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Selected factors limiting the microbial degradation of recalcitrant compounds

Miguel A. Providenti, Hung Lee and Jack T. Trevors

University of Guelph, Department of Environmental Biology, Guelph, Ontario, Canada NIG 2W1

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SUMMARY

The focus of this review is to examine some of the reasons biodegradation may not take place in the environment even though its occurrence in the laboratory has been demonstrated. Some approaches for dealing with chemical persistence will be discussed. In addition, the potential of bioremediation as an in situ clean-up technology will be considered.

INTRODUCTION

The biological degradation of toxic, xenobiotic compounds previously believed to be resistant to natural processes has been recognized for several decades. As a result of these findings, interest in biological remediation of polluted soil and water has increased. Biological remediation, as opposed to physical or chemical processes, is considered a safe, efficient, and less expensive method of removing harmful pollutants, either through transformation to non-toxic products or through complete mineralization to water, inorganic minerals, and CO_2 (via aerobic degradation) or sometimes CH_4 (via anaerobic degradation) [124].

Bioremediation techniques, as reviewed by Morgan and Watkinson [124,125], include inoculation of soil with previously isolated microorganisms known to degrade certain pollutants, stimulation of indigenous microbial populations through nutrient amendments and/or increased aeration, and addition of cometabolic substrates. Although some success has been achieved in biodegradation of fuel and petroleum contaminated sites, optimization of biodegradation to remediate sites polluted by other organic compounds is still in the developmental stage.

ENVIRONMENTAL PARAMETERS

Proper environmental conditions are fundamentally important to microbial growth and survival, and its importance to biodegradation in nature cannot be overstated. If environmental conditions such as pH, temperature, water activity, aeration (for aerobic degradation), and redox potential (for anaerobic degradation) are not adequate, microbial growth and survival will be adversely affected. Consequently, biodegradation may not occur at optimal rates. In soil systems, soil type and hydration also play a role in determining the efficiency of biodegradation. The general effects of environmental parameters on soil microorganisms have been covered [139] and the importance of these parameters to biodegradation specifically in both aqueous and soil systems has been reviewed [103,124,125]. Numerous factors are summarized in Table 1, but some points which merit further comments follow.

Oxygen is used by organisms not only as the terminal electron acceptor in aerobic respiration, but also as a substrate in oxygenase-catalyzed biodegradative reactions. These include ring cleavage and hydroxylation of cyclic aromatic compounds, and oxidation of aliphatic chemicals [37,128]. Proper aeration is therefore essential if aerobic, catabolic reactions are to occur.

Biodegradative reactions can also occur in anoxic environments where oxygen may not be essential or may be inhibitory. For example, biodegradation of organic contaminants has been shown to occur under denitrifying [23,46,84, 117], methanogenic [22,48,54,55,60,62,99], sulphate-reducing [71,97,98,147,165], and iron-reducing conditions [109,110].

Halogenated hydrocarbons may play the role of alternate electron acceptor when they undergo reductive dechlorination, or substitution of Cl with H, in an anaerobic environment [39,40]. In support of this view are the results of Mohn and Tiedje [121] and Dolfing [38] who showed that in the bacterium *Desulfominile tiedjei* DCB-1 [34], reductive dechlorination of 3-chlorobenzoic acid is coupled to ATP formation and cell growth, suggesting a new form of chemotrophy in an anaerobic environment. For more information, see Mohn and Tiedje [122].

Under anaerobic conditions, an electron donor has to be present for reductive dechlorination to occur. The electron

Correspondence to: H. Lee or J.T. Trevors, University of Guelph, Department of Environmental Biology, Guelph, Ontario, Canada NIG 2W1.

TABLE 1

Effect of selected environmental parameters on microorganisms and contaminants

Parameter	Effect of microorganisms	Effect on contaminants
Soil moisture	inadequate hydration depresses microbial metabolism and microbial movement through soil surplus water limits oxygen transport	inadequate hydration decreases contaminant as well as nutrient transport through soil
Soil type	depending on soil type and hydration, microbial and movement affected	depending on soil type, contaminant sorption varies
Aeration	oxygen often limiting in soil and aqueous systems. Necessary for aerobic respiration	oxygen a substrate in several catabolic reactions of various contaminants
Redox potential	for anaerobic metabolism to occur, alternate electron acceptors are necessary	
pH	microbial activity dependent on pH	water solubility and sorption to soil and sediment can vary with pH
Temperature	microbial metabolism varies with temperature	temperature can affect contaminant solubility, sorption, viscosity, and volatilization

donor may be provided by endogenous or exogenous sources. For example, reductive dechlorination of tetrachloroethylene and trichloroethylene by methanogenic enrichment cultures was enhanced by the presence of glucose, methanol, formate, or acetate [36,55], with methanol providing the greatest enhancement. Similarly, dechlorination of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) added to a methanogenic acquifer sample was enhanced by various short-chain alcohols or acids [63]. Nies and Vogel [132] also observed dechlorination of polychlorinated biphenyls (PCBs) in the presence of acetate, acetone, methanol, or glucose by microorganisms enriched from anaerobic sediments. However, these additional substrates may not always be necessary for metabolism of PCBs.

Dechlorination can transform persistent compounds to those that may be more amenable to microbial degradation. Subsequent degradation can occur under either aerobic or anaerobic conditions. The potential for exploiting reductive dechlorination to enhance biodegradation of highly chlorinated compounds is discussed later.

Approaches to enhance biodegradation of pollutants in situ by optimizing environmental conditions, as well as potential problems associated with these techniques, have been reviewed by Morgan and Watkinson [124,125]. Their comments and those made by others are briefly summarized here. Generally, care must be exercised to ensure that contaminants, nutrients, and bacterial inocula, if any, do not leach from the site and spread through the environment [124,125]. Recirculation of ground water being treated with microorganisms is an option. Water and inorganic nutrients, such as ammonium, phosphate, and essential minerals can be easily provided. The pH can also be adjusted, provided the soil site or water volume is not too large. Temperature is difficult to manipulate and in situ bioremediation efforts will be limited by seasonal fluctuations in temperature. Small field plots can be enclosed in a temporary green-house type structure for bioremediation to occur all year round in colder climates.

Aeration of soil can be improved by tilling [16], composting with bulking agents such as organic crop or forestry residues [124], or venting [81]. Tilling also mixes degrading microorganisms throughout the top portion (about 0.5 m) of soil. Venting of soil may remove volatile contaminants from soil [81]. There may be limitations with these approaches. For example, aeration of contaminated, deep subsurface soils would not be improved by surface tillage. Bulking agents may provide easily metabolized carbon and energy sources that may decrease biodegradation [124]. In aqueous systems, aeration can be improved by direct sparging with air or oxygen, or addition of hydrogen peroxide which forms water

and oxygen on decomposition. However, hydrogen peroxide reacts with organic matter and can inhibit microbial activity [124,125].

Anaerobic degradation in subsoils or aqueous systems may be enhanced by adding suitable electron acceptors. For example, amendment with nitrate or nitrous oxide may allow denitrification-coupled degradation to occur [100]. The use of mixed oxygen–nitrate amendments is also possible. This approach may be useful in situations where aerobic biodegradation is present but limited. Following initial aerobic degradation, an alternate electron acceptor could be provided to allow complete biodegradation to occur. Hutchins [85] studied a mixed electron acceptor system for biodegradation of different monocyclic aromatic compounds by aquifer microorganisms. Microbial degradation was measured under 3 conditions: (i) limited-aerobic; (ii) denitrifying (with either nitrate or nitrate oxide); and (iii) limited-aerobic but supplemented with nitrate. The latter condition improved biodegradation of benzene, which was found to be recalcitrant under either limited-aerobic or denitrifying conditions.

In summary, environmental conditions affect biodegradation at two levels; through their influence on microbial numbers and activity, and their influence on the physical/chemical properties of the pollutants. The effect of various environmental parameters can be interactive, rendering a predictive model difficult. Optimization of environmental conditions is crucial to any bioremediation effort.

LIMITED AVAILABILITY OF CONTAMINANT

Low aqueous solubility

Limited availability of many environmental pollutants to microorganisms is a major factor that affects biodegradation. Even if the capacity to degrade is present and environmental conditions are adequate, inability of microorganisms to acquire target compounds limits degradation.

There is some experimental evidence to indicate that low aqueous solubility may limit growth on and biodegradation of hydrocarbons. For example, Chen et al. [31] noted that the degree of anaerobic degradation of polychlorinated biphenyl congeners in sediment samples declined with decreasing solubilities of the congeners. However, lower solubility correlates with increased chlorination of PCBs, which in turn correlates with increased recalcitrance. In a study of the biodegradation of several hydrocarbons, Thomas et al. [167] found the growth rate of an enrichment culture depended on the amount of dissolved naphthalene or 4-chlorobiphenyl present. In an earlier study, growth rates of *Nocardia* and *Pseudomonas* strains on selected aromatic hydrocarbons such as anthracene, phenanthrene and naphthalene were related to aqueous solubilities of these hydrocarbons [194]. Hydrocarbons with lower aqueous solubilities supported slower growth. In subsequent studies, naphthalene and bibenzyl [192], and phenanthrene [193] were found to be utilized only in their dissolved forms. In these studies, bacterial growth was independent of the amount of solid hydrocarbons present. However, some researchers found the amount of solid hydrocarbon influences the growth and biodegradation rates. For example, Keuth and Rehm [95] studied the growth on and mineralization of phenanthrene by *Arthrobacter polychromogenes*. They found that increasing phenanthrene crystal concentration from 75 to 300 mg L^{-1} led to 20% and 100% increase in rates of growth and specific mineralization, respectively. They surmised the diffusion time between phenanthrene and degrading microorganism decreased due to the larger surface area encountered as the amount of phenanthrene crystals increased.

The spontaneous dissolution rate of hydrocarbons has been suggested as one factor that limits their biodegradation. Stucki and Alexander [164] found the growth of *Flavobacterium* and *Beijerinckia* sp. on phenanthrene was dependent on the spontaneous dissolution rate of this compound. However, this is not always the case with all water insoluble substrates. For example, with octadecane, the biodegradation rate by a mixed bacterial culture in a liquid medium was 200 times faster than the spontaneous dissolution rate [167]. These researchers suggested factors other than dissolution rate may be limiting. One possibility is that substrates may not be adequately emulsified. This results in lower surface areas for contact with microbial cells. However, microorganisms that adhere to hydrophobic compounds may solubilize them, thus allowing cellular uptake and degradation.

One approach to improve uptake and possibly biodegradation is to increase solubilization of substrates. In a laboratory study, Guerin and Jones [70] observed increased biodegradation of phenanthrene by a *Mycobacterium* sp. in the presence of synthetic surfactants such as Tween compounds. Improved mineralization was also seen using synthetic emulsifiers such as β-cyclodextrin with a co-culture of *Arthrobacter* and *Pseudomonas* strains for 4-chlorobiphenyl [80], and sodium ligninsulfonate with *Pseudomonas* sp. 7509 for PCB mixtures [105].

Synthetic dispersants have been used with variable results in marine environments for cleanup of petroleum spills. Some synthetic dispersants were toxic to microorganisms to varying degrees and their presence often decreased biological degradation of petroleum [103,171]. The reader is referred to Trett et al. [171] and Leahy and Colwell [103] for more information. In laboratory studies, Laha and Luthy [101, 102] observed a similar effect. These investigators demonstrated that phenanthrene mineralization decreased when various synthetic non-ionic surfactants (an alkylethoxylate, two different alkylphenol ethoxylates, two Tween compounds, CHAPS, and octylglucoside) were added to soil slurry systems. This inhibition occurred when surfactants were present at concentrations above their critical micelle concentration (CMC). The authors suggested that the cause for the inhibition was a limited amount of available phenanthrene because of strong partitioning into surfactant micelles [102]. When surfactants were diluted to sub-CMC concentrations, inhibition of mineralization was reversed [101]. However, the mineralization rate was not improved. This suggests that biodegradation was limited by partitioning of phenanthrene into micelles, rather than toxic effects

exerted by the surfactants. Availability and uptake by microbial cells may be decreased even though solubilization increased [101]. In contrast, other studies have found that some non-ionic surfactants (Alfonic 810-60, Novel II 1412-56) can improve the mineralization of phenanthrene and biphenyl in soil slurries by both inoculated and indigenous degrading organisms [14,15].

Several researchers have investigated the use of microbially produced emulsifiers and surfactants to enhance biodegradation of hydrophobic compounds. One advantage of bioemulsifiers or biosurfactants is their biodegradability, lower toxicity, and possibly greater effectiveness in some applications [144,180]. Oberbremer et al. [134] studied the effect of different biosurfactants on biodegradation of a hydrocarbon mixture containing tetradecane, pentadecane, hexadecane, 1,2,4-trimethylcyclohexane, pristane, phenyldecane, and naphthalene in a soil slurry. Under oxygen limited conditions, the addition of several glycolipid surfactants led to 45–140% increase in hydrocarbon degradation, with sophorose lipids providing the greatest enhancement. Jain et al. [89] observed increased biodegradation of tetradecane, hexadecane and pristane in soil samples amended with partially purified biosurfactants produced by *P. aeruginosa* UG2. Zhang and Miller [197] found that increasing amounts of *P. aeruginosa* ATCC 9027 rhamnolipid biosurfactant improved dispersion and mineralization of octadecane in a mineral salts medium. In contrast, crude oil mineralization by pure and mixed cultures generally decreased following pretreatment with the bioemulsifier emulsan produced by *Acinetobacter calcoaceticus* RAG-1 [52]. These researchers suggested several reasons for this effect, including inhibition of microbial adhesion to emulsified oil or masking of oil by emulsan, which may prevent solubilization by microbially produced surfactants. Also, Falatko and Novak [47] noted either an inhibition or no improvement of petroleum hydrocarbon biodegradation following addition of biosurfactants, depending on carbon sources used during biosurfactant production. Biosurfactants produced in a glucose–vegetable oil medium proved inhibitory to biodegradation while biosurfactants produced in a gasoline medium were not.

In laboratory studies, both biological and chemical surfactants have been used with success to wash hydrocarbons from soil in slurry or column systems. The biosurfactants produced by *P. aeruginosa* UG2 have been shown to increase the partitioning of several structurally distinct hydrophobic compounds, including two PCBs, into the aqueous phase in soil slurries [18,179]. The glycolipid biosurfactant produced by *P. aeruginosa* SB30 improved removal of weathered oil from gravel contaminated by the Exxon-Valdez spill [73]. The biosurfactants produced in activated sludge amended with either a glucose–vegetable oil mixture or gasoline were also effective at removing toluene, *m*-xylene, 1,2,4-trimethylbenzene, and naphthalene from sand held in columns [47]. In other studies, biodegradable synthetic surfactants (for example, ethoxylated alcohols, ethoxylated nonylphenols, anionic sulfates, anionic sulfonates, Tween compounds, and SDS) have been used to remove various hydrophobic contaminants from soil held in columns [1,2, 12,43,47,86] and soil slurries [14,15,101,102]. The use of surfactants, in particular biosurfactants, may be a promising method with which to wash water-insoluble pollutants ex situ from soil. However, their use in situ may cause leaching of hydrophobic pollutants into the ground water by increased mobilization. Surfactants and their possible applications in bioremediation are also discussed in a later section.

Uptake limitations

The efficiency of pollutant and nutrient uptake and subsequent growth of microorganisms depends on the state of the nutrient sources. For hydrophobic compounds, uptake can be enhanced if they are available in a dissolved, solubilized, or emulsified state. However, little is known about how recalcitrant hydrocarbons are taken up by microorganisms. It is presumed that these compounds may enter cells through passive diffusion, facilitated diffusion, and/or active transport mechanisms. Closely related to limitations of low aqueous solubility and inadequate transport is the molecular weight(s) of compound(s). Depending on arrangement of atoms, a large molecule may not easily traverse cellular membranes. More research on uptake mechanisms of hydrophobic, xenobiotic compounds is necessary.

Whether xenobiotic compounds enter passively, by facilitated diffusion, or by active transport merits attention. Problems at this level would be limiting if the degradative enzymes were located intracellularly [118]. Little information exists on cellular location of numerous catabolic enzymes. Elucidation of degradative mechanisms at subcellular and molecular levels will be valuable in biodegradation studies. For example, in a study of the biodegradation of several isomeric aminobenzene sulfonates by *Alcaligenes* sp. strain 0–1, Thurnheer et al. [168] suggested the existence of a selective permeability barrier at the cell membrane to some of these compounds. This was shown by the ability of intact cells previously induced on benzene sulfonate to utilize 2- but not 3- and 4-aminobenzene sulfonates, even though all these compounds can be desulfonated by cell extracts. It was concluded that transport of these compounds into microbial cells was the initial step in their degradation.

There is some experimental evidence that insufficient transport can limit initial steps of biodegradation of some xenobiotic compounds. For example, hydrolytic dechlorination, the first step in biodegradation of 4-chlorobenzoic acid (4-CBA) by coryneform bacterium NTB-1, previously identified as *Alcaligenes denitrificans* [68], did not occur under anaerobic conditions [177], even though cell extracts contained dehalogenating activity [68]. Groenewegen et al. [67] reported subsequently that negligible uptake of 4-CBA by strain NTB-1 under these conditions was the reason for the inability of this strain to dechlorinate 4-CBA. Uptake of 4-CBA was shown to be induced by 4-CBA and dependent on energy generated by the proton motive force. Under anaerobic conditions, 4-CBA transport activity resumed if an alternative electron acceptor such as nitrate was provided [67,68].

Miller and Bartha [118] have also suggested that assimi-

lation of recalcitrant hydrocarbons may be transport-limited. These researchers used lecithin-liposomes to deliver the solid alkane octadecane to *Pseudomonas* sp. The microorganism took up about 18-fold more octadecane over a 1-h incubation period when it was liposome-carried compared to when unencapsulated. However, their study did not provide evidence for transmembrane transport as a limiting factor in degradation. Octadecane was believed to partition into the lipid bilayer and not into the aqueous centre of liposomes [118]. Following fusion between cells and vesicles, substrates were not released directly into the cytoplasm but remained inserted in the membranes. Presumably, alkanes can diffuse out of membranes into the cytoplasm. If the degradative enzymes are located in the cytoplasm, substrates would be metabolized. However, this system may be an excellent method for studying possible transport limitations of hydrophilic xenobiotic compounds which will likely associate with aqueous regions of vesicles. Following fusion, these compounds will be delivered directly to the cytoplasm.

Sorption to soil and sediment matter

Uptake and biodegradation of many pollutants can be limited by sorption of compounds to soil or sediment components. Sorption binds contaminants and removes them from the dissolved state [44]. If compounds are strongly sorbed, they may be unavailable to microorganisms, thereby limiting their biodegradation. Therefore, the fate of contaminants in soils and sediments is influenced by the competing processes of biodegradation and sorption [69]. It is noteworthy that sorption refers to both adsorption and absorption. Adsorption occurs on surfaces (for example, between a charged compound and clay), while absorption describes sorption beyond the surface into a separate portion defined by the surface (for example, partitioning into organic matter) [44].

Many studies on factors affecting xenobiotic sorption to soils and sediments have focused on pesticides. Some researchers have also examined factors affecting sorption of other organic contaminants [92–94]. Sorption of PCBs has been studied in detail [35,86,133], as has that of polycyclic aromatic hydrocarbons (PAHs) [41,114]. In general, soil sorption of neutral, hydrophobic compounds is dependent on soil and sediment organic content, as measured by the amount of carbon present [44]. A useful parameter for describing sorption of chemicals to soil and sediments is its organic carbon partition coefficient (K_{oc}), which is correlated to its octanol–water partition coefficient (K_{ow}) [44,92–94]. For more specific K_{oc} data of organic contaminants, refer to Karickhoff [92–94], Paya-Perez et al. [140], and Means et al. [114]. Some factors affecting the degree of xenobiotic sorption are summarized in Table 2.

Sorption was reported to protect 2,4-dichlorophenoxyacetate (2,4-D) [135,66] and 1,2-dibromoethane (1,2-EDB) [163] from biodegradation in soil samples. Weissenfels et al. [186] found that sorption of several PAHs decreased the amount biodegraded in soil samples from contaminated sites. Also, Mihelcic and Luthy [117] observed that the desorption rate was one factor affecting biodegradation of naphthalene and acenaphthalene in a soil–water system. Al-Bashir et al. [6] reported sorption of naphthalene was highly irreversible, depending on soil organic content. The desorption rate also controlled mineralization in a flooded soil system under denitrifying conditions. Manilal and Alexander [112] suggested a similar correlation for aerobic mineralization of phenanthrene in soil; sorption increased as the percentage of organic matter increased and biodegradation consequently decreased. For example, for 49% of the phenanthrene to be mineralized, 11 days elapsed in soil containing 6.9% organic matter versus 4 days in a mineral salts medium. However, sorption can mitigate some toxic effects of pollutants in soils [103,186] and in bioreactors [133] by decreasing bioavailable concentrations.

Hydrophobic pollutant desorption may be enhanced if soil is washed with surfactants. Many researchers have shown enhanced mobilization of various organic compounds from soil columns and slurries using both microbially produced [18,47,73,179] and synthetic surfactants [1,2,14,15,43,86,101, 102]. Several studies have investigated the effect of various synthetic surfactants not only on contaminant desorption from soil but also on microbial mineralization. In one set [14,15], two different non-ionic alcohol ethoxylate surfactants at relatively low sub-CMC concentrations (0.01–100 μg surfactant ml^{-1} solution for desorption studies, 10–100 $\mu g\ g^{-1}$ air-dried soil for mineralization studies), desorption and mineralization of phenanthrene and biphenyl were studied in both mineral and organic soils. In the mineral soil, desorption of both compounds was found to be variable depending on the type and concentrations of the surfactants used. For example, about 18–20% of the added phenanthrene was desorbed by 100 $\mu g\ ml^{-1}$ Alfonic 810–60. At 0.1–10 $\mu g\ ml^{-1}$ concentrations, the amount desorbed was about 10%, similar to that without the surfactant. However, at 0.01 $\mu g\ ml^{-1}$, this surfactant decreased desorption to only 5%. A similar trend was found with the detergent Novel II 1412–56. Phenanthrene mineralization was enhanced about 2-fold by 10 $\mu g\ g^{-1}$ of Alfonic 810–60, but inhibited about 70% by 100 $\mu g\ g^{-1}$ of this detergent. In the organic soil, desorption was not improved for either compound but mineralization was generally enhanced by the surfactants depending on concentrations used. Similar results were obtained in another study investigating the effect of these two surfactants on the desorption and mineralization of the two model contaminants in aquifer sand and silt loam slurries [15]. The mechanism by which the surfactants improved microbial mineralization without increasing desorption is not known [14]. One possibility is that surfactants adsorbed to surfaces of degrading microorganisms, promoted microbial sorption to soils, and subsequently enhanced biodegradation [113]. In contrast, Laha and Luthy [101,102] found that various nonionic surfactants enhanced phenanthrene desorption from soil slurries but inhibited phenanthrene mineralization by inoculated organisms.

It is noteworthy that in most of the desorption studies cited, soils were spiked with contaminants and desorption from soils was tested immediately after spiking. The ease with which hydrocarbons can be removed from soils may

TABLE 2

Parameters affecting sorption of contaminants to soil and sediments

Parameter	Effect on sorption[a]
Soil type	
organic matter	sorption increasing wih increasing organic matter content
clay	sorption increasing with increasing clay content
sand	sorption decreasing with increasing sand content
Soil properties	
surface area and cation exchange capacity (CEC)	the greater the surface area and CEC, the greater the adsorption
Environmental parameters	
temperature	sorption decreasing with increasing temperature
soil moisture	sorption decreasing with increasing hydration
pH	sorption can vary with pH, often showing a plateau within a certain range
Nature of contaminant	sorption increasing with increasing hydrophobicity

[a] It is noteworthy that these are general effects. PCB sorption, for example, is independent of temperature [94].

change with ageing of soil samples [88]. A study using aged soils would mimic recovery from and possibly remediation of soils polluted for years to decades. Harvey et al. [73] used gravel samples which had been contaminated with oil for 4–5 months before washing with biosurfactants produced by *P. aeruginosa* SB30. The best enhancement in oil removal (2–3-fold greater than water control) occurred with a 1–2 min contact time at 60 °C using 1% (w/v) biosurfactant solution. However, the results obtained using gravel may be different from those using soil samples composed of sand, silt, and clay particles as well as organic matter. It is also noteworthy that the mode of application of hydrocarbons to soil or sediment samples may affect their sorption and recovery when using surfactants. For example, hydrocarbons may be evenly distributed throughout the samples or applied to the surface of the samples. In the latter case, a gradient may be created. There is a paucity of knowledge on how hydrocarbon distribution affects contaminant recovery when using surfactants.

Several problems encountered with surfactant washing of contamined soil should be noted. First, surfactants can adsorb to soil components [1,47,108,176]. Second, some ionic surfactants may precipitate [87]. Third, surfactant micelles and dispersed soil hydrophobic matter can clog soil pores and prevent free movement of surfactants through matrices [12]. For surfactant washing of soil to be effective, these problems must be minimized. Another concern with surfactant washing in situ is the possibility of increased mobility and leaching of contaminants into groundwater [89, 179].

It should be noted that a certain fraction of many contaminants do not desorb easily from soil [35,143,174]. Also, some contaminants form covalent bonds with soil organic matter and are bound irreversibly [125]. These persistent fractions are recalcitrant and removal may not be possible.

Restricted microbial movement

Restricted microbial movement through soil has been suggested as a limiting factor in biodegradation [64]. Factors affecting microbial movement in the environment include soil type [53], water activity [139], water flow [173], ionic strength of carrying fluid [59], and cell number, cell size, and cell surface characteristics [53,58]. Restricted microbial movement can affect biodegradation of soil contaminants because substrates must be available and accessible either to microorganisms or their extracellular enzymes for metabolism to occur. Movement is important for microorganisms to reach interfaces where nutrients and other microorganisms aggregate [181]. For biodegradation of poorly soluble compounds to occur, close contact between cells and pollutants may be necessary. For example, Rosenberg and Rosenberg [152] showed that at low cell densities, adhesion of *A. calcoaceticus* RAG-1 to hexadecane was necessary for growth to occur. Foght and Westlake [51] also demonstrated a role of bacterial adherence in the metabolism of several PAHs and aromatic heterocyclics on solid media.

Microorganisms in soil are not easily dispersed and cannot move from one site of contamination to another. Frequent tilling and mixing of soils, used often to increase aeration, will aid in the spread of degrading microorganisms. Loading of soil with water will also increase microbial movement. However, the danger of contaminant leaching should not be overlooked with this approach. Moreover, aeration of soil

may be decreased. Surfactants can increase solubilization and mobilization of insoluble pollutants, as discussed above. Substrates may enter the aqueous phase and become more available to microorganisms.

METABOLIC LIMITATIONS

Biodegradation in the environment is limited by metabolic barriers not encountered in, or predicted by, controlled experiments in the laboratory. Assuming environmental conditions are adequate and contaminants are accessible, several metabolic barriers may limit degradation.

Structural constraints

One barrier to biological degradation is inability of degradative enzymes to catabolize target xenobiotic compounds due to structural constraints. Some compounds, because of substitution patterns, are not amenable to microbial degradation and can persist in the environment. The chlorine substitution pattern as a limiting factor in biodegradation of halogenated organic compounds has been reviewed by Neilson [128]. In general, the greater the degree of chlorination, the greater the resistance to aerobic degradation. On the other hand, anaerobic dechlorination occurs readily for highly chlorinated compounds [128], probably because reductive dechlorination yields energy for growth. The position of the chlorine substituent in aromatic compounds may affect degradation more so than the numbers of chlorines [128]. Structure–biodegradability relationships have been described for polycyclic aromatic hydrocarbons (PAHs). For example, Bossert and Bartha [21] observed that chemicals possessing four or more fused rings were resistant to microbial degradation and tended to persist.

Several approaches have been proposed for improving biodegradation of some structurally recalcitrant pollutants. Some researchers [3,49,145] have suggested the use of alternating anaerobic–aerobic cycles to enhance biodegradation of PCBs and hexachlorobenzene. The initial anaerobic phase allows reductive dechlorination of highly chlorinated compounds to occur, thus removing inhibitory chlorine substituents. The less chlorinated molecule is more readily mineralized aerobically. This approach may be useful also with other chlorinated compounds. Another approach suggested for degradation recalcitrant hydrocarbons, such as benzo[*a*]pyrene (BaP) and other large PAHs, is a photolytic pretreatment of these compounds followed by biodegradation [119]. Photolysis converted BaP to polar materials which were more amenable to mineralization. Several recently isolated microorganisms have been shown to possess the ability to degrade high molecular weight PAHs and may be useful in their biodegradation. For example, a *P. paucimobilis* strain isolated by Mueller et al. [127] and an *A. denitrificans* strain isolated by Weissenfels et al. [185] are able to utilize fluoranthene as sole sources of carbon and energy. A *Mycobacterium* sp. isolated by Heitkamp et al. [76] cometabolizes pyrene in the presence of alternate organic carbon sources such as peptone, yeast extract, or starch [75].

Many other structurally recalcitrant compounds will be difficult to degrade biologically unless microorganisms are isolated or genetically-engineered with novel degradative capabilities. Physical or chemical methods may be required for their destruction.

Cometabolic requirements

One metabolic barrier to microbial degradation is lack of catabolic enzyme induction. Insufficient induction may result from a cometabolic requirement by some degraders. Cometabolism has been defined as the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound [33]. Cometabolism and its relationship to biodegradation of xenobiotic substances have been reviewed by Janke and Fritsche [90]. Cometabolites are believed to supply energy and reducing equivalents which support microbial growth and allow degradation of non-growth substrates [90]. The cometabolite may also induce production of catabolic enzymes which recognize contaminants and catalyze their transformation.

There are numerous reports of a cometabolic requirement for biodegradation. The reader is referred to the review by Janke and Fritsch [90] for examples; some will be mentioned here. Aerobic degradation of PCB may require a cometabolite. In laboratory studies, biphenyl [5], 4-chlorobiphenyl [57], benzoate, 2-chlorobiphenyl or 4-chlorobiphenyl [138] have been used as cometabolites by various microorganisms. Aerobic degradation of persistent chlorinated phenols (CP) like 3,5-DCP, 3,4,5-TCP, and 2,3,4,5-TeCP requires PCP as a cometabolite [107]. Aerobic degradation of 1- and 2-chloronaphthalene requires naphthalene [126]. Aerobic degradation of high molecular weight PAHs such as dibenzothiophene, pyrene, and fluoranthene requires an alternate carbon source such as glucose [51,75,185]. Aerobic degradation of one and two carbon chlorinated aliphatics has been shown to occur with cometabolic oxidation of methane [104,136,171], propane [50,183], formate [136], phenol or toluene [129,130], and ammonia [13].

One approach to satisfy a cometabolic requirement is to supply the cometabolite. In laboratory studies, biphenyl amendment was one of the most useful ways to enhance PCB mineralization in aerobic soil samples [26] and PCB degradation in anaerobic sediment samples [149]. Aerobic degradation of chlorinated biphenyl in a liquid medium was shown to occur with benzoate as the cometabolite [138]. Amendment with benzoate also allowed cometabolic degradation of the resulting CBAs which often accumulate during PCB degradation [138]. In the open environment, enhancement of PCB biodegradation by these methods may not be suitable since the cometabolites are themselves toxic. This is also the case with cometabolites needed for degradation of chlorinated naphthalene [126] and persistent chlorinated phenols [107]. For degradation of chlorinated aliphatic hydrocarbons, methane can be supplied. Semprini et al. [158] showed a 20–30% enhancement of trichloroethylene (TCE) biodegradation following methane and oxygen injection into test plots contaminated with the pollutant. Transformation of less chlorinated ethenes was also stimulated, with the percent transformed increasing as the degree of

chlorination decreased. Propane and formate could also be supplied to stimulate biodegradation of chlorinated aliphatics. However, Henry and Grbić-Galić [78] cautioned that large scale application of formate may be expensive. Also, continued addition of formate may select for non-methanotrophic microorganisms able to grow on formate but unable to degrade chlorinated aliphatic hydrocarbons.

One approach to overcome the requirement of cometabolites may be to select for mutants whose expression of the degradative enzymes is constitutive or, at least, inducible without the obligate presence of a possibly toxic cometabolite. Growth substrates will still have to be present since degradation of the contaminant may not provide energy to the microorganism. For example, Mondello [123] constructed a recombinant *Escherichia coli*, designated FM 4560, with the biphenyl degrading genes isolated from *Pseudomonas* strain LB400. This recombinant microorganism was able to degrade a variety of PCBs to CBAs and BAs without the obligate presence of biphenyl. When succinate was provided as a carbon source, the recombinant *E. coli* was superior to LB400 in degrading PCB mixtures. Using a similar approach, Winter et al. [191] constructed 2 recombinant *E. coli* strains containing the toluene monooxygenase genes from *P. mendocina* KR-1. Strain FM5 contains the recombinant plasmid under the control of the temperature sensitive P_L promoter while strain HB101 carries the plasmid under the *tac* promoter. Using radiolabelled substrate, TCE mineralization was readily demonstrated by both *E. coli* strains without the obligate presence of toluene as the growth substrate.

Cometabolic degradation of PCBs, chlorinated phenols, and chlorinated aliphatics in large scale fermenters and reactors has been investigated [4,11,107,141,142]. These studies suggest another approach to enhance biodegradation of compounds whose mineralization may require toxic cometabolites. Of particular interest is the two-stage, dispersed-growth bioreactor proposed by Alvarez-Cohen and McCarty [11] for the biodegradation of TCE and other chlorinated aliphatic compounds. In this system using a mixed methanotrophic culture, cells are first fed CH_4 in a growth chamber to induce production of methane monooxygenase activity and to accumulate endogenous reductant supplies. The cells are then transferred to another vessel where biodegradation of the chlorinated aliphatic contaminants occurs in the absence of methane. Cells were able to degrade repeated doses of TCE. However, they lose this degrading activity over time [9]. When cells are no longer able to degrade the contaminants, they are discarded. This two-stage system alleviates several potentially limiting factors which may be encountered during TCE biodegradation in single-chamber reactors. By using pre-induced cells, it avoids competitive inhibition between the growth substrate (CH_4) and the cometabolite (TCE), both of which are metabolized by methane monooxygenase (MMO). By continuously replacing non-degrading cells, degradation of contaminants is maintained. Factors affecting the degradation ability of cells include conditions under which they are stored or handled preceding biodegradation, initial cell density at the start of biodegradation, amount of exogenous reducing compound (in the form of formate) supplied during biodegradation, and the production of metabolites toxic to the microorganisms during TCE biodegradation [9]. Product toxicity as a limiting factor during the biodegradation of chlorinated aliphatic compounds is discussed later in this review.

Preferential metabolism of alternate carbon sources

Preferential metabolism of alternate carbon sources by degrading organisms may limit biodegradation of some contaminants. For example, Swindoll et al. [166] observed that addition of easily degradable carbon sources, such as amino acids or glucose, inhibited aerobic mineralization of ethylene dibromide, *p*-nitrophenol, phenol, and toluene in subsurface soil samples. Mihelcic and Luthey [116] found that under denitrifying conditions biodegradation of naphthalene and acenaphthene in a soil slurry system was inhibited because of the presence of easily available, naturally occurring organic carbon.

However, alternate carbon sources may not necessarily inhibit contaminant degradation. They may have no effect [96,136] or may improve biodegradation depending on culture conditions. For example, PCP degradation by mixed microorganisms in an anaerobic, fixed-film reactor was enhanced by supplementation with 1 g L^{-1} glucose [77]. In another study, addition of 0.1 g L^{-1} glucose decreased the acclimation period before onset of PCP biodegradation by *Flavobacterium* sp. under various nutrient limitations [169]. The acclimation period preceding aerobic degradation of PCP by *Flavobacterium* sp. was shortened also by addition of glutamate [65,170]. The reason for this effect is not known, although these alternate carbon sources were thought to not only stimulate growth of PCP degrading organisms, but also help mitigate toxic effects of PCP at high concentrations [65,170]. In another study, glucose at low concentrations (0.45 g L^{-1}) was shown to enhance phenanthrene mineralization in batch culture of *A. polychromogenes* but to inhibit at higher concentrations (3 g L^{-1}) [95].

Little can be done to improve biodegradation of xenobiotic compounds in soil and aquatic environments if preferential metabolism of alternate carbon sources occurs. However, in a controlled environment (e.g. a bioreactor), preferential metabolism may be minimized by varying the concentrations of alternate carbon sources so as not to limit biodegradation.

Inhibition of metabolism

Another metabolic barrier to contaminant biodegradation is inhibition of mineralization. This can be caused by some chemical(s) or toxin(s) already present in the environment or produced by microorganisms.

Inhibition of degradation can be caused by the contaminants being present at toxic levels. For example, at 500 ppm, PCP mineralization was suppressed in soil samples inoculated with *Flavobacterium* sp. [32]. However, at 100, 50, and 10 ppm, 60–70% of added PCP was mineralized within one week, although initial mineralization rates decreased with increasing PCP concentration. These authors suggested that inhibition due to toxic substrate concentrations can be alleviated by first leaching a heavily contaminated soil site,

followed by biodegrading the leachate. An alternative is to select for degrading microorganisms with greater tolerance to high levels of contaminants.

Inhibition of biodegradation can also occur due to production of toxic compounds during metabolism of the target contaminant(s). Some toxic compounds may be partial degradation products that accumulate and are more toxic than the parent compound(s). The toxic compounds may be lethal not only to the degrading organism but to other microorganisms as well. Studies by Liu et al. [106] and Ruckdeschel et al. [154] showed that some partial degradation products of 2,4-dinitrotoluene (2,4-DNP) and PCP, respectively, are more toxic to different bacterial species than parent substrates. Short et al. [159] found that inoculation of 2,4-dichlorophenoxyacetate (2,4-D) amended nonsterile soils with recombinant, 2,4-D degrading *P. putida* PPO301 cells resulted in accumulation of the more toxic metabolite 2,4-dichlorophenol (2,4-DCP). Concentration of 2,4-D (initially at 600 ppm) decreased rapidly by more than 50% in the first 6 days and slowly thereafter such that about 90% was degraded after 50 days. On the other hand, concentration of 2,4-DCP increased slowly in the first 6 days to 20 ppm, then increased quickly to 70 ppm by day 10 and remained relatively unchanged for 30 days before declining to about 40 ppm at day 50. Fungal colony-forming units declined rapidly after day 6 to non-detectable levels by day 18, a time course which coincides with the rapid increase in 2,4-DCP levels. It was noted also in plate assays that 10–25 $\mu g\ ml^{-1}$ 2,4-DCP inhibited growth of several fungal strains.

Metabolite inhibition was suggested by Haugland et al. [74] as the cause for the diminished ability of a two-member bacterial consortium to degrade a mixture of 2,4-D and 2,4,5-trichlorophenoxyacetate (2,4,5-T). The coculture consisted of a 2,4-D degrading *A. eutrophus* JMP134 and a 2,4,5-T degrading *P. cepacia* AC1100 strains. When supplied individually to the coculture, both 2,4-D and 2,4,5-T were completely degraded within 24 h. However, only about 50% of each compound was degraded by the coculture over 24 h when supplied as a mixture. The 2,4,5-T degrading *P. cepacia* AC1100 metabolized 2,4-D incompletely and the accumulated partial degradation products were postulated to affect the viability and degrading ability of the coculture.

In another study, Zhang and Wiegel [198] found that anaerobic degradation of 2,4-dichlorophenol (2,4-DCP) by a six-member community could be inhibited by 4-chlorophenol (4-CP), a toxic metabolic intermediate in 2,4-DCP degradation. 4-CP can accumulate because its reductive dechlorination to phenol was the rate-limiting step in the pathway. Degradation of 2,4-DCP and other intermediates such as phenol and benzoate was found to be completely inhibited by 4-CP at 4.5, 1.5, and 3 mM, respectively.

The biodegradation of chlorinated aliphatic compounds, in particular TCE, can be subject to metabolite toxicity. Cometabolism of TCE by the ammonium utilizing *Nitrosomonas europaea* [148], methane utilizing *Methylosinus trichosporium* OB3b [137], mixed methanotrophic cultures [9,10,78], propane utilizing *Myobacterium vaccae* [183], and toluene degrading *P. putida* F1 [182] may result in formation of products toxic to the degrading microorganisms. Toxicity was demonstrated by loss of cell viability and decrease in metabolism of the respective growth substrates. Rasche et al. [148], Wackett and Householder [182], and Oldenhuis et al. [137] showed that some cellular proteins were covalently modified by reactive TCE transformation products. This may inactivate enzymes and decrease cell viability. Epoxides and/or acyl chlorides have been suggested as the reactive intermediates responsible for protein modification [137, 148,182]. Covalent modification of enzymes specifically responsible for initial transformation of the chlorinated hydrocarbons was suggested for ammonia mono-oxygenase [148] and demonstrated for soluble methane mono-oxygenase [137]. De novo synthesis of proteins reversed the inactivation by TCE cometabolism [137,148]. Carbon monoxide, another transformation intermediate of TCE cometabolism, was shown by Henry and Grbić-Galić [79] to inhibit aerobic degradation of TCE by a *Methylomonas* sp.

One approach to overcome metabolite inhibition by accumulation of toxic partial degradation intermediates may be to use microbial consortia selected for the ability to degrade the metabolites. However, this approach may not necessarily be effective as shown in the studies of Zhang and Wiegel (1990) and Haugland et al. (1990), discussed above. Another approach is to clone genes responsible for degradation of toxic metabolite(s) into a suitable microbial host capable of degrading the parent substrate(s). Degradation may be more efficient since all metabolic reactions can be carried out by the recombinant microorganism. Moreover, potentially toxic metabolites are not released. For example, Haugland et al. [74] were able to overcome the problems encountered during the degradation of a 2,4-D and 2,4,5-T mixture by transferring the 2,4-D degradative plasmid of *A. eutrophus* JMP134 into 2,4,5-T degrading *P. cepacia* AC1100. The novel strain constructed, designated RHJ1, was capable of degrading both substrates, individually and when present together in a mixture.

Contaminant biodegradation may also be inhibited by the presence of toxic metals in soil and aquatic environments. The toxicity of numerous metals to microorganisms is well known. The mechanisms of metal toxicity may include interactions with protein sulfhydryl groups [120,161], with electron transport chains, inhibition of enzymes, binding to nucleic acids and membranes, and inhibition of cell division [83,162]. Their possible negative effect on contaminant biodegradation should not be overlooked. This is especially relevant for heavy metals as they are often present in toxic waste sites or industrial sewage [189,190]. In one study, Said and Lewis [155] investigated the effect of different heavy metals (Cu, Hg, Zn, Cd, and Cr) on aerobic biodegradation of 2,4-D methyl ester (2,4-DME) by aquatic microorganisms. Zn, Cu and Cd, at greater than 7.0, 30, and 100 μM, respectively, inhibited 2,4-DME degradation rate in sediment samples by more than 10% as well as increased the half-life of 2,4-DME by 10%. Higher concentrations of these metals (790 μM Zn, 170 μM Cu and 240 μM Cd) were needed to cause a doubling of the half-life of 2,4-DME.

Some metals may affect biodegradation by inhibiting the

synthesis of degradative enzymes. For example, in *M. trichosporium* OB3b, Cu at concentrations greater than 0.25 μM decreases synthesis of soluble methane monooxygenase, the enzyme responsible for biodegradation of chlorinated aliphatic compounds [175]. In addition to direct toxicity to degrading microorganisms, some metals may affect biodegradation by complexing (or chelating) contaminants. Madsen and Alexander [111] reported that mineralization of organic compounds such as oxalate, citrate, nitrilotriacetate and EDTA by both single or mixed bacterial cultures was affected when these compounds complexed to metals such as Ca, Mg, Fe, and Al. Brynhildsen and Rosswall [27] also observed that citrate mineralization by a *Klebsiella* sp. was decreased by chelation with metals such as Cd, Cu, Mg, and Zn. Chemical complexation to certain metals is common for organic compounds containing amino or carboxyl groups [111]. However, complexation to organic compounds may mitigate the toxic effects of some metals by lowering the amount available to organisms [83,155]. Some metals such as Ca and Fe may also affect contaminant biodegradation by decreasing the amount of phosphate available to microorganisms [150].

Toxic metal inhibition may be overcome by cloning genes responsible for microbial resistance to selected metals into degrading microorganisms. Spontaneous metal-resistant mutants may also be selected for in laboratory experiments. Some genes responsible for metal resistance are carried on plasmids or transposons [17,25,28,83,115,120,131,160,172] which may facilitate gene transfer between microorganisms or genetic engineering. Mechanisms by which bacteria resist metals include exclusion from the cell interior, binding of metal to intracellular structures, specific metal efflux pumps, and transformation of metals [17,25,28,83,115,120,131,160, 172].

The presence of some inorganic nutrients has also been shown to inhibit anaerobic biodegradation. For example, Gibson and Suflita [62] found that sulphate inhibited anaerobic reductive dechlorination of several chloroaromatics. The mechanism of inhibition by sulphate was not understood. However, inhibition may be alleviated by sulphate removal via dissimilatory sulphate reduction, a condition mimicked by flooding samples with acetate. In a subsequent study, these authors suggested that sulphate reduction competed with reductive dechlorination for available electrons [63].

Goldstein et al. [64] and Zaidi et al. [195] have suggested that some natural antibacterial compounds present in the environment may limit the success of inoculation to enhance biodegradation. Inhibition by natural antibacterial toxins may be overcome by selecting for bacteria able to tolerate these toxins [195].

Many industrial wastes and polluted sites contain mixtures of different organic and inorganic chemicals. Different contaminants, when present together, can interact and affect biodegradation. The simultaneous presence of different toxic organic compounds may inhibit biodegradation even though, individually, each compound can be degraded. One example was discussed above for the aerobic biodegradation of a 2, 4-D and 2,4,5-T mixture by a two member consortium [74]. Another example is provided by Alvarez and Vogel [8] who investigated substrate interactions during aerobic biodegradation of benzene, toluene, and *p*-xylene by mixed and pure cultures. In a mixed culture, *p*-xylene increased the lag phase before benzene and/or toluene degradation began; however, the degradation rate of benzene was unaffected while that of toluene was decreased. In contrast, degradation rate of *p*-xylene was improved in the presence of toluene. Benzene and toluene, when present together, did not affect each other's degradation. Results with pure cultures of *Pseudomonas* sp. strain CFS-215 or with *Arthrobacter* sp. strain HCB were similar.

OTHER LIMITING FACTORS

Low concentration of contaminants

Another limiting factor to biodegradation may be the low concentrations of contaminant(s) in the environment. The contaminant concentrations that are of interest are the amounts that are bioavailable. Even if the total amount of contaminants present is high, they may not necessarily be available to degraders. For example, in aqueous environments, poor solubility of contaminants may contribute to low bioavailable concentrations. In soils or sediments, sorption of contaminants can decrease the amount of contaminants available to degraders.

Research by Alexander's group has shown that the mineralization rates of many organic compounds at low concentrations (e.g. pg to ng ml^{-1}) cannot be extrapolated from rates obtained at higher concentrations (e.g. $\mu g\ ml^{-1}$) [19,20,82,153,184]. Threshold concentrations necessary for biodegradation depended upon the contaminants as well as the nature of the degrading microorganisms (i.e. whether they were of oligo-, meso-, or eutrophic origin) [20]. Also, certain compounds (benzoate, phenylacetate, 2,4-D) are transformed at higher concentrations (ppm range) but mineralized with little assimilation of the carbon by the organisms, at lower concentrations [82,153,184]. The reader is referred to a review article by Alexander [7] for a more thorough discussion of this topic.

Several studies suggested low contaminant concentration as one factor affecting biodegradation. Goldstein et al. [64] and Zaidi et al. [196] suggested low concentrations as one likely reason for failure of inoculation with degrading microorganisms to enhance mineralization of phenolic compounds in soil and water samples. Wiggins and Alexander [187] also suggested low contaminant concentration as one factor lengthening the acclimation period before mineralization of different xenobiotic compounds by indigenous microbial communities. Studies by Zaidi et al. [196] also showed that experimental conditions used to enrich for degrading microorganisms (e.g. high substrate concentration and pH 7) may select for species unable to metabolize the target compound under conditions which differ from those used for enrichment. Therefore, when enriching for contaminant degrading microorganisms in select environments, it

may be advantageous to use conditions that mimic those found in the targeted environment.

Little can be done to overcome low contaminant as a limitation if the total amount present is very low. Some method of concentrating the contaminant may be useful in this regard. In situations where contaminant concentrations are high but not bioavailable because of low solubility or strong sorption, surfactants may be used to increase solubilization and possibly the bioavailability of contaminants.

Protozoan predation

Biodegradation and, more specifically, inocula added to soil can be affected by protozoan predation. Protozoa, which actively graze on bacteria in the environment [45], may reduce the population of degraders to a level where mineralization is adversely affected. Predation was suggested by Goldstein et al. [64], Zaidi et al. [195], and Ramadan et al. [146] as one reason for failure of inoculation with degrading microorganisms to enhance biodegradation. Wiggins et al. [188] suggested predation as one factor that lengthened the acclimation period preceding contaminant mineralization in aquatic environments. The effect of predation may be overcome by providing conditions for optimal growth of degrading microorganisms. For biodegradation to occur at a suitable rate, a balance must be reached between microbial growth and protozoan predation. When using indigenous microorganisms for biodegradation, it is possible to stimulate microbial growth by nutrient amendments or by providing optimal environmental conditions. When using non-indigenous microorganisms, a large inoculum size may play a role in overcoming predation. The microbial population added must be sufficiently large to ensure survival from protozoan predation [146] and assure an adequate rate of degradation. Inoculum size is discussed later.

Genetic exchange

Exchange of genetic material has been suggested as a factor affecting the acquisition of biodegradative capabilities in nature [115]. Many genes encoding for degradative enzymes are found on plasmids [29,72,115,156]. Boyle [24] and Seech and Trevors [157] have reviewed factors that affect interbacterial exchange of genes in the environment and how this exchange relates to the evolution of degradative capabilities. Plasmid transfer can occur in non-sterile soils [157] and the frequency of this event often depends on the size and ratio of donor and recipient cells. This ratio is dependent on interacting biological, physical and chemical factors which affect microbial growth, survival and activity [24,157]. Plasmid acquisition has been shown to increase the degradative capabilities of bacteria towards several chlorinated, organic compounds [24]. Plasmids can mobilize both plasmid and chromosomal genes, thereby increasing their distribution in the environment. The reader is referred to these two reviews for more details on the topic. Some authors have suggested that the ability of different bacterial strains to degrade PCBs [56] and haloalkanes [178] was acquired through horizontal genetic transfer.

Natural genetic exchange and genetic engineering are useful tools for creating recombinant microorganisms with broader degradative capabilities [61,88]. Genetic engineering may also eliminate the need for consortia since all the metabolic steps in the degradative pathway of a contaminant are combined into one microorganism. This topic has been covered by Sayler et al. [156] and Rojo et al. [151], and the reader is referred to their reviews for a discussion of potential problems with this approach. However, little is known about the expression of recombinant genes when microorganisms are placed in an environmental matrix such as soil. This area requires additional research, especially if recombinant organisms are to be safely and successfully used in environmental applications.

Inoculum size and physiological status

The density of degrading microorganisms can play a role in the rate and extent of biodegradation. Chen and Alexander [30] and Wiggins et al. [188] showed that the length of time required before 2,4-D and *p*-nitrophenol mineralization in lake water reflected the interval needed by an initially small, indigenous population (e.g. 10^1–10^3 cells ml^{-1}) to reach a sufficiently large size (e.g. 10^4–10^5 cells ml^{-1}). In situations where the indigenous population of degraders is small, inoculation with an active population of degrading strains is an option for enhancing the biodegradation rate and reducing the acclimation period. For example, in the study described by Chen and Alexander [30], inoculation with an active degrading population eliminated the acclimation period. In another study, Edgehill and Finn [42] observed that increasing the inoculum size of PCP-degrading *Arthrobacter* sp. from 10^4 to 10^6 cells g^{-1} soil decreased the time necessary for 90% of the PCP to be degraded from 100 to 24 h. They also found that adding 10^6 *Arthrobacter* cells g^{-1} dry soil reduced the half-life of PCP from 2 weeks to less than 1 day. Crawford and Mohn [32] investigated the biodegradation of PCP in soil inoculated with a PCP-degrading *Flavobacterium* strain. They found that in the first two days after inoculation, PCP degradation rate was directly correlated with the inoculum size in the range of 10^4 to 10^6 cells g^{-1} soil.

Closely associated with inoculum density is the physiological status of introduced degrading microorganisms. To prepare cells for soil inoculation, it may be necessary to culture cells in a medium containing the target contaminants to induce the appropriate biodegradative enzymes. It may also be desirable to subject cells to environmental conditions which mimic those found in target sites.

CONCLUSIONS

The ability to metabolize pollutants is present in many microorganisms. However, suboptimal environmental conditions, limited availability of contaminants, metabolic constraints, low concentrations of contaminants in the environment, and protozoan predation of degrading microorganisms can limit biodegradation. Since every contaminated site is unique, factors that limit microbial degradation must be

carefully assessed for each individual site before any attempt at bioremediation. Moreover, interactions between some of these factors are possible and each factor should not be considered in isolation.

Some approaches exist for overcoming limiting factors to contaminant biodegradation. Problems with microorganisms may be overcome by (a) isolating or genetically engineering better degrading strains or consortia; (b) improving formulation and inoculation methods for introduced microorganism; and/or (c) improving degradative activity by indigenous microorganisms. Problems with contaminant availability may be partially overcome by using surfactants. If environmental conditions are inadequate and limit degradation, the use of bioreactors may be considered. Bioreactors could provide a controlled environment where aeration, moisture, and temperature are optimized.

Bioreactors may help in other ways. For example, cometabolites necessary for degradation of some compounds may be supplied more efficiently (e.g. methane for chlorinated aliphatics). Since a bioreactor can be designed to provide a high degree of containment, dispersal of microorganisms can be minimized. Conceivably, bioreactors can be used to treat soil, leachate from a site, surfactant-mobilized contaminants, or sub-surface contamination not easily treated by in situ technology.

Previous research has uncovered many factors which limit biodegradation of contaminants in the environment. However, much remains unknown about the nature and/or mechanism underlying these limiting factors both in situ and ex situ. More research is necessary to understand these limiting factors and to provide the scientific basis with which to address these problems.

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