electron carrying molecules such as NADH. These carriers transport electrons from an electron donor to the terminal electron acceptor (Table 1) through an electron transport chain. This generates a proton (H^+) gradient across an energy transducing membrane, which is dissipated by an enzyme to generate energy as adenosine triphosphate (ATP) molecules, the "currency" of energy in the cell.

Microorganisms need appropriate environmental conditions to survive and grow. These conditions include appropriate pH, temperature, oxygen, nutrients, and lack of inhibiting or toxic compounds (Cookson 1995; Thomas and Ward 1989). Typically, bioremediation is most efficient at a pH near 7. However, bioremediation can be achieved between pH values of 5.5 and 8.5. Most bioremediation systems operate over a temperature range of 15^0C to 45^0C. Aerobic microorganisms need a certain

amount of oxygen not only to survive, but also to mediate their reactions. Generally, oxygen concentration greater than 2 mg/L is required for aerobic microorganisms to efficiently degrade organic pollutants. Microorganisms need nutrients for their growth. The major nutrients needed are identified with the generalized biomass formula ($C_{60}H_{82}O_{23}N_{12}P$) and include carbon, hydrogen, oxygen, nitrogen and phosphorous. The actual quantity of these nutrients depends on the biochemical oxygen demand (BOD) of the contaminated soil. Generally, the C to N to P ratio (by weight) required is 120:10:1. Other nutrients such as sodium, potassium, ammonium, calcium, magnesium, iron, chloride and sulfur are needed in minor quantities, in the concentration range of 1 to 100 mg/L. In addition, traces (less than 1 mg/L) of nutrients such as manganese, cobalt, nickel, vanadium, boron, copper, zinc, various organics (vitamins) and molybdenum are needed. One must be careful that toxic substances do not exist that will produce adverse conditions for bioremediation. High concentration of any contaminant can frequently be toxic to microbes. Some contaminants even at low concentrations may be toxic to microbes. Generally, toxicity concerns are addressed by dilution or acclimated microbes, or by induced bioavailability limitations. It is also desirable to maintain the soil moisture level between 40 to 80% of field capacity. Different classes of organic pollutants have different microbial degradation pathways and thus different considerations for bioremediation strategies. We discuss here in detail the biodegradation of five major classes of pollutants: petroleum hydrocarbons, chlorinated aliphatics, PCBs, explosives, and pesticides/herbicides.

Petroleum hydrocarbons and PAHs

Interest in the biodegradation mechanisms and environmental fate of petroleum hydrocarbons and PAHs is prompted by their ubiquitous distribution in the environment and their deleterious effects on human health. Aliphatic petroleum hydrocarbons are short or branched chain alkanes and comprise the light fraction of refined oil. Monoaromatics in this light fraction include the class of compounds sometime referred to as "BTEX", for benzene, toluene, ethylbenzene and *ortho, para,* and *meta* xylene. PAHs constitute a large and diverse class of organic compounds consisting of three or more fused aromatic rings in various structural configurations PAHs are formed through industrial, and diagenetic processes, as well as by incomplete combustion of organic matter (ATSDR 1990). Primary sources for entry into the environment are via emissions from combustion processes or from spillage of petroleum products. Pollution of soil by tar oil from coal gasification facilities is the source of considerable PAH contamination in the U.S., as well as in other countries (ATSDR 1990). Anthropogenic sources such as vehicles emissions, heating and power plants, industrial and combustion processes are considered to be the principal sources to the environment on a mass basis (Kanaly and Harayama 2000).

The biodegradation of monoaromatic BTEX and PAHs by microorganisms is the subject of many reviews (Cerniglia 1984; Kanaly and Harayama 2000) and the biodegradation pathways of these aromatics are well documented and typically require oxygen to initiate the biodegradation process. Many microorganisms, including bacteria, algae and fungi possess degradative enzymes for the transformation of PAHs. However *in situ* microbial metabolism of aromatics is limited principally by two factors: absence of metabolic capabilities and the generally low solubility of these compounds resulting in low bioavailability (Makkar and Rockne 2003). Although they generally persist longer in soils than other hydrocarbons, high molecular weight (HMW) Aromatics are slowly removed from contaminated soils, suggesting that biodegradation pathway do exist (Kanaly and Harayama 2000). In the last fifteen years, research pertaining specifically to the bacterial biodegradation of PAHs composed of more than three rings has advanced significantly. Bacterial isolates, which can attack HMW PAHs, have been reported (Kanaly and Harayama 2000). In addition, many HMW PAHs are also susceptible to at least partial degradation by bacteria using other lower molecular weight hydrocarbons for carbon and energy.

The biochemical pathways for the biodegradation of both aliphatic and aromatic compounds have been well described (Cerniglia 1984). Bacteria under aerobic conditions can degrade most aliphatics and PAHs with less than five rings. It is evidenced that the initial step in aerobic catabolism of a monoaromatic or PAH molecule by bacteria occurs via oxidation of a single ring to a dihydrodiol. These dihydroxylated intermediates may further be metabolized via ring cleavage, resulting in intermediates that are further converted to carbon dioxide. Similarly, aliphatic hydrocarbons are also attacked with oxygen as a reactant. The oxygen is inserted into the end carbon by an monooxygenase enzyme leading to production of an acid. The fatty acid is then degraded piecemeal through a process termed *beta* oxidation resulting in two-carbon fatty acids being released.

In general, HMW PAHs are slowly degraded by indigenous microorganisms and may persist is soils and sediments (Kanaly and Harayama 2000). The recalcitrance of these pollutants is due in part to a strong adsorption of HMW PAHs to soil organic matter and low solubility, which results in decreased bioavailability for microbial degradation (Shor, Liang et al. 2003). Microorganisms show fundamental differences in the mechanisms of aromatic metabolism used. Bacteria initiate the oxidation of aromatics by incorporating both atoms of molecular oxygen into aromatic ring to produce a *cis*-dihydrodiol, which is then dehydrogenated to give catechols. Some bacteria also have been reported to attack PAHs with a methane monooxygenase (Rockne, Stensel et al. 1998). In addition, alkanes, BTEX and PAHs are now known to be degraded anaerobically (Chee-Sanford, Frost et al. 1996; Rockne and Strand 1998; So and Young 1999; Rockne, Chee-Sanford et al. 2000).

Fungi metabolize aromatics and aliphatics in pathways similar to those used by mammalian cytochrome P-450 enzyme systems (such as exist in the human liver) (Cerniglia 1984). Although a diverse group of fungi oxidize aromatics to dihydrodiols, only a few have the ability to mineralize to CO_2 (Cerniglia 1984). Most of the studies on PAH biodegradation by fungi are done using *Phanerochaete chrysosporium*. The first step in degradation of PAHs by *P. chrysosporium* involves the extra-cellular enzymes lignin peroxidase, manganese peroxidase and other H_2O_2 producing peroxidases which are sent outside the cell to attack macromolecules in wood. These extra-cellular enzymes have the remarkable ability to oxidize an astounding variety of organic compounds such as PAHs, lignin, cellulose, PCBs, and even dioxins (Bumpus and Aust 1987; Aust 1990; Bonnarme and Jeffries 1990).
It is generally accepted that the low level of bioavailability is the most important factor involved in the slow degradation of HMW PAHs and petroleum hydrocarbons. This is because these compounds possess low water solubility and therefore partition onto soil mineral surfaces and sorbs to available organic materials (Rockne, Shor et al. 2002). A wide diversity of research has focused on bioavailability limitations and ways of overcoming them, over the last decade (Makkar and Rockne 2003).

Chlorinated aliphatics

Chlorinated hydrocarbon contaminants such as trichloroethene and tetrachloroethene (TCE and PCE, respectively), have been identified as a major threat to groundwater resources and comprise the two most prevalent groundwater contaminants in the United States (Mackay, Roberts et al. 1985; Barbash and Roberts 1986). Sites contaminated with these compounds are particularly difficult to manage because, when released into the environment as nonaqueous phase liquids (NAPLs), large quantities of organic contaminants may be trapped in soils and aquifer materials, resulting in continuous groundwater contamination sources as they slowly dissolve into groundwater over periods of decades.

Table 2. Mode of bioremediation for several organic soil pollutants amenable to biodegradation.

Compound Class	Bioremediation mode	Representative microorganism(s)	Established technology?
Petroleum hydrocarbons	Primary: aerobic oxidation	*Pseudomonas* spp., multiple aerobic heterotrophs	Yes
PAHs	Primary: aerobic oxidation	Many species of aerobic heterotrophs	Yes
	Secondary: fungal/anaerobic	*Cunninghamella elegans*, *Phanerochaete chrysosporium*	No
Petroleum hydrocarbons: BTEX	Primary: aerobic oxidation	Multiple aerobic heterotrophic spp.	Yes
	Secondary: anaerobic oxidation	TOL4 and other denitrifying spp.; sulfate reducing spp.	Some
Chlorinated aliphatics	Primary: anaerobic reductive dehalogenation	Mixed anaerobic cultures. Complete dechlorination of PCE/TCE requires the presence of *Dehalococcus ethenogenes*	Yes
	Secondary: cometabolic aerobic oxidation	*Methylosinus* and *Methlylococcus* spp (methane)	Some
		Pseudomonads (toluene and phenol)	
		Nitrosomonas spp. (ammonia)	
Highly chlorinated PCBs	Primary: reductive dechlorination	Mixed anaerobic consortia	No
Mono- and di-chlorinated PCBs	Primary: aerobic oxidation	*Burkholderia* Str. LB400	Some
Explosives	Primary: reduction of nitro groups	Mixed anaerobic consortia	Yes
Pesticides and herbicides	Multiple modes; primarily aerobic for organo-phosphates and non-chlorinated compounds	Multiple aerobic and anaerobic heterotrophs; *Flavobacterium* spp., *Arthrobacter* spp.	Yes

PCE and TCE comprise the two most common groundwater contaminants in the United States, comprising the majority of all superfund sites (Barbash and Roberts 1986). It is estimated that cleanup costs with current technology could cost in the tens of billions of dollars in the USA alone (USEPA 2000). TCE and PCE are chlorinated solvents comprised of ethene with three or four chlorines. They are used for a variety of applications such as degreasers, industrial solvents, and dry cleaning agents. They are ideal solvents because they are relatively chemically inert to decomposition due to their high degree of chlorination. However, their chemical stability makes them environmentally persistent. For example, PCE cannot be degraded aerobically (with oxygen) and TCE can only be biodegraded aerobically by a small group of microorganisms in a fortuitous process known as co-metabolism (Table 2). The human

health effects of these compounds are well known. Both PCE and TCE have relatively low acute toxicity thresholds and both are putative human carcinogens. Repeated exposure to low levels of TCE in drinking water has been linked to cancer clusters in various localities (USEPA 2000).

Because of their relatively low aqueous solubility, these compounds can typically exist in the groundwater as a distinct phase, termed a non-aqueous phase liquid (NAPL), depending on the magnitude of the source. When in a NAPL, cleanup is complicated by the continuing source downstream represented by the difficult to remove NAPL. Continuous extraction and cleaning of the groundwater or soil-vapor in the contaminated plume is generally ineffective because the NAPL "source zone" can continue to be a source of contamination for 100's of years. Therefore, current approaches to TCE/PCE contaminated groundwater cleanup have focused on source-zone cleanup. Successfully demonstrated technologies for removing the source zone include complete excavation and NAPL vacuum extraction, as well as *in situ* treatments such as bioremediation, surfactant and/or solvent-assisted *in situ* washing/solubilization, and various combinations of these technologies (e.g. "bioslurping"). One of the main problems associated with *in situ* remediation of chlorinated solvent source zones is the residual contaminant and extraction solvent or surfactant left behind after clean up.

Although both PCE and TCE are stable aerobically, it has been known since the 1980's (Freedman and Gossett 1989) that these compounds can be degraded anaerobically (without oxygen) through a process called reductive dechlorination mediated by a reductive dehalogenase enzyme (RDase). In reductive dechlorination, microbes utilize PCE or TCE (the electron acceptor) to oxidize a reduced organic compound (the electron donor) in the same way humans use oxygen to oxidize the food we eat (Figure 3). Although a variety of organic acids can support reductive dechlorination, the "true" electron donor is often hydrogen produced from the fermentation of simple organic acids (Fennell, Gossett et al. 1997). Thus, efficient stimulation of this activity in situ requires the addition of electron donors such as lactic acid (sometimes referred to as hydrogen releasing compounds or HRC). Reductive dechlorination activity is typically quantified by chemical analysis of PCE and one or more of its dechlorination products: trichloroethene (TCE), dichloroethene (typically 1,2 *cis*, cDCE), vinyl chloride (VC), and ethene. Variation in the ratios of the various daughter products is often used to argue that *in situ* reductive dechlorination is occurring. In order for reductive dechlorination to occur, three components must be in temporal and spatial proximity: dechlorinating microorganisms, electron donor(s), and electron acceptor (PCE or metabolites).

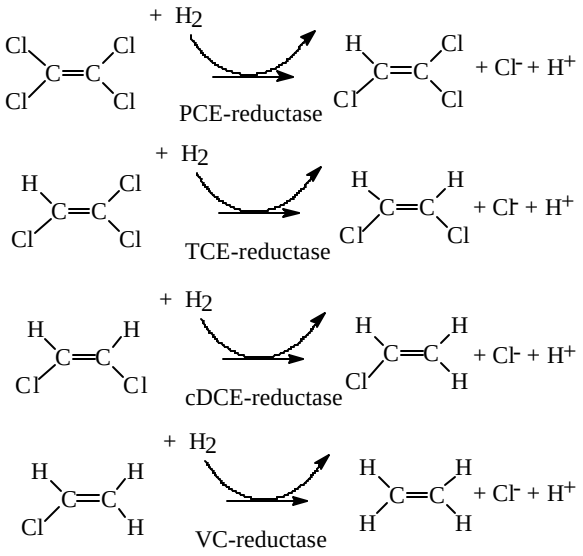

Figure 3. Stepwise reductive dechlorination of PCE to ethene mediated by anaerobic microorganisms. In each step, hydrogen is the electron donor for dechlorination mediated by the appropriate reductase enzyme.

PCBs

PCBs are common contaminants in soils and sediments impacted by electrical transformer production and associated leaks. Highly chlorinated PCBs are completely resistant to aerobic attack. However,

microbially-mediated reductive dechlorination of PCBs is an established field, having first been discovered in the mid-1980s. The activity is catalyzed by bacterial consortia that couple the reduction of chlorines on the PCB to the oxidation of an external electron donor under anaerobic conditions, releasing chloride ions. Although in theory any chlorine position can be dechlorinated, due to enzymatic capability (and possible stearic hindrance) most observed dechlorination activity follows a select group of pathways. The reduction pattern can be influenced by a variety of factors, including chlorine substitution pattern and environmental conditions (Bedard and Haberl 1990). There is evidence that temperature may play a key role in the selection of microorganisms and/or consortia that mediate these processes (Bedard, Bunnell et al. 1996).

It has been found that addition of co-substrates has accelerated PCB dechlorination activity through a stimulation or "priming" of the microbes responsible for PCB reductive dechlorination. Perhaps one of the more successful applications of this type of biostimulation has been the addition of less toxic poly brominated biphenyls (PBBs) to stimulate PCB dechlorination (Bedard, Van Dort et al. 1998). It was found that PBBs are readily debrominated at high rates by sediment enrichments that have been previously contaminated with PCBs.

Once sufficiently dechlorinated, mono- and di-chlorinated biphenyls are known to be aerobically degraded by bacteria such as *Burkholderia* Str. LB400 (Maltseva, Tsoi et al. 1999). This bacterium can break one ring with oxygen (similar to PAHs, see above) and proceed to mineralize the compound. Because this activity is limited to only mono- and di-chlorobiphenyls, researchers have proposed a sequential anaerobic/aerobic treatment process for PCBs (Maltseva, Tsoi et al. 1999; Master, Lai et al. 2002). First reductive dechlorination is stimulated through addition of electron donors, and, potentially, biostimulants such as PBBs, resulting in a buildup of mono and dichlorobiphenyls. Then, the contaminated soil is treated aerobically resulting in complete degradation of the PCB.

Explosives

Trinitrotoluene (TNT), RDX, and HMX are highly oxidative explosives used in a wide variety of commercial and military processes. Like chlorinated aliphatics, explosives contain highly oxidizing groups (typically nitro groups) that are amenable to reduction by the addition of electrons. Indeed, it is the combination of these oxidants together with the carbon skeleton that gives an explosive the capability for explosive autocatalytic oxidation.

Addition of readily fermentable/degradable sugars such as molasses has been shown to stimulate the reductive removal of nitro groups from trinitrotoluene (TNT) and RDX (see Figure 1). Removal of these groups leaves either an aromatic (TNT, Tetryl) or cyclic hydrocarbon that is much less toxic and more easily degradable oxidatively (see above) (Cataldo, Harvey et al. 1990; Kitts, Cunningham et al. 1994). Others have shown that HMX is completely mineralized to carbon dioxide and nitrous oxide by anaerobic microorganisms (Regan and Crawford 1994).

Pesticides and herbicides

Pesticides and herbicides comprise a large number of compounds with different chemical structures (Figure 1). These compounds can broadly be organized into chlorinated pesticides (DDT, heptachlor, dieldrin, chlordane), nitrogen-containing aromatics (such as atrazine), and organo-phosphates (malathion and parathion). The chlorinated pesticides are very recalcitrant to biodegradation and are generally referred to as persistent. Many are comprised of exotic structures that are completely xenobiotic and highly chlorinated. Although partial reductive dechlorination of some compounds are reported (e.g. reduction of DDT to DDE), many of these pathways are dead end and do not lead to significant detoxification.

The triazines are degraded through both aerobic and anaerobic processes. Typically, this follows a de-alkylation (removal of the alkyl groups), followed by hydrolysis of the chloro group. Frequently, triazines like atrazine can persist in the environment, particularly the hydrolyzed analogs (Anderson, Kruger et al. 1994).

Organo-phosphates have a similar structural motif: a central phosphoro group with two attached sulfur, oxygen, or nitrogen atoms and three ether-linked methyl or ethyl chains (Figure 1). The general biodegradation pathways for these compounds are breaking of the ether linkages through hydrolytic reactions (Anderson, Kruger et al. 1994). Previously produced insecticides and herbicides were more persistent than currently manufactured compounds, typified by these more readily hydrolyzed substituents.

SYSTEM DESIGN AND IMPLEMENTATION

General Design Approach

Figure 4 shows the general approach to the application of bioremediation. This approach can be followed for both in-situ and ex-situ conditions. In-situ bioremediation allows treatment of a large volume of soil at once and it is mostly effective at sites with sandy soils. In-situ bioremediation techniques can vary depending on the method of supplying oxygen or electron donors to the organisms that degrade the contaminants. Three commonly used in-situ methods include bioventing and injection of hydrogen peroxide or oxygen releasing compound (ORC) for aerobic treatment and injection of HRC for anaerobic treatment.

- Bioventing- These systems deliver air from the atmosphere into the soil above the water table through injection wells placed in the ground where the contamination exists (Figure 5). An air blower may be used to push in or pull out air into or from the soil through the injection wells. Air flows through the soil and the oxygen present in the air is used by the microorganisms. Nutrients may be pumped into the soil through the injection wells. Nitrogen and phosphorus may be added to increase the growth rate of the microorganisms (Hinchee and Miller 1990; USEPA 1992; USEPA 1993b).
- Injection of Hydrogen Peroxide or Oxygen Release Compound (ORC)- This process delivers oxygen to stimulate the activity of naturally occurring microorganisms by circulating hydrogen peroxide (in liquid form) or ORC through contaminated soils to speed up the bioremediation of organic contaminants. ORC is a patented formulation of magnesium peroxide which when moist releases oxygen slowly (Oencrantz et al. 1995). Since it involves putting a chemical (hydrogen peroxide or ORC) into the ground (which may eventually seep into the ground water), this process is used only at sites where ground water is already contaminated. A system of pipes or a sprinkler system is typically used to deliver hydrogen peroxide to shallow contaminated soils. Injection wells are used for deeper contaminated soils.
- Injection of Hydrogen Releasing Compound (HRC)- This process is similar to addition of ORC except hydrogen-producing compounds are typically more soluble. A major concern here is the cost; the hydrogen yield per cost is critical for the typically large volumes of contaminated groundwater needing treatment. Typical HRCs include molasses and other sugar-like derivatives, solubilized chitin-based compounds, lactic acid and polymerized poly-lactates. HRC injection is gaining widespread acceptance by the regulatory community because of its proven success in the field and the fact that most HRCs are completely non-toxic (USEPA 2000). One caution, however, is that the addition of large amounts of HRC will result in highly anaerobic groundwater conditions (and attendant nuisance issues like high sulfide concentrations) far downstream of injection and may require aeration prior to any subsequent use.

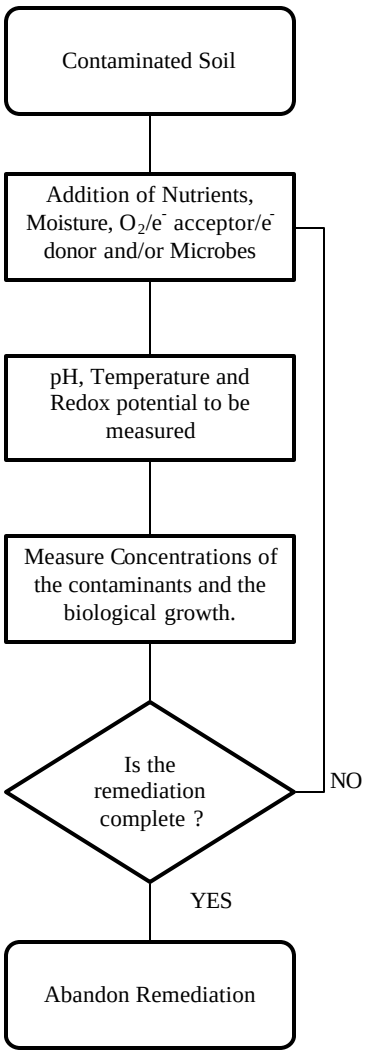

Figure 4. Decision tree showing general approach to bioremediation.

Ex-situ bioremediation involves excavation of the contaminated soil and treating in a treatment plant located on the site or away from the site. This approach can be faster, easier to control, and used to treat a wider range of contaminants and soil types than in-situ approach. Ex-situ bioremediation can be implemented as slurry-phase bioremediation, or solid-phase bioremediation (USEPA 1988a; USEPA 1994b).

In *slurry-phase bioremediation*, the contaminated soil is mixed with water to create a slurry. The slurry is aerated, and the contaminants are aerobically biodegraded. The treatment can take place on-site, or the soils can be removed and transported to a remote location for treatment (USEPA 1990). The process generally takes place in a tank or vessel (a "bioreactor"), but can also take place in a lagoon (Figure 6A). Figure 6B presents a schematic of the process. Contaminated soil is excavated and then screened to remove large particles and debris. A specific volume of soil is mixed with water, nutrients, and microorganisms. The resulting slurry pH may be adjusted, if necessary. The slurry is treated in the bioreactor until the desired level of treatment is achieved. Aeration is provided by compressors and air spargers. Mixing is accomplished either by aeration alone or by aeration combined with mechanical mixers. During treatment, the oxygen and nutrient content, pH, and temperature of the slurry are adjusted and maintained at levels suitable for aerobic microbial growth. Natural soil microbial populations may be used if suitable strains and numbers are present in the soil. More typically, microorganisms are added to ensure timely and effective treatment. The microorganisms can be seeded initially on start-up or supplemented continuously throughout the treatment period for each batch of soil treated. When the desired level of treatment has been achieved, the unit is emptied. The treated soil is then dewatered and backfilled in excavations. The wastewater is treated and disposed or recycled, and a second volume of soil is treated.

In *solid-phase bioremediation*, soil is treated above ground treatment areas equipped with collection systems to prevent any contaminant from escaping the treatment. Moisture, heat, nutrients, or oxygen are controlled to enhance bioremediation for the application of this treatment. Solid-phase systems are relatively simple to operate and maintain, require large amount of space, and cleanups require more time to complete than slurry-phase processes. There are three different ways of implementing solid-phase

bioremediation: contained solid phase bioremediation, composting, and land farming (USEPA 1993a; USEPA 1988a; Cookson 1995).

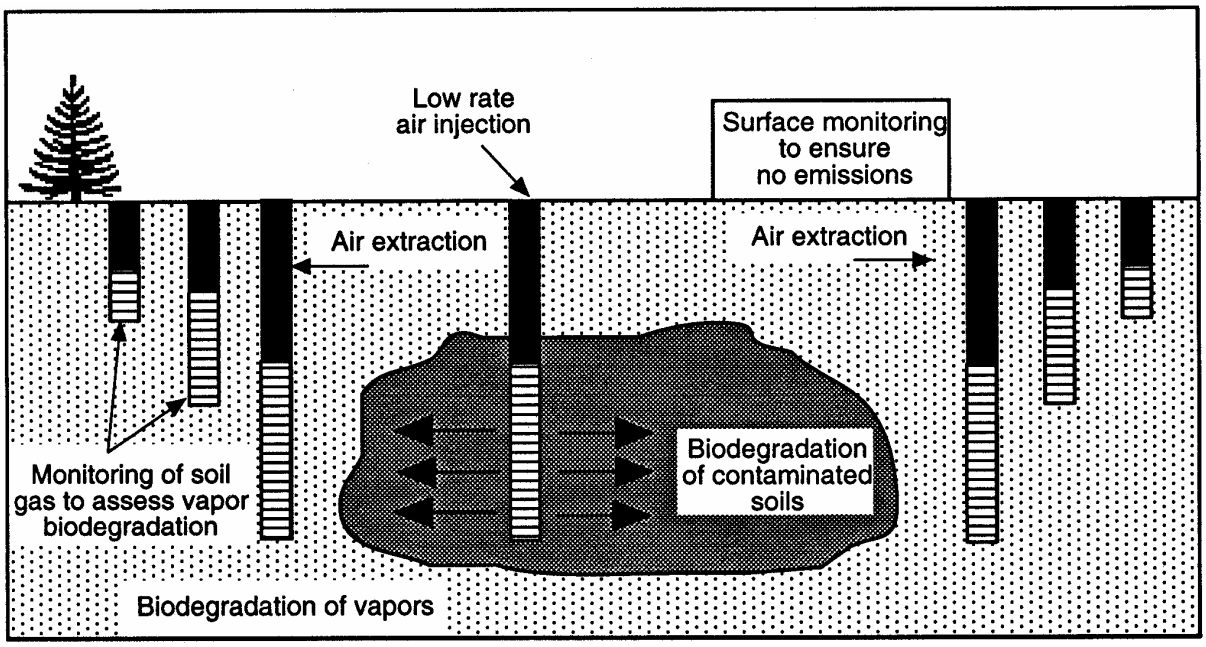

Figure 5. Schematic showing soil-cross section of the bioventing remediation process (USEPA 1992).

- In *contained solid phase bioremediation*, the excavated soils are not slurried with water; the contaminated soils are simply blended to achieve a homogeneous texture. Occasionally, textural or bulk amendments, nutrients, moisture, pH adjustment, and microbes are added. The soil is then placed in an enclosed building, vault, tank, or vessel (Figure 7). The temperature and moisture conditions are controlled to maintain good growing conditions for the microbial population. In addition, since the soil mass is enclosed, rainfall and runoff are eliminated, and VOC emissions can be controlled. Mechanisms for managing/controlling flammable or explosive atmospheres and special equipment for blending and aeration of the soil may be required.

- *Composting*, if carried out in an enclosed vessel, is similar to contained solid phase bioremediation, but it does not employ added microorganisms. Structurally firm material may be added to the contaminated material to improve its handling characteristics, and the mixture may be periodically stirred or mixed to promote aeration and aerobic degradation. If necessary, moisture may also be added. Usually composting is conducted outdoors rather than in an enclosed space. The two basic types of unenclosed composting are open and static windrow systems (Figure 8). In *open windrow systems*, the compost is stacked in elongated piles. Aeration is accomplished by tearing down and rebuilding the piles. *In static windrow systems*, the piles are aerated by a forced air system. Composting is commonly a less controlled process than other forms of bioremediation (with the possible exception of land farming). The waste is not protected from variations in natural environmental conditions, such as rainfall and temperature fluctuations.

- The *land farming* process involves spreading the contaminated soil in fields or limited treatment beds. The soil is spread in thin lifts up to 1/2-inch thick. Conventional construction and/or farm equipment may be used to spread the soil. The soil is tilled periodically thereby providing oxygen. Microorganisms, nutrients, and moisture may also be added. Clay or plastic liners may be installed in the field prior to placement of the contaminated soil, which act to retard or prevent migration of

contaminants into underlying and adjacent clean soils, groundwater, and surface water. Treatment is achieved through biodegradation, in combination with aeration and possibly photo oxidation in sunlight. These processes are most active in warm, moist sunny conditions. Treatment is greatly diminished or even completely arrested during winter months when temperatures are cold and snow covers the ground.

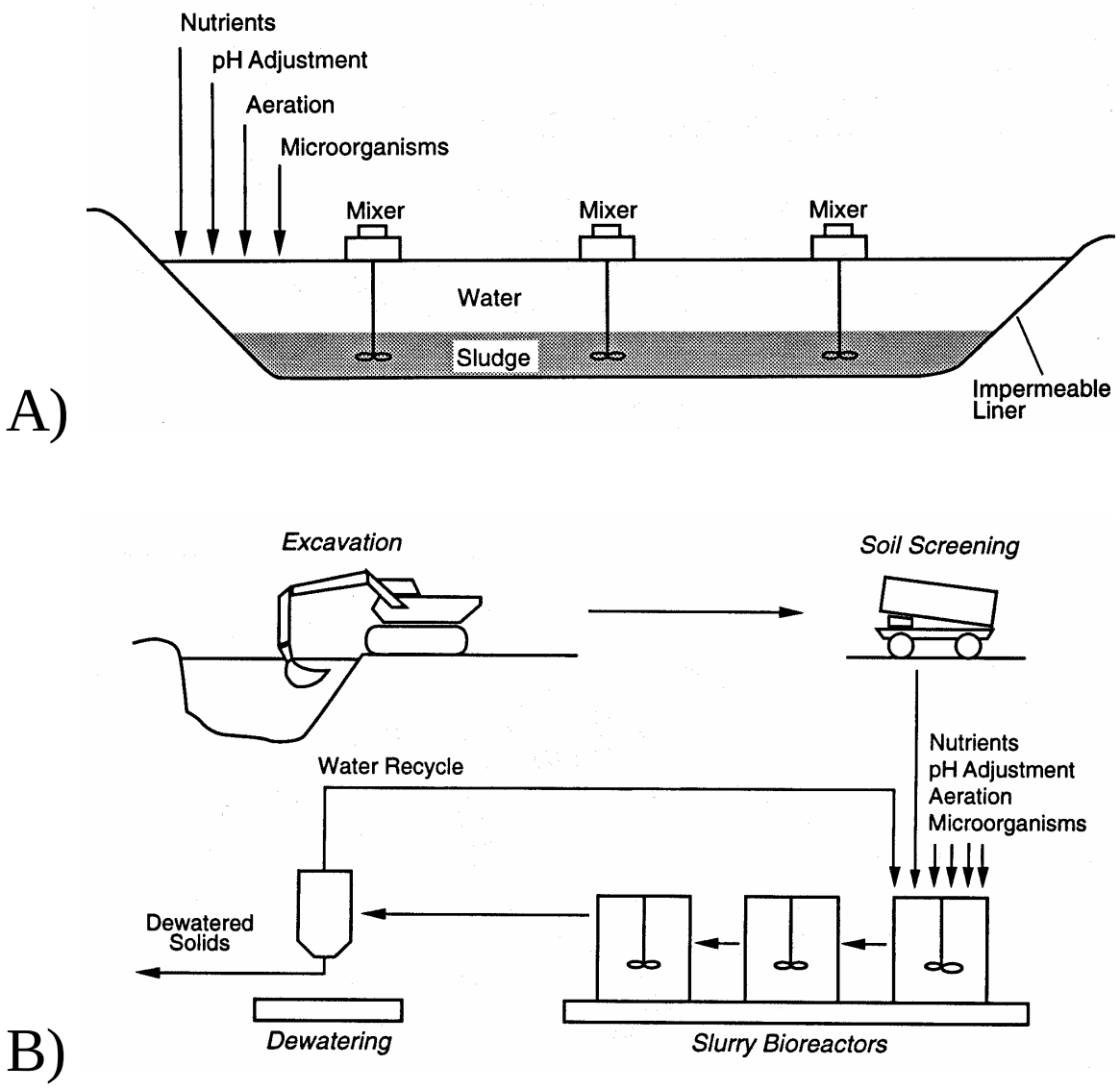

Figure 6. Ex-situ slurry-phase bioremediation in (A) lagoons, and (B) above-ground reactors (USEPA 1993a).

Natural Attenuation

In the natural attenuation process, native microorganisms occurring in the soil (yeast, fungi, or bacteria) degrade the contaminants for their survival. It has been demonstrated that certain types of petroleum hydrocarbons are easily degraded by these naturally occurring microorganisms and that natural attenuation, or intrinsic remediation, of these contaminants may be sufficient for risk management at

many sites (USEPA 2000). If effective, intrinsic remediation is an environmentally friendly alternative to active treatment technologies such as pump-and-treat or in-situ flushing. Chlorinated hydrocarbons, PAHs, and PCBs are less-easily degraded than petroleum hydrocarbons; however, intrinsic reductive dechlorination of PCE and TCE to ethene or ethane by anaerobic bacteria has been demonstrated in the field (USEPA 2000).

Current regulations for natural attenuation require extensive monitoring to prove that the contaminants are indeed being degraded. This requirement has led to the process being called *monitored natural attenuation*, or MNA. Although MNA may be thought to be a cheaper alternative, frequently the monitoring costs are substantial, and may exceed active bioremediation costs which occur over a shorter time frame (USEPA 2000).

Selection of Equipment

The equipment required for in-situ bioremediation are basically those which involve the injection of nutrients into the soil, which may consist of spraying or sprinkling equipment, injection wells or extraction wells (Thomas and Ward 1989; USEPA 1992). They need to be in sufficient number to ensure that the required amount of moisture and nutrients reach the contaminated zone. In the case of ex-situ bioremediation, a bioreactor with adequate volume to hold the contaminated soil is needed or adequate space is needed to spread the contaminated soil (Cookson 1995; USEPA 1990; USEPA 1993a). The volume of bioreactor is roughly estimated using:

$$\frac{dC}{dt} = \frac{r_0}{K_{sd}\ X_s + 1} \qquad (1)$$

$$\frac{\mathrm{dM}}{\mathrm{dt}} = \mathrm{V}\ \mathrm{r}_0 \qquad (2)$$

Where V=volume of the reactor, r_0 the rate of contaminant biodegradation, C=the mass concentration of soluble contaminant, M=the mass of contaminant to be put in the reactor, t=the time in days, K_{sd}=the soil distribution coefficient, and X_s=the mass concentration of solids (contaminated soils).

Monitoring equipment is also needed to check contaminant spreading and also to verify contaminant degradation. Equipment is necessary for the measurement of concentrations of microbes, nutrients, carbon dioxide; and oxygen (aerobic treatment), ORP or TEAP (anaerobic treatment). A common technique to measure the in situ TEAP is to simply measure the hydrogen (H_2) concentration, which has been shown to be an accurate predictive tool. Excavating equipment is also necessary for excavating the contaminated soil from the site for the ex-situ treatment.

Selection of Operational Parameters

Besides the microbes, the following parameters are monitored and maintained at certain levels: nutrients, oxygen supply, temperature, pH, and moisture content. The addition of organic nutrients is often required to maintain the microbial ecology. Usually, initial laboratory tests are conducted to find the amount of carbon, nitrogen, and phosphorus required for the type and concentrations of contaminants at that particular site. But, an approximate estimate of nitrogen, phosphorus, and oxygen uptake rates can be made using the following equations (Cookson 1995):

$$\mathrm{r_N} = \frac{0.06\ \mathrm{r_0}}{1 + 0.05\ \tau} \qquad (3)$$

$$\mathrm{r_p} = \frac{0.06\ \mathrm{r_0}}{1 + 0.05\ \tau} \qquad (4)$$

$$r_{oxygen} = 0.06\, r_0 \left(1 - \frac{0.06}{1 + 0.05\,\tau}\right) \qquad (5)$$

Where r_N=the rate of nitrogen uptake, r_p=the rate of phosphorus uptake, r_{oxygen}=the rate of oxygen uptake rate, r_0=the rate of biodegradation, and τ=the solids residence time.

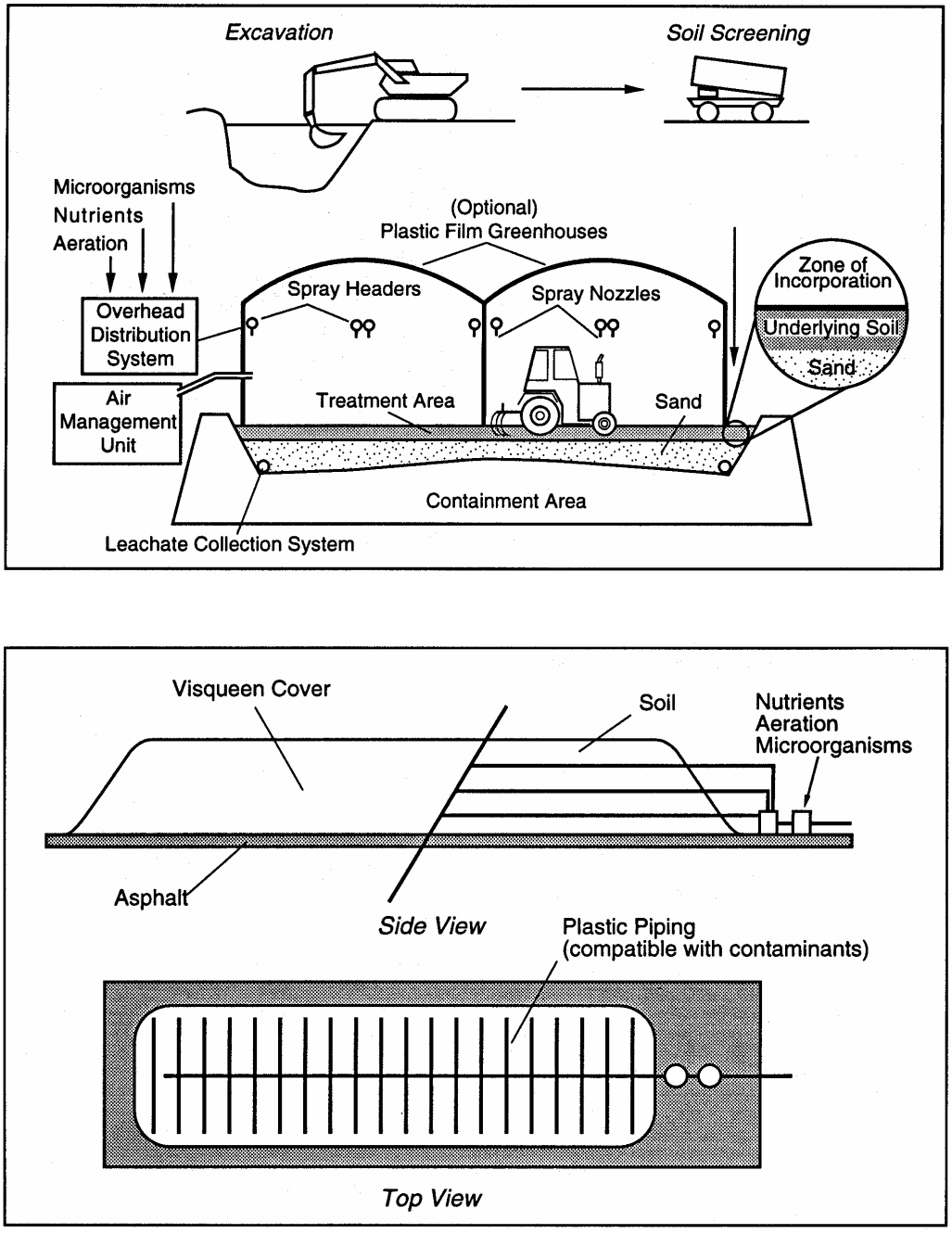

Figure 7. Contained ex-situ solid phase bioremediation (USEPA 1993a).

The oxygen supply is maintained by either pumping in or extracting out air through the contaminated zone through injection wells. Generally, the concentration of oxygen is maintained between 2 to 20 mg/L depending on the type and concentration of the contaminants and also on the soil type. The temperature of the bioreactor needs to be controlled if remediation is carried out in extreme weather conditions. Generally, the temperature is maintained between 25°-40° C to ensure microbial activity. Since many soils are acidic in nature, pH adjustment is often needed. This is generally done by adding calcium or calcium/magnesium-containing compounds to the soil. The optimum moisture content required is generally obtained from pilot scale tests done before the actual remediation is started and spraying or sprinkling equipment or injection wells are installed accordingly.

MODIFIED/COMPLEMENTING TECHNOLOGIES

Different technologies use bioremediation as a modification to that technology or as complementing technology. Soil vapor extraction is combined with bioremediation in that the extracted organic vapors are passed through the soil zones where microbial activity is high or passed through biofilters or biostrippers (so-called "bioslurping") which contain microbial populations in chambers and contaminated vapor extracted is passed through the biofilters to facilitate degradation. Soil washing and bioremediation may be combined in that the soil may be washed first with some reagent and then the effluent and the silty and clayey materials are mixed to form a slurry which is then treated in a bioreactor for bioremediation. This process will reduce the volume of soil to be treated and in turn reduce the total costs involved in remediation.

ECONOMIC AND REGULATORY CONSIDERATIONS

This technology is found to be economical compared to the other technologies especially when the contaminants involved are organic compounds and the soils have sufficient hydraulic conductivity to allow nutrient and oxygen flow through it. The costs involved in the treatment vary from as low as $30 per cubic yard of soil to as high as $750 per cubic yard (USEPA 1985).

From regulatory perspective, the air emissions have to be within the limits specified in the regulations, the seepage of the contaminants into the groundwater during remediation must be controlled, and care has to be taken that the volatile organic compounds do not enter into the atmosphere while excavating the soil for ex-situ remediation. As discussed above, MNA has extensive regulatory controls to ensure active remediation is occurring.

Bioremediation has been the subject of intense research for the past few years to address issues such as:

- Evaluation of suitable microbes, nutritional requirements, lag times, and degradation rates (in the field) for various types of contaminants;
- Optimization of environmental conditions, and stimulation of favorable growth conditions under site-specific variations;
- In-situ methods for an efficient monitoring process;
- Mass balance of electron donors and acceptors within a given system;
- Impact on aquifer permeability due to enhanced bioremediation;
- Enhancing bioavailability of hydrophobic compounds through addition of surfactants, biosurfactants, and/or co-solvents
- Enhancing bioremediation of soils of low permeability;
- Understanding bioaugmentation in soils of low permeability;
- Understanding bioaugmentation, including which organics degrade specific types of contaminants;
- Effect of hydraulic conductivity on microbial activity; and
- Techniques to minimize well fouling.

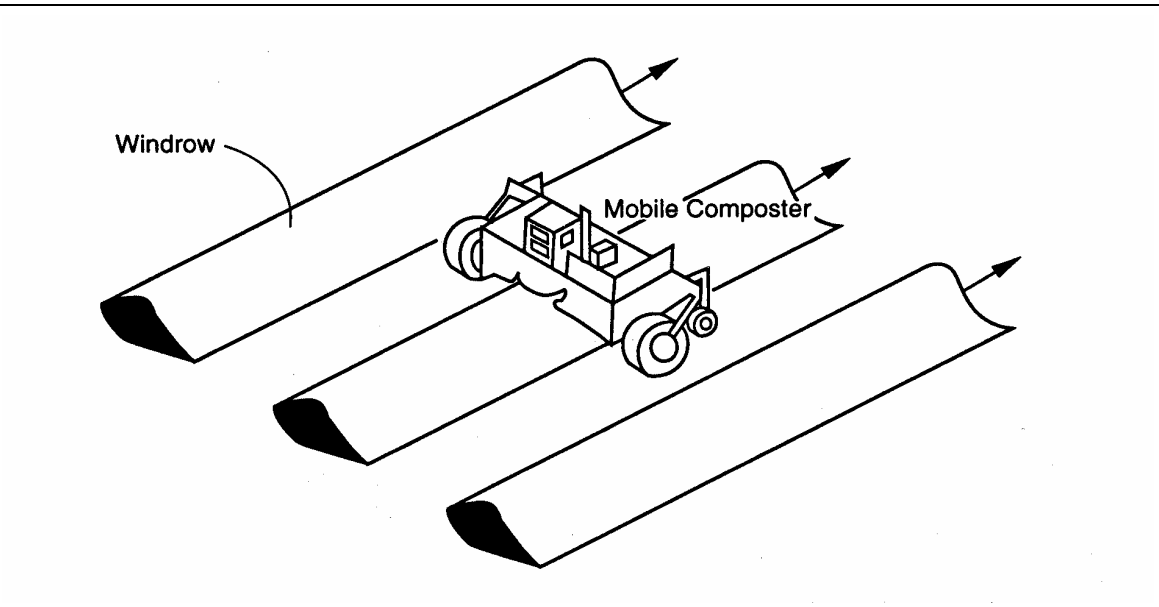

Figure 8. Ex-situ composting: open and static windrow systems (USEPA 1993a).

CASE STUDIES

The efficiency for cleanup of the remediation technology used at a particular site is very important while considering the costs involved in the remediation and the effect of contamination on the environment. Several case studies have been reported where bioremediation has been used to remediate site contamination (FRTR 1995a, (USEPA 2000). To illustrate the performance and cost issues, three full-scale remediation studies are presented below.

Brown Wood Preserving Superfund Site

For approximately 30 years, the Brown Wood Preserving site in Live Oak, Florida was used for the pressure treatment of lumber products with creosote. Occasionally, penta-chloro phenol was also used for this purpose. Lumber was treated in two cylinders and the wastewater from the treatment was dumped into a lagoon. The lagoon and the soils (which included clayey soils to fine sand) around it were consequently contaminated with high levels of organics, which consisted of PAHs found in creosote. These contaminants included benzo[a]anthracene, benzo[a]pyrene, benzo[b+k]fluoranthene, chrysene, dibenz[a,h]anthracene and indeno[1,2,3-cd]pyrene. The concentrations of these PAHs ranged from 100 to 208 mg/kg. Consequently, a study was conducted and land treatment technology was selected to cleanup the contaminated soils which were stockpiled during the interim removal activities. A clean-up goal of 100 mg/kg of summation concentrations of six PAHs was set.

Construction of the land treatment area included installation of a clay liner, berm, run-on swales, and a subsurface drainage system. A retention pond was set up for run-off control. A portable irrigation system was used for maintaining optimum moisture during remediation. The first lift was inoculated with PAH-degrading microorganisms and the lifts cultivated every two weeks. The soil moisture content was maintained at 10%. Land treatment of the contaminated soils was performed form January 1989 to July 1990. Approximately 8100 cubic yards of soil was treated in three lifts. The concentration of PAHs was

found to be varying between 23 to 92 mg/kg after treatment. The cleanup goal was achieved within a period of 18 months. The total treatment cost for this site was $565,400, or approximately $70 per cubic yard of soil which is low compared to other treatment technologies.

French Limited Superfund Site

The French Limited Superfund site in Crosby, Texas was a former industrial waste disposal facility where an estimated 70 million gallons of petrochemical wastes were disposed in an unlined lagoon between 1966 and 1971. The primary contaminants at the site included benzo[a]pyrene, vinyl chloride, and benzene. The contaminant concentrations ranged from 400 mg/kg to 5000 mg/kg. Initial studies were conducted and slurry phase bioremediation was selected as the treatment technology for the cleanup of the contaminated soils.

The treatment was done in two cells each having a capacity of 17 million gallons. An innovative technology called the Mixflo system was used for aeration (to maintain a dissolved oxygen concentration of 2 mg/L) which minimized the air emissions during the treatment. Approximately 300,000 tons of soil and sludge was treated during the cleanup operation. The cleanup took approximately 11 months time and the concentrations after the treatment ranged from 7 to 43 mg/kg. The total cost was approximately $49,000,000, which included costs for treatment, pilot studies, technology development, and backfill of the lagoon.

Avco Lycoming Superfund Site

The Avco Lycoming Superfund site was a fairly small (<30 acre) facility used since the 1920s for a variety of industries. The primary contaminants at the site include the chlorinated solvents PCE, TCE and *cis* dichloroethene (cDCE), as well as hexavalent chromium and cadmium. The site was identified in the 1980s as the source of contamination to the local water utility and a Record of Decision (ROD) was issued in 1990 for pump and treat cleanup and investigation of innovative in situ bioremediation technologies. The soil consisted of sandy silt with hydraulic conductivity of 0.2 to >20 ft/day (USEPA 2000).

Bench and pilot scale studies showed the potential for injection of molasses as a feasible technology to induce in situ reductive dechlorination of the PCE, TCE and cDCE, and transformation of the hexavalent chromium. Treatment goals were <5 µg/L for TCE, <70 µg/L for cDCE, <2 µg/L for vinyl chloride (VC) and <32 µg/L for hexavalent chromium.

The treatment was by done mixing molasses with potable water in a batch tank, followed by injection into the reactive zone. The results showed establishment of completely anaerobic conditions within 18 months throughout the entire reactive zone; even in areas that were fully aerobic prior to the cleanup. By year two (results are still forthcoming), nearly all of the monitoring wells showed levels of the contaminants below cleanup goals, including hexavalent chromium. Levels of cDCE increased in response to reduction of the PCE and TCE on site, as expected from reductive dechlorination, and these levels soon decreased as further reduction of the cDCE commenced. The total cost was reportedly $220,000 for construction and $50,000/yr for operation and maintenance. Pilot plant costs were <$150,000.

CONCLUSION

Bioremediation is a rapidly establishing technology for contaminated soil and groundwater treatment. For some compounds, it may be the best technology for treatment, particularly in sites where it is difficult to access the contamination such as in deeper aquifers. Although nearly all organic pollutants can be biodegraded in the laboratory and may all be suitable for bioremediation at some sites, a few stand out as

having been clearly demonstrated to be efficiently treated by in situ or ex situ bioremediation. The key factors are ease of transport for any amendments to the site of action, ease of biodegradation, low toxicity, and high bioavailability. Given these factors, the classes of organic contaminants best suited for bioremediation include: chlorinated aliphatics, explosives, BTEX, and petroleum hydrocarbons.

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