INTERNATIONAL JOURNAL OF CHEMICAL REACTOR ENGINEERING

Volume 1 2003 *Review* R3

Transport and Reaction Processes in Bioremediation of Organic Contaminants. 1. Review of Bacterial Degradation and Transport

David C. Bressler* Murray R. Gray†

*University of Alberta, dbressle@ualberta.ca

†University of Alberta, Murray.Gray@UAlberta.ca

ISSN 1542-6580

Transport and Reaction Processes in Bioremediation of Organic Contaminants. 1. Review of Bacterial Degradation and Transport

David C. Bressler and Murray R. Gray

Abstract

Bioremediation of contaminants in soil and water involves a complex interplay between transport processes and biological reactions. Equilibrium physical factors such as aqueous solubility, soil adsorption, and phase partitioning indicate the transport processes that can limit bioremediation, namely the rates of interfacial transport and availability of contaminants to microbes. The physical properties of hydrophobic contaminants and the properties of biological membranes can be considered simultaneously by constructing a simple model for flux across from the aqueous phase to the cell interior. This simple model helps to reconcile the observed maximum biodegradation rates of different priority pollutants. This flux model for bioremediation suggests that the inhibition of biotransformation by alkyl substitution of aromatics may be due to transport kinetics rather than steric hindrance at the active enzymes. This report links the solubility of contaminants to the kinetics of transport across cell membranes, and thus suggests a mechanism which can control the overall activity of bioremediation processes for complex mixtures of contaminants.

KEYWORDS: bioremediation, bioreactor, metabolism, priority pollutants, PAH, dibenzothiophene, trichloroethylene, phenol, aromatics

1. INTRODUCTION

Wide ranges of organic compounds are generated by chemical and refining process industries that are undesirable in the environment. When these compounds contaminate cooling or other process wastewaters, or when they are spilled during transport or handling, remediation is required. The physical, chemical and biological properties of soil and water contaminants are diverse, giving a range of toxicity characteristics and persistence in the environment. Amongst the technologies available for conversion, removal, or sequestration of these contaminants, biological treatment is attractive because it can offer a combination of low cost and permanent removal. Although phytoremediation of soil and wetland treatment of contaminated water are becoming important technologies, this review will focus on microbial remediation from the viewpoint of chemical reaction engineering. This paper considers the properties of organic contaminants and how they interact with microbes, and presents some general guiding principles to the fundamental steps in bioremediation. To illustrate the behavior of a wide range of contaminants, we selected a representative set of organic compounds listed by the Environmental Protection Agency of the United States as priority pollutants, including aromatic hydrocarbons, chlorinated solvents, and oxygenated organic compounds. All of these compounds can be degraded by biological activity, either by transformation to other species or mineralization to carbon dioxide and soluble salts. The second paper in the series considers a case study of bioreactor performance in soil remediation, with a particular emphasis on enhancing the availability of contaminants to an active microbial population.

2. TRANSPORT AND SOLUBILITY LIMITATIONS ON BIOREMEDIATION

Many biological agents are metabolically capable of transforming or mineralizing chemical compounds which are considered environmental contaminants. Unfortunately, in order for a microbial cell to metabolize a compound, the enzymes of the cell must gain direct access to the compound. With the exception of some extracellular enzymes from fungi, this access requires that the compound must either cross the cell membrane of the bacterial cells, or at least directly contact membrane-bound enzymes. Except for the special case where cells attach directly to the interface between the aqueous phase and an organic contaminant phase, the compound must be in a solubilized state in an aqueous environment. There are three main mass transfer limitations on the accessibility of environmental contaminants to bacterial cells. The first is compound availability, particularly in the presence of soil where the contaminant may be sorbed to natural organic matter. The second is the presence of multiple phases, such as oil or dense organic liquids, either in a contaminated soil or in water, that control partitioning and release of contaminants. The final transport process is the crossing of the bacterial membrane itself, to the interior of the cell where many active enzymes for transformation or mineralization are located.

2.1 Physical Properties of Contaminants

Solubility and adsorption characteristics are two major properties of an environmental contaminant that greatly influence the overall potential rate of bioremediation. The data of Table 1 summarize the physicochemical properties of a variety of contaminants identified by the United States Environmental Protection Agency as high priority contaminants. These contaminants were selected as representatives of the main classes of organic contaminants in terms of their physical and biochemical behavior. The aqueous solubility of a contaminant plays a great role in determining the potential for bioremediation of a contaminated site. The data of Figure 1 demonstrate the relationship between aqueous water solubility and $log\ K_{ow}$ (the octanol-water partition coefficient) for a variety of environmental contaminants. If a compound is very polar, then it would be expected to have a high water solubility. The octanol-water partition gives a good overall estimate of compound polarity, and indicates the solubility of a contaminant in biological membranes and lipids. The data of Figure 1 clearly demonstrate the direct correlation between $log\ K_{ow}$ and aqueous solubility.

Contaminants with low aqueous solubility pose a significant problem for bioremediation. If a contaminant has extremely low polarity, then the compound is relatively insoluble in an aqueous environment. Compounds of this nature also tend to have high adsorption rates to various solid matrices in the environment. Figure 1 demonstrates that the experimental soil adsorption of contaminants (i.e. K_{oc}) correlates directly with K_{ow} and inversely with aqueous solubility. When a compound sorbs strongly to soil organics, the variability of soil types in the environment can make extrapolations of biodegradation rates from one site to another impossible.

Table 1. Experimental properties and predicted toxicity of a range of common environmental contaminants. The biodegradation rates listed are maximum first-order primary biodegradation rates compiled by Aronson and Howard (1997) and Aronson *et al.* (1999) from laboratory microcosm studies. Physical properties are from the Environmental Fate Database (2003) and Pearlman *et al.* (1984). Fish toxicity was determined by the EPA profiler (Environmental Science Center, 2003).

	Aerobic Biodegradation Rate, d^{-1}	Anaerobic Biodegradation Rate, d^{-1}	log K_{ow}	Maximum log K_{oc}	Water Solubility (mg/L)	Fish Toxicity* ChV (mg/L)
Aromatics						
Toluene	42.5	3.36	2.73	2.57	526	3
Naphthalene	3.36	0.0057	3.3	4.43	31.9	1.2
Benzene	3.3	0.071	2.13	1.99	1750	7.6
Fluorene	0.33	Not Available	4.18	3.45	1.83	0.28
Pyrene	0.143	Not Available	4.88	5.08	1.35	0.055
Benzo(a)anthracene	0.116	Not Available	5.84	Not available	0.011	0.019
Benzo(a)pyrene	0.057	Not Available	5.97	6.70	0.0016	0.006
Fluoranthene	0.045	Not Available	4.95	4.62	0.2424	0.055
Chrysene	0.037	Not Available	5.664	5.12	0.0063	0.019
Halogenated						
Dichloromethane	0.533	Not Available	1.25	1.45	13,200	30
Tetrachloroethylene	0.0011	0.0033	3.4	2.32	150	N.D.
Vinyl Chloride	Not Available	0.12	0.6	1.47	2763	N.D.
Oxygenated						
p-cresol	19.0	0.11	1.94	3.81	21.520	0.12
Phenol	7.99	Not Available	1.46	3.49	82,800	0.19
Acetone	7.3	Not Available	-0.24	1.26	Miscible	480
Methyl Ethyl Ketone	1.4	Not Available	0.29	0.72	223,000	220
Methanol	0.693	0.88	-0.77	0.95	Miscible	590

(*Fish chronic toxicity value. This is the same as a chronic no-effect-concentration (NEC) and the geometric mean of the maximum allowable toxicant concentration (MATC).)

The behavior of the contaminants in Table 1 spans the range from compounds that are predominantly wastewater/groundwater contaminants, such as methanol, to low solubility components such as PAHs which are more commonly encountered as soil contaminants. The high values of K_{oc} indicate that PAHs will be relatively immobile in groundwater. Conversely, the high water solubility of the oxygenated compounds usually leads to problems with containment such as large contaminant plume sizes and contaminated groundwater tables.

The chemical structure of the contaminant determines its partitioning characteristics, so that homologous series of compounds can give very different behavior. The data of Table 1 show that addition of an alkyl carbon groups to moderately soluble compounds, such as benzene and phenol, will significantly increase the K_{ow} and decrease the corresponding aqueous solubility. The data of Figure 2 show the trends for alkyl substitution of polycyclic aromatic hydrocarbons (PAHs). The more soluble naphthalene follows the same trend as benzene and phenol, with reductions in solubility with increasing substitution. When the solubility is extremely low, as in 4 and 5 ring PAHs, then the addition of alkyl groups has much less impact, and can even increase solubility. These data indicate that different members of homologous series of compounds can behave quite differently, so that lumping of components for the purposes of kinetic analysis must be considered very carefully.

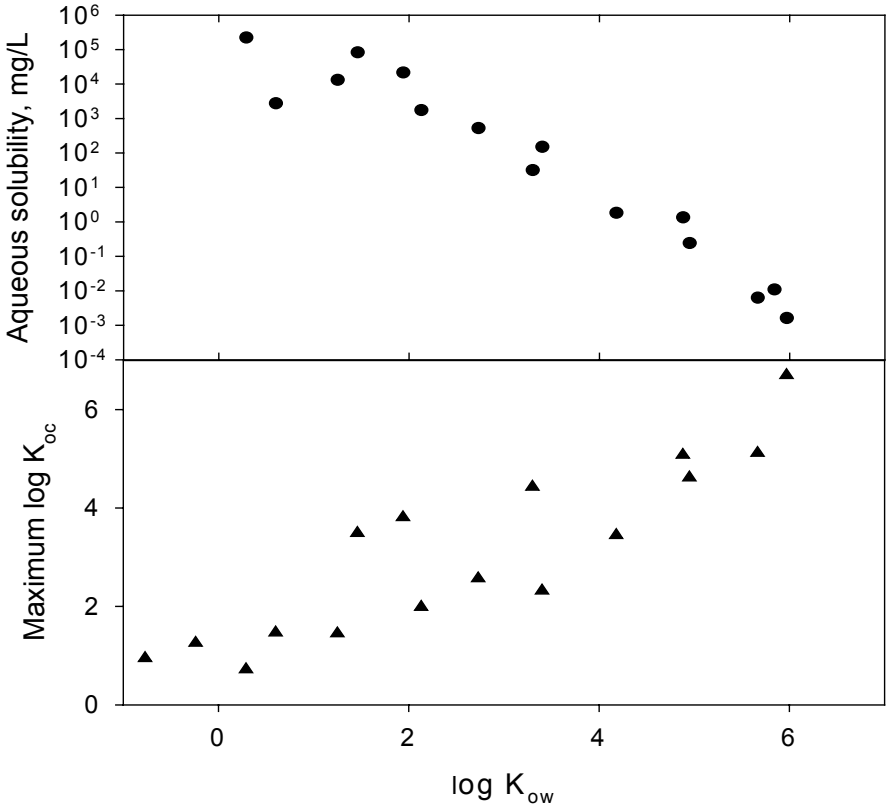

Figure 1. Correlation of experimental water solubilities and soil organic carbon partition coefficients for environmental contaminants (listed in Table 1) with octanol-water partition coefficient. K_{oc}= (μg adsorbed/g organic carbon)/(μg/mL solution))

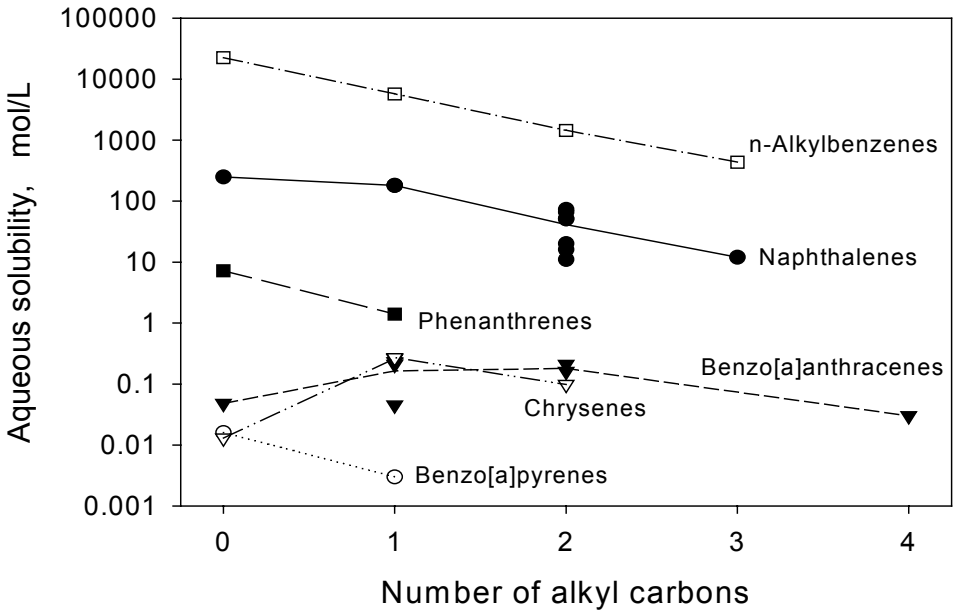

Figure 2. Effect of alkyl substitution on aqueous solubility of aromatic hydrocarbons. Data points are from Pearlman *et al.* (1984), and the lines show trends in solubility using the averages of each set of isomers.

The biological degradation characteristics vary significantly with the physicochemical properties of contaminants, as illustrated in Figure 3. The data on biodegradation rate are taken from a variety of microcosm studies, but they illustrate the main trends in rates of microbial degradation. The maximum rates of aerobic biodegradation are usually an order of magnitude greater than the anaerobic rates. The anaerobic degradation of contaminants is much less likely, therefore, to be subject to mass transfer limitations. Exceptions to this generalization include compounds such as tetrachloroethylene which are chlorine-substituted and demonstrate potential metabolic limitations and very low biodegradation rates in general. The data of Figure 3 suggest that compounds with intermediate values of log K_{ow}, in the range of 1-3.5, tend to give the highest rates of aerobic and anaerobic biodegradation. If a compound has very low water solubility, then the maximum rate of bioremediation may be dictated solely by mass transfer limitations. Polar contaminants are usually readily accessible to biological agents, but as shown in Figure 3, the rates of biodegradation can be lower than some less soluble compounds. If highly water-soluble compounds are found to persist in the environment it can usually be attributed to toxicity, or due to other metabolic limitations which will be discussed below.

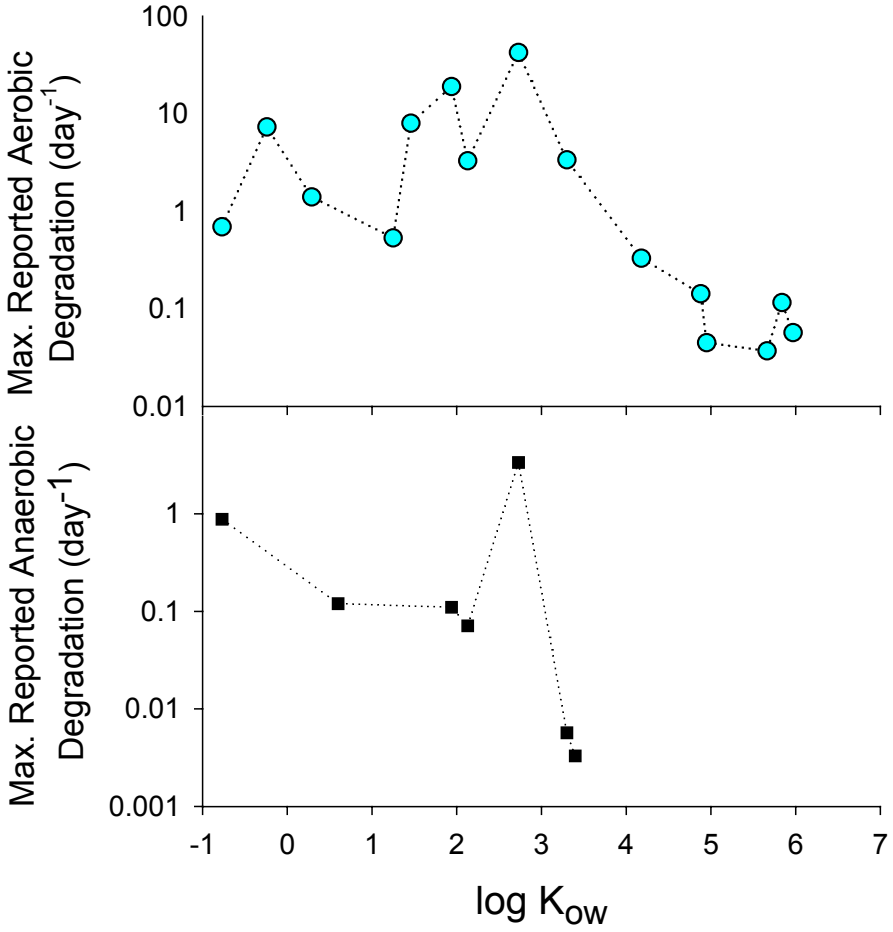

Figure 3. Correlation of reported maximum aerobic and anaerobic biodegradation rates for known environmental contaminants listed in Table 1 to their octanol-water partition coefficients.

2.1.1 Multiple Phases: As discussed above, if a contaminant has low water solubility then there is a limit to how much of the contaminant is accessible to bioremediation. Any contaminant not dissolved in an aqueous environment would be considered to be in a separate phase. In some cases, the contaminant would simply exist as a distinct liquid, solid or crystalline phase. In these cases, the mass transfer limitations on bioremediation would be dictated by solubilization rates of the contaminant. If, however, the contaminant was dissolved in a second nonpolar liquid phase, such as crude oil or creosote, then a host of other factors have to be considered including mixing and viscosity of the hydrophobic phase, solute equilibrium between phases, and interfacial properties of the two phases.

2.1.2 Membrane Crossing: Assuming that a contaminant has some degree of water solubility with a corresponding concentration in an aqueous environment, the next obstacle to bioremediation is accessing the enzymatic machinery of the cell. Most bacterial systems for remediation of chemical contaminants rely on enzymes found within the bacterial cell. These enzymes most often rely on cell-associated cofactors as either part of the enzyme machinery or as sources of oxidizing or reduction potential. There are a few examples of membrane bound enzymes, usually bound to the inner side of the cell membrane, therefore, most bacterial mineralization pathways rely on at least one intracellular enzyme that is separated from the surrounding environment by cell walls and outer membranes.

In order for a contaminant to gain entry into the microbial cell, it must pass through both the cell membrane and other cell-wall associated obstacles. The cell membrane itself poses a significant barrier to mass transport. The cell membrane is composed of a lipid bilayer which can be characterized as having three unique polarity domains. The outside of both sides of the membrane are considered fairly polar due to the presence of polar head groups on the phospholipids that compose the majority of the membrane. The center region of the bilayer is nonpolar in nature due to the fatty acid moieties of the phospholipids. In addition to the polarity obstacles of the cell membrane, there are often other membrane associated proteins, peptidoglycan, and even outer membranes that must be crossed (in the case of gram-negative bacteria). In order for a contaminant to make it through all the cell barriers and gain access to the inside of the cell, it must either be selectively taken into the cell, or have the right combination of polarity, size, and functional groups to allow it to get across the cell membranes. Figure 3 demonstrates that there appears to be a "window" in the range of *log* K_{ow} coefficients that allows optimal biodegradation under aerobic conditions. Values above 3 demonstrate reduced biodegradation. This limitation is usually attributed to the poor aqueous solubilities and adsorption properties of these compounds, but the repulsion of these very nonpolar compounds by the polar regions of the cell membrane probably also plays a significant role. Interestingly, values below 1 also demonstrate reduced rates of biodegradation. This could be attributed to a reduced ability of these extremely polar compounds to diffuse across the nonpolar regions of the cell membranes of typical microorganisms.

The flux of components across the bacterial membrane can be considered by a simple flux equation as follows:

$$F = P\left[C_e - C_i\right] \tag{1}$$

where P is the permeability of the membrane per unit area and C_e and C_i are the external and intracellular concentrations respectively. For the specific case where crossing the cell membranes limits the rate of biodegradation, the active enzymes will significantly reduce the intracellular concentration so that that $C_i \cong 0$, and the flux of contaminant into the cell will be limited by permeation through the membrane. The permeability of bilipid membranes can be estimated from the oil-water partition coefficient and the molecular weight of the compound (Stein, 1969):

$$P = 0.03\frac{K_{oil-water}}{MW^{1/2}} \tag{2}$$

The oil-water partition coefficient is typically 1/10 of the octanol-water partition coefficient. From equations (1) and (2), the maximum membrane flux can be estimated:

$$F^{\max} = 0.003\frac{K_{ow}C_{aq}}{MW^{1/2}} \tag{3}$$

where C_{aq} is the aqueous solubility of the contaminant at saturation. The data from Table 1 were used to calculate the maximum fluxes for each of the contaminants, which are plotted against the maximum aerobic biodegradation rate in Figure 4. In the case of the highly soluble oxygenated compounds, microbes are normally inhibited by concentrations well below the aqueous solubility limit. Consequently, the maximum flux of these compounds was estimated using the maximum concentration where biodegradation has been reported, rather than the aqueous solubility.

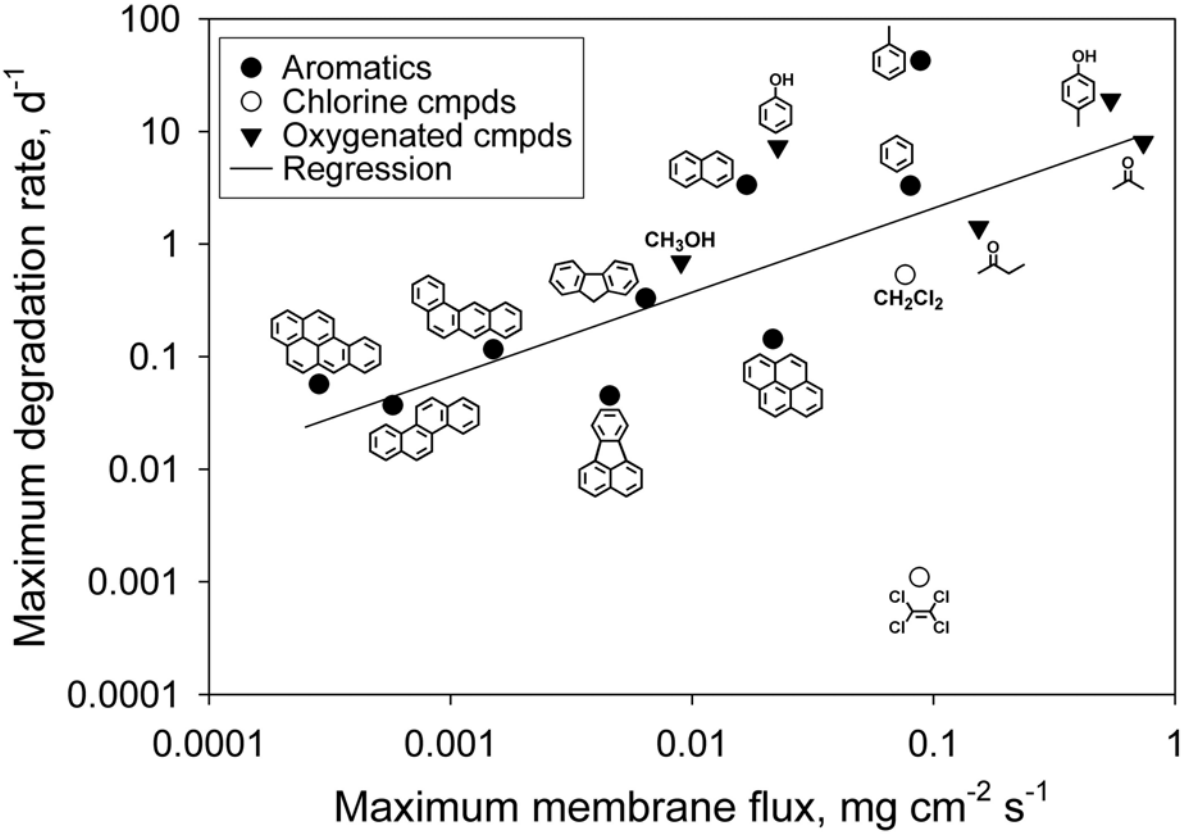

Figure 4. Correlation between maximum aerobic degradation rate and maximum membrane flux. The maximum flux was estimated from equation (3) and the property data of Table 1.

The data of Figure 4 suggest that the broad trends of maximum possible bioremediation rate can be correlated with an estimate of membrane flux, as calculated from the physicochemical properties of contaminants. The regression in Figure 4 was statistically significant, but the outliers from the main trend are extremely interesting. If we assume that the regression represents compounds with biodegradation rates that are limited by membrane flux, then points well above the regression trend may indicate compounds that are transported by some mechanism other than diffusion across the lipid bilayer. Similarly, compounds with rates well below the trend would indicate limitations in the rate of enzymatic conversion, rather than membrane permeation. In addition, the inclusion of biodegradation rates from lab microcosm experiments introduces the possibility of some degree of soil adsorption by the contaminants studied. However, a plot of maximum biodegradation rate against K_{oc} would give similar trends to the plot of biodegradation rate against K_{ow}, demonstrating that soil desorption is no more effective than K_{ow} at reconciling the various results.

The three compounds that fall well above the trend in Figure 4 are toluene, with a maximum aerobic degradation rate of 42.5 d^{-1}, naphthalene with a rate of 3.36 d^{-1}, and phenol with a rate of 7.99 d^{-1}. Given the similarity between toluene and benzene, the high degradation rate of toluene is not likely due simply to passive diffusion through porins, because we would expect benzene to have similar transport properties. The high rate of toluene, naphthalene, and phenol degradation suggests more selective uptake, possibly by active transport against the concentration gradient. Many nutrients are actively transported into the cells using energy requiring protein pumps. Some of these pump proteins are active against a wide range of compounds, while others can have very selective target compounds. These pumps have specific rates and could play a role in concentrating contaminants intracellularly, against the concentration gradient. Complicating matters of mass transport is the fact that membrane-bound pumps often pump contaminants out of the cell as a detoxification mechanism, even though the cell can biodegrade the contaminant (Bugg *et al.*, 2000). Protein pumps that pump out from the cell interior are also active in some bacteria for removing toluene (Ramos *et al.*, 1998) and antibiotics (Nikaido, 1994), but no pumps for entry of

hydrophobic compounds into cells have been identified as yet. In many cases as a hydrophobic compound is concentrated inside the cell, it is stored in the form of an inclusion body, which can be a hydrophobic phase within the cell surrounded by a single phospholipid layer. The uptake of n-alkane hydrocarbons, for example, is selective to specific components and is likely active against the local concentration gradient (Kim *et al.*, 2002). Such pumping and inclusions have not been identified for aromatic compounds, but this mechanism has the potential for the use of bacterial cells as concentration agents to reduce the aqueous concentration of contaminants.

Compounds that fall well below the correlation curve in Figure 4 may give lower rates than expected due to limitations in the performance of the intracellular enzymes. Degradation of these compounds would then be limited by reaction rate rather than by flux across the membrane. The chlorinated compound tetrachloroethylene lies furthest below the trend, consistent with the difficulty in degrading this compound enzymatically under aerobic conditions. This compound was the only one in the representative set with significantly higher rates of anaerobic degradation than aerobic degradadation (Table 1), consistent with enzyme-limited rates of aerobic removal. The PAH that lies furthest below the trend is pyrene, which gave similar degradation rates to the 5-ring PAHs even though its flux was higher. In one study, pyrene was found to degrade more slowly than some other PAHs due to a significant lag in the biological activity, presumable due to a lag in the synthesis of key enzymes (Karimi Lotfabad and Gray, 2002). When biodegradation of compounds such as tetrachloroethylene and pyrene is slow, then they should be converted at rates below the maximum diffusive flux across the membrane, below the trend line in Figure 4.

The limited data for anaerobic biodegradation rates were also plotted versus maximum membrane flux, as in Figure 4, but there was no significant correlation. This lack of correlation is consistent with the generally low rates of anaerobic conversion, which make mass transfer limitations unlikely.

Extremes in combinations of size, polarity, or charged groups ensure that simple diffusion alone could not allow significant biodegradation rates for some compounds. For example, there are numerous reports of biodegradation and mineralization for 2-, 3- and even four-ringed polyaromatic hydrocarbons, but only select polyaromatic hydrocarbons with 5 rings have been shown to be subject to biotransformations. Biodegradation of larger compounds has not been reported by bacterial systems. It is not unreasonable to predict that the currently known classes of bacterial dioxygenases should be able to oxidize these larger structures, so it is possible that there is a finite physical size of a compound which is able to cross the bacterial membrane at a rate that allows detectable conversion. The data of Figure 4 do not eliminate this possibility, but they suggest that the low aqueous solubilities of higher PAHs would be quite sufficient to account for extremely low (i.e. undetectable) rates of conversion.

3. BIOLOGICAL LIMITATIONS ON BIOREMEDATION

3.1 Metabolic Limitations

Assuming that a bacterial agent has the ability to bring the contaminant inside of the cell (to the biodegradation machinery) there are still significant biological factors that can influence the kinetics of biodegradation and mineralization. These factors can loosely be designated as either metabolic or growth limitations of the selected bioremediation agent. Metabolic factors include enzymatic limitations of the organism due to substrate recognition and steric hindrance of the substrate. Growth limitations can be thought of as any other factors that affect the growth and proliferation of the biocatalytic bacterial population.

3.1.1 Large Polycyclic Aromatics: One potential metabolic limitation to bioremediation is the molecular size of the contaminant to be remediated. Bioremediation of an organic compound relies on a complex biochemical pathway involving a series of enzyme-catalyzed reactions. Substrate recognition by the active sites of the various enzymes is required for the catalysis to occur. Even if a high molecular weight compound was able to get inside the cell, one potential limitation may be that the structure may be too large to gain access and interact with the enzyme's active site. This type of steric hindrance would be expected to increase with increasing compound size. This principle may explain the relative recalcitrance of large molecular weight polycyclic aromatic hydrocarbons. Pollard *et al.* (1994) reported that weathered oils demonstrated increased asphaltene content that was thought to be due not only to condensation reactions and loss of the more volatile components, but to selective biodegradation of the lower molecular weight components as well.

Samata *et al.* (2002) recently summarized the current known potential for bioremediation of the larger polycyclic aromatic hydrocarbons. At the time of their review, the biodegradation of the four-ring compounds fluoranthene, pyrene, chrysene, and benz[*a*]anthracene had been demonstrated and investigated to various degrees. The authors also summarized the known biodegradation potential of a few five- and six-ringed structures as well. Most of the studies involving compounds in that size range simply demonstrated removal of the starting compounds, with a few demonstrating release of $^{14}CO_2$ from radiolabeled substrates (labeled at only one carbon atom) (Ye *et al.* 1995; Kelley and Cerniglia 1995). This loss of the contaminants, in their original form, would likely be due to simple oxidations such as oxidation to alcohols and not necessarily to mineralization. In many cases where biotransformation of these large structures has been shown in the presence of alternate carbon sources, co-oxidation may be responsible for the observed transformations. In most of the reported investigations, the metabolites were never identified and mineralization was not shown. Benzo[*a*]pyrene, one of the most intensively studied five-ring structures, is know to abundantly exist in coal tar and yet has never been shown to serve as a sole carbon or energy source for any microorganism (Samata *et al.* 2002). However, this compound was reported to be mineralized by a microbial consortia grown on diesel fuel (Kanaly, *et al.* 2000). As an upper limit, direct biodegradation of six-ring structures or larger has never been demonstrated. Increasing recalcitrance attributed to increasing molecular size is also demonstrated by the environmental persistence of the high molecular weight components, including resins and asphaltenes, found associated with weathered petroleum and bitumen deposits (Pollard *et al.* 1994).

3.1.2 Addition of Functional Groups: One major limitation to bioremediation of environmental contaminants is the heterogeneity of compounds present and wide range of substitution found in the environment. Typically when research is conducted to develop bioremediation agents, model compounds are chosen for strain selection and enrichment. The use of model compounds allows for the selection of isolates able to perform desirable catalytic conversions and mineralization of selected chemical classes. Unfortunately, often when these isolated strains are tested against more complex mixtures such as those found in contaminated sites, the strains show poor activity. Research has shown that compound substitution results in decreased activity or even complete elimination of activity. One well-known example of this trend is the bioremediation of dibenzothiophene and its alkylated derivatives. The parent compound dibenzothiophene has been studied extensively and been reviewed by Bressler *et al.,* (1997) and Kropp and Fedorak (1998). At least three mechanistically independent biodegradation pathways are known for this compound. Other investigations into the biodegradation of methylated and dimethylated derivatives of dibenzothiophene clearly demonstrate that even the smallest amount of substitution results in drastic reduction of biodegradation efficiency (Kropp *et al.,* 1997). This finding is most often attributed to steric hindrance of the enzyme-substrate interaction. Bulky side groups are thought to prevent the compounds from efficiently entering the biocatalytic enzymes active sites, thus blocking metabolism. Substitution at specific sites on the compound may also change the properties of the compound such that the activation energy required for specific bond cleavage may increase prohibiting further metabolism. As indicated by the data of Figure 2, however, alkyl substitution of moderately soluble compounds can substantially reduce the aqueous solubility, thereby limiting the flux of the compound to the exterior of the cell. Dibenzothiophene has an aqueous solubility of 5.6 μmol/L (Pearlman *et al.,* 1984). With an aqueous solubility in the order of magnitude of the phenanthrene series, the membrane flux of dibenzothiophene would be predicted to decrease significantly with even limited methyl substitution. It is quite probable that both steric factors and reduced flux likely play a role in the low rates of bioconversion of alkyl dibenzothiophenes.

In general, the more severe the substitution, the greater the inhibition to metabolism. The desulfurization of dibenzothiophene and related compounds was the focus of the development of a bioupgrading process by Energy Biosystems in the United States. A major limitation of their developed biocatalysts was their inability to desulfurized various substituted dibenzothiophene derivatives (Monticello, 1998). Because fossil fuels have a significant proportion of the organic sulfur incorporated into substituted thiophene structures, the limitation presented a formidable obstacle to making these biocatalytic processes competitive in the marketplace. Recent investigations have looked to overcome the obstacle of substitution using directed evolution and gain-of-function mutation with some degree of success in this process (Arensdorf *et al.,* 2002). New mutants were identified capable of desulfurization of aliphatic sulfur compounds and increased activity towards selected methylated dibenzothiophenes. Mutation may be successful in alleviating some of the sterically hindered enzymatic activity, but would not be expected to greatly increase the membrane flux and therefore would never allow activity similar to that of the unsubstituted parent compound. However, if active transport components capable of increasing membrane flux were introduced, both obstacles to metabolism could be removed.

3.1.3 Gene Regulation: Bacterial metabolism can be thought of as a complex network of interrelated biochemical pathways. Some of the pathways are tightly regulated while others have somewhat more relaxed control. The problem that metabolic regulation poses for bioremediation is that contaminants are most often found in complex mixtures in the environment, not as pure compounds. It is not uncommon to find catabolic repression in which one component of a contaminant mixture represses the activity of the biocatalyst towards a compound it would otherwise biodegrade as a sole carbon source. It is even possible for metabolites of biodegradation pathways to suppress induction of essential degradative enzymes (Allard and Neilson, 1997). In some cases, mutation and genetic engineering can often be used to help alleviate this metabolic repression. Unfortunately, once these biocatalysts are released to the environment, there is selective pressure against these modified microorganisms.

A second issue related to metabolic regulation is the requirement for inducers in some biodegradation systems. There are many examples of biocatalysts that have the metabolic potential to biodegrade selected contaminant compounds due to their wide substrate specificity. Unfortunately, these contaminants are not recognized as substrates by the cells and therefore do not stimulate transcription and translation of the biodegradative enzymes required. In these cases, other substrates, which are recognized, must be added in order to induce the biodegradative pathways (Allard and Neilson, 1997).

3.1.4 Xenobiotics: In the previous section the effect of substitution on biodegradation was discussed. The previous focus was on the presence of carbon-based substitution. However, there is a second class of substitution that has a drastic effect on biodegradation rates. Synthetic substituents such as halogens, sulfur bridges, and nitrogen functionalities offer further complications. Due to the fact that halogenated compounds are relatively uncommon in the environment, the enzymatic ability to remove the substituent may be rare or even nonexistent, even though steric hindrance may not be a problem. This observation is supported by Figure 4 which demonstrates that tetrachloroethylene was biodegraded at much lower rates than would be expected based on the calculated maximum membrane flux.

The danger presented by these xenobiotic compounds is that they often cause toxic effects at all levels of the trophic food chain (Limber and Betts, 1995). Limbert and Betts (1996) suggested that this lack of activity is due to the premise that there are only a few distinct natural metabolic reaction mechanisms. The authors presented the argument that if a compound does not fit into an existing pathway developed over the course of natural evolution, or can be converted to an intermediate in these pathways, then any transformation reactions would be fortuitous and random. This principle explains the initial relative recalcitrance of several well-known environmental xenobiotic contaminants including trichloroethylene (TCE), polychlorinated biphenyls (PCBs), and trinitrotoluene (TNT). Fortunately, many enzymes from existing pathways have relatively wide substrate specificity. It is believed that enzymes with wider substrate specificity are able to bind to xenobiotic compounds which are analogous to natural substrates and if the substitution of the xenobiotic does not substantially alter the charge distribution of the enzymes active site, the enzyme can catalyze its specific reaction (Grady, 1985). This catalysis of a reaction with an analogous substrate has been termed fortuitous or gratuitous metabolism and is thought to be the major mechanism by which xenobiotics are biotransformed (Limbert and Betts, 1995).

One example of a well-studied xenobiotic is TCE. It is among the most prevalent environmental contaminants in groundwater in North America. It is a good solvent and is used to clean grease from machinery in manufacturing processes. TCE is subject to reductive dechlorination by anaerobic bacteria to produce vinyl chloride which is a potent human carcinogen. He *et al.* (2003) suggest that complete anaerobic degradation may be possible by isolating an anaerobic culture capable of detoxifying vinyl chloride. Due to the significant obstacles to the anaerobic process, previous research on TCE removal has been focused on oxidative biological methods of TCE remediation in soil and water. Various oxidative pathways for TCE biodegradation have been reported that rely on initial oxidation by monooxygenase and dioxygenase enzymes with wide specificities (University of Minnesota Biocatalysis/Biodegradation Database, 2003).

Within the last decade, more recent investigations have revealed that this conversion by non-selective enzymes is just one of four possible dehalogenation processes. Janssen *et al.* (2001) recently re-evaluated the dehalogenation of xenobiotics and presented three other mechanisms. Their classifications were based on more recent investigations by other groups who investigated the structure, genetic regulation, and biochemical properties of novel dehalogenase enzymes. These more recent investigations revealed that halogenated compounds can serve as carbon source and oxidizable substrate with either oxygen or nitrate as an electron acceptor due to these novel

enzymes. Aerobic processes have been shown to be possible for a wide range of substrates including 1,3-dichloropropylene and 1,2-dibromoethane as well as the chlorinated aromatics pentachlorophenol and 4-chlorobenzoate. For many of these reactions, novel enzymes are required. Examples include dichloroacetate halidohydrolase and haloacid dehalogenase which demonstrate selective dehalogenation in TCE biodegradation. A second mechanism presented by Janssen *et al.* (2001) was the metabolism of halogenated compounds during anaerobic fermentation in which a dehalogenated intermediate serves as an electron acceptor. Examples given include trichloroethylene, tetrachloroethylene, and orth-chlorophenol. The final type of transformation presented was when the halogenated compound is dehalogenated during the co-metabolism of another substrate.

Reviews by Limert and Betts (1996) and Allard and Neilson (1997) further discuss the limitations of remediation of chlorinated compounds and other xenobiotic and in detail. A feature of xenobiotic remediation is that many of the xenobiotics are degraded by consortia comprised of different species that carry out sequential reactions in order to achieve complete compound mineralization. Strain selection, genetic engineering and directed evolution of novel enzymatic activities hold promise for the development of future bacterial strains for the bioremediation of xenobiotics.

3.2 Growth Limitations

In addition to metabolic limitations on biocatalytic systems, there are also issues involved with the general propagation and proliferation of the biocatalytic agent. Microbial cell-based biocatalytic systems generally follow one of two scenarios. The first is the inoculation of a relatively small number of biocatalytic cells into a contaminated system. Whether they are able to proliferate through the metabolism of the contaminant alone or require additional supplements, the cells can be considered to be for the most part in an exponential growth phase. This type of system is preferential because the culture is self-sustaining and requires substantially less in terms of cultivation requirements. The second type of system is where a large culture of bioremediation agent is grown separately and then inoculated into a contaminated system. This augmentation approach often incorporates strains or populations that do not grow on the selected contaminant and tend to display more biotransformation than mineralization. These inoculations tend to be much more limited in application and relatively more expensive. It is usually preferential to have an actively growing biocatalytic system where the contaminant is continuously completely removed from the system. Whenever the contaminant serves as a primary carbon and energy source, various factors can have negative effects on the growing biocatalyst and must be taken into consideration during process design.

3.2.1 Environmental conditions: Microorganisms are known to have somewhat restricted growing conditions. Cells have temperature ranges in which they are able to metabolize and grow. At temperatures which are too high, the cells are killed mainly due to protein and enzyme denaturation and membrane damage (Pollard *et al.,* 1994). At temperatures that are too low, the metabolic activity of the cells can be reduced drastically due to decreased kinetic rates, and membrane gelling causes ceased transport across (Pollard *et al.,* 1994). This principle would be expected to be even more critical to processes aimed at bioremediating contaminants with extremely low initial flux rates across the biocatalyst cell membranes. Processes limited by membrane flux would be especially vulnerable to decreased process temperatures. At temperatures below freezing, ice crystal formation can destroy cellular structure and cohesion causing cell death. Temperature has much greater implications for bioremediation processes that rely on in situ methodologies. In countries such as Canada, where ambient temperatures approach or go below freezing, temperature has a great effect on the efficacy of bioremediation processes. This principle is even more important in contaminated sites such as the Arctic and Antarctic where the temperature rarely exceeds the melting point and permafrost is a permanent feature of the environment. The effect of temperature on biodegradative processes can be demonstrated by the persistence of contamination in these environments of otherwise easily degraded contaminants (Filler *et al*. 1991). In order for more rapid bioremediation in these extreme environments, engineering considerations must be made for increasing the localized temperature at the contaminated site. A recent example of this principle is the construction of heated biopiles in the remediation of a diesel fuel- contaminated soil in Prudhoe Bay Alaska (Filler *et al*, 2001). Through an integrated approach using thermal insulation, microbial monitoring, and optimization of power sources, the research effort was able to demonstrate ex situ bioremediation of soil to levels of contaminant below applicable cleanup standards. In order to achieve this success, they increased the process temperature to ranges between 0.5ºC to 7.8ºC, above the freezing point of water. The observed increase in bioremediation activity could be attributed to higher microbial growth rates, increased metabolic flux of the contaminants into the biocatalyst, and greater enzymatic rates.

Other environmental conditions that must be taken into consideration include forces such as osmotic strength (salinity), oxygenation levels, soil compaction, and moisture content. Of these parameters, salinity and oxygen content have the greatest affect on bioremediation agent growth. Most bacterial cells, with the exception of some halophilic or salt tolerant organisms, are unable to grow at salt concentrations much above 3-5%. Unfortunately, any contaminated site with high salt content poses intrinsic difficulties for any bioremediation agent. Oxygen content also plays a very specific role in the types of organisms that can be used in bioremediation processes. The presence of oxygen in the remediation process infers that the process will be oxidative in nature and rely generally on aerobic bacterial strains. Typical processes occur in situ within the first few feet of the soil surface, or in any aerated process. A lack of oxygen, such as found in deep soil in situ processes, infers an anaerobic process relying on pathways utilizing reductive steps and the use of water as an oxygen donor. Generally the potential metabolic pathways between the two oxygenation conditions are quite different, giving different potential products and process efficiencies. Perhaps the biggest distinction between aerobic and anaerobic process is the general drastic differences in time required for the processes to occur. Aerobic processes generally yield a much greater potential energy yield for bacterial growth per unit of substrate and thus aerobic processes tend to occur much more rapidly. Most anaerobic processes are much slower, but often have the advantage of having unique reaction mechanisms and can be used in situ in the deep subsurface with no process cost associated with aeration. The data of Table 1 and Figure 3 demonstrate that both aerobic and anaerobic microorganisms are capable of degrading a wide range of environmental contaminants, but that aerobic rates are much higher for many compounds of interest.

Often associated with the environmental conditions at the contaminated site is the potential for weathering. Weathering can be thought of as the selective loss of compounds from a contaminant mixture due to factors such as volatilization, abiotic reaction, and selective biodegradation. Weathering usually results in a selective loss of the easily degradable compounds, which tends to leave behind refractory residues that are resistant to further microbial attack (Bossert and Bartha, 1984). In a weathered contaminated site, the residual compounds tend to have low Henry's constants (K_H), high octanol-water coefficients, and high soil organic carbon-water partition coefficients (Pollard *et al.,* 1994). The residues are non-volatile (or semi-volatile) and partition preferentially to the oil phase, soil organic matter, and solid surfaces. Because biodegradation relies on aqueous phase properties, bioavailability is restricted severely in weathered contaminated sites (Pollard *et al.,* 1994)

3.2.2 Toxicity: One of the major limitations to the application of bioremediation agents to contaminated environments is the limited growth of most biocatalysts in toxic environments. The simplest example is when the target contaminant is itself toxic. In many cases initial biotransformation greatly reduces the toxicity of many contaminants (Seymour *et al.* 1997). Often a biocatalyst is able to utilize and detoxify a contaminant if the concentration is below the toxic threshold, but at higher concentrations the growth and viability of the biocatalyst are altered. This can be a problem is the target compound is found at high concentrations in a contaminated site. To utilize biodegradation in this scenario, usually the contaminant would have to be extracted and diluted prior to biodegradation at a much greater expense. A second toxicity problem that can be associated with a contaminant is if biodegradation produces metabolites with increased toxicity. A common example of this principle is the biodegradation of TCE (He *et al.*, 2003), where the initial anaerobic biodegradation produces vinyl chloride, which exhibits greatly increased toxicity. For this reason, anaerobic biodegradation of TCE has been problematic.

Most contaminated sites do not contain only a single contaminant; rather, they contain a wide variety of chemical agents, many of which have significant toxicities. Many sites are contaminated with halogenated xenobiotics and toxic inorganic components as well. Examples of these scenarios include military sites, landfills, and industrial waste disposal sites. The consequence of this is that in order for a biodegradative microorganism to be effective in removing a contaminant, it must be extremely resistant to the other toxic agents found at the site. In many cases these additional contaminants are not well characterized and the effect they will have on the microorganism is not known until actual application. Thus there exists some uncertainty when applying biodegradative agents to new environments. For example, it is quite possible for bacteria to oxidize other contaminants in a site while biodegrading the targeted contaminant. As a result, early oxidative biotransformations produce metabolites with increased polarities and water solubilities. Consequently, these biotransformed compounds, if not further degraded, have the potential to have greater mobility in the soil and water and thus expand the area contaminated. The associated risk is the contamination of adjacent previously uncontaminated water bodies and tables.

3.3.2 Abiotic reactions: One complication in bioremediation is the potential reactivity of biodegradation metabolites. If a biodegradation process produces reactive intermediates, these products may undergo side reactions producing terminal products that may be recalcitrant or have increased toxicity and genotoxicity. An example of this principle is the biodegradation of dibenzothiophene. Three metabolites described from the biodegradation of dibenzothiophene include benzothiophene sulfoxide, benzothiophene-2,3-dione, and 3-hydroxy-2-formylbenzothiophene. Research by Kropp *et al*. (1994) demonstrated that benzothiophene sulfoxide underwent an abiotic Diels-Alder condensation reaction to form benzo[*b*]naphtho[1,2-*d*]thiophene. Subsequent investigations by Bressler and Fedorak (2001a; 2001b) demonstrated that both 3-hydroxy-2-formylbenzothiophene and benzothiophene-2,3-dione also independently underwent rapid abiotic condensation reactions resulting in larger molecular weight products including thioindigo and 2-oxo-2-(2-thiophenyl)ethanoic acid disulfide respectively. These abiotic reactions were demonstrated to occur at high enough rates in laboratory experiments to dominate the mass balance of actual product formed under typical growth conditions. The extensive prior literature had missed the production of these products due to their larger sizes and associated analytical difficulties. Before bioremediation of dibenzothiophene contamination should be attempted with any biocatalyst, the recalcitrance and toxicity of these prevalent condensation products should be assessed. Most other biodegradative pathways have the same potential for abiotic side reactions and the prevalence and consequences of each should be investigated prior to field trials of any process.

3.3.3 Energy metabolism: In order to grow and propagate, microbes require energy from the conversion of carbon sources to more oxidized forms, whether by aerobic growth with oxygen as the terminal electron acceptor or by anaerobic growth with electron acceptors such as sulfate or nitrate. The lower the flux of a contaminant, the lower the available energy from that compound. The data of Figure 4 illustrate that multi-ring PAHs can give fluxes that are orders of magnitude below more accessible substrates. The minimum flux to cell survival, without cell division and growth, is the flux required to maintain the basal metabolism. If the cells cannot obtain this minimum amount of energy, then they cannot grow. This factor may contribute to the inability of microorganisms to degrade benzo[*a*]pyrene as sole carbon and energy source, as noted above, but to allow co-metabolism in the presence of other richer carbon sources in a controlled laboratory setting. In soil remediation, however, such co-metabolism of residual low concentrations may be limited by transport of contaminants out of the soil matrix. Attempts to stimulate degradation by adding more readily available compounds can merely give growth and degradation of the added material, rather than general enhancement of activity against target contaminants (Gray *et al.*, 2000).

3.3 Other Biological Factors

There are numerous other process specific considerations that may arise during bioremediation processes. Two important cases are the presence of biofilms, and the formation of surfactants and emulsifiers that change the non-aqueous solubility.

3.3.1 Biofilm formation: Wolfaardi *et al*. (1995) suggested that the when certain bacterial isolates generate biofilms, they may have increased attachment to solid matrices. This principle would result in localization of the biomass and could be used to retain the biocatalyst in the process, as in rotating biological contactors. Unfortunately, this would mean that for *in situ* remediation, the biocatalyst would not be expected to penetrate effectively through the pores of the soil or aquifer soil without some form of mechanical assistance. Hydrophobic bacteria can attach to surfaces such as oil or solid PAH, which provides a locally higher supply of nutrients to the attched bacteria. Propagation of attached bacteria to give a biofilm can inhibit the mass transfer into the aqueous phase, so that the overall rate of biodegradation is reduced (Mukherji and Weber, 1998). Consequently, efficient bioreactor operation must occur in one of two regimes to avoid limitations due to mass transfer. The first is to disperse the contaminant phase to give a large interfacial area. This condition is intrinsic to contaminated soil, where coating of contaminants on soil particles provides a large area. Given sufficient area, the degradation of contaminants will be limited more by transport within the non-aqueous phase than by interfacial transport. This case is considered in depth in the second paper of this series. Under these circumstances, quiescent conditions to promote biofilm formation will neither aid nor hinder the bioremediation because the rate-limiting step is unaffected (Gray *et al.*, 2000). The second regime for effective bioremediation occurs when agitation disperses the non-aqueous phase and prevents the accumulation of static biofilms. This condition would be satisfied in agitated vessels, as in the activated sludge process for wastewater treatment.

3.3.2 Role of surface-active compounds: As discussed in Section 1.1.2, the flux of contaminant to cells is partly limited by solubility in the aqueous phase. Addition of surfactants offers the possibility of enhancing this solubility, and considerable research has been conducted both on synthetic surfactants and biosurfactants in bioremediation of soil and in bioremediation of oil spills in water. As reviewed by Volkering *et al.* (1998), the literature is replete with contradictory findings on surfactant use, due partly to the complex physical and biological impact of changing the interfacial compositions in a bioremediation environment. Careful experimental protocols are required to separate physical changes due to surfactants from toxicity (in the case of synthetics) and actual improvement in bioremediation.

The addition of surfactant, either exogenously or by biosynthesis by microorganisms, profoundly affects the dispersion of contaminant phase and its interfacial area, even at concentrations well below the critical micelle concentration. Lowering the interfacial tension aids in mobilization of non-aqueous phase liquid in soil *in situ*, and provides emulsification of liquids in agitated bioreactors. The adsorption of surfactants to hydrophobic surfaces also helps to disperse solids, such as fine particles of contaminated soil or solid PAHs, in the aqueous phase. Such physical changes can easily account for many of the benefits of surfactant addition or formation by bacteria *in situ*. The adsorption of the surfactants to interfaces and surfaces also attenuates surfactant in the subsurface, an effect that is well recognized in the literature both on *in situ* remediation of soil and in the related literature on surfactant-aided recovery of crude oil.

In some cases, addition of surfactants can reduce the rate of bioremediation. The most common causes are toxicity and the use of surfactant as a growth substrate, which reduces the biological activity towards the targeted contaminants (Volkering *et al.*, 1998). In the case of bacteria attached to the oil-water interface, the addition of even low concentrations of surfactants can disperse the bacteria into the aqueous phase (Volkering *et al.*, 1998, Stelmack *et al.*, 1999). Disruption of biofilm formation may either hinder or enhance the process, depending on the regime of bioremediation as discussed above. Where bacteria rely on surface attachment for effective transport, addition of surfactants can give severe inhibition of biodegradation (Volkering *et al.*, 1998).

The most controversial aspect of surfactant addition is changes in interfacial transport processes and cell uptake. Above the critical micelle concentration, surfactants can increase the apparent solubility of hydrophobic compounds by association with the surfactant micelles. Controlled studies of biodegradation of solid PAHs have generally shown that micellar material does not enhance the rate of biodegradation, mainly because the rate limiting step is dissolution of the solid phase, rather than transport through the aqueous phase (Volkering et al, 1998). Enhancement of dissolution is possible, but the important role of surfactants in dispersing hydrophobic solids makes it very difficult to distinguish changes in effective surface area from changes in actual dissolution kinetics. Experiments with controlled interfacial area, rather that suspended solid crystals, would be required to distinguish the two mechanisms. In the case of liquid-phase contaminants, mechanisms of pseudo-solubilization have been proposed to aid in transport to cells, but the dominant role of surfactants is clearly in emulsifying hydrophobic liquids, as discussed above. In bioremediation processes, the main potential benefit of surfactants is clearly linked to the modification of the physical state of the contaminants, balanced against the possible toxic and inhibitory effects of synthetic surfactants.

4. CONCLUSIONS

A wide variety of factors that have been shown to affect the overall bioremediation of contaminated sites. These factors can contribute to either physical or metabolic limitations to bioremediation, and their complex physical and biological interactions tend to make each bioremediation application unique. While the effects of physical factors such as aqueous solubility, soil adsorption, and phase partitioning have been recognized for many years, the implications of cellular transport processes such as membrane flux have not been explored in the past literature. Based on a flux model for bioremediation, the inhibition to biotransformation of compounds such as dibenzothiophene caused by substitution may due to transport limitations on cell uptake, as well as steric hindrance of enzyme function. The effect of alkyl substitution in reducing the aqueous solubility of aromatic compounds can be profound because decreased solubility has been shown to result in decreased availability to the biocatalyst. This report links this decreased solubility to decreased metabolic flux across the biocatalyst cell membranes, and thus contributes another mechanism which may control the overall activity of bioremediation processes for complex mixtures of contaminants.

ACKNOWLEDGEMENTS

The authors are grateful for financial support from Syncrude Canada Ltd. and the Natural Sciences and Engineering Research Council of Canada, under the Industrial Research Chair in Advanced Upgrading of Bitumen.

NOTATION

C_{aq} = aqueous solubility, mg·cm^{-3}
C_e = extracellular concentration, mg·cm^{-3}
C_i = intracellular concentration, mg·cm^{-3}
F^{max} = maximum membrane flux, mg·cm^{-2}·s^{-1}
K_{oc} = soil-organic carbon-water partition coefficient
$K_{oil\text{-}water}$ = oil-water partition coefficient
K_{ow} = octanol-water partition coefficient
MW = molecular weight
P = membrane permeability, cm·s^{-1}

REFERENCES

Allard, A.-S., Neilson, A.H. "Bioremediation of Organic Waste Sites: A Critical Review of Microbial Aspects" Int. Biodeterior. Biodegrad., Vol. 39, No. 4, 253-285 (1997).

Arensdorf, J.J., Loomis, A.K., DiGrazia, P.M., Monticello, D.J., Pienkos, P.T. "Chemostat Approach for the Directed Evolution of Biodesulfurization Gain-of-Function Mutants", Appl. Environ. Microbiol., Vol. 68, No. 2, 691-698 (2002).

Aronson, D., Howard, P.H. "Anaerobic Biodegradation of Organic Chemicals in Groundwater: A Summary of Field and Laboratory Studies", Environmental Science Center, Syracuse Research Corporation, Retrieved Febuary 20, 2003, http://esc.syrres.com/demos/ratecon.pdf, (1997).

Aronson, D., Citra, M., Shuler, K., Printup, H., Howard, P.H. "Aerobic Biodegradation of Organic Chemicals in Environmental Media: A Summary of Field and Laboratory Studies", Environmental Science Center, Syracuse Research Corporation, Retrieved Febuary 20, 2003, http://esc.syrres.com/demos/AERBIO.pdf (1999).

Bossert, I., Bartha, R. "The fate of petroleum in soil ecosystems", in "Petroleum Microbiology", R.M. Atlas Eds., Macmillan Publ., New York, 435-473 (1984).

Bressler, D.C., Norman, J.A., and Fedorak, P.M. "Ring Cleavage of Sulfur Heterocycles: How does it Happen?", Biodegradation, Vol. 8, No. 5, 297-311 (1997).

Bressler, D.C., Fedorak, P.M. "Purification, Stability and Mineralization of 3-Hydroxy-2-formylbenzothiophene, a Metabolite of Dibenzothiophene", Appl. Environ. Microbiol., Vol. 67, No. 2, 821-826 (2001a).

Bressler, D.C., Fedorak, P.M. "Identification of Disulfides from the Biodegradation of Dibenzothiophene", Appl. Environ. Microbiol. Vol. 67, No. 11, 5084-5093 (2001b).

Bugg, T., Foght, J.M., Pickard, M.A., Gray, M.R. "Uptake and Active Efflux of Polycyclic Aromatic Hydrocarbons by Pseudomonas fluorescens LP6a", Appl. Environ. Microbiol. Vol 66, 5387-5392 (2000).

Environmental Protection Agency, "EPI Suite v3.10", Retrieved February 20, (2002) http://www.epa.gov/oppt/exposure/docs/episuitedl.htm, (2000).

Environmental Science Center, "PBT Profiler Ver 1.201" Office of Pollution Prevention and Toxics , U.S. Environmental Protection Agency, Retrieved Feb 20, 2003, http://www.pbtprofiler.net/notice.asp, (2003).

Filler, D.M., Lindstrom, J.E., Braddock, J.F., Johnson, R.A., Nickalaski, R. "Integral Biopile Components for Successful Bioremediation in the Arctic", Cold Reg. Sci. Tech., Vol. 32, No. 2-3, 143-156 (2001).

Grady, C.P. Jr. "Biodegradation: Its Measurement and Microbiological Basis", Biotechnol. Bioeng., Vol. 27, No. 5, 660-674 (1985).

Gray, M.R., Banerjee, D.K., Dudas, M.J., Pickard, M.A. "Protocols to enhance biodegradation of hydrocarbon contaminants in soil", Bioremediation J. Vol 4, 249-257 (2000).

Janssen, D.B., Oppentocht, J.E., Poelarends, G.J. "Microbial Dehalogenation", Curr. Opin. Biotech., Vol. 12, No. 3, 254-258 (2001).

Kanaly, R.A., Bartha, R., Watanbe, K., Harayama, S. "Rapid Mineralization of Benzo[a]pyrene by a Microbial Consortium Growing on Diesel Fuel", Appl. Environ. Microbiol., Vol. 66, No. 10, 4205-4211 (2000).

Karimi Lotfabad, S., Gray, M.R. "Kinetics of biodegradation of mixtures of polycyclic aromatic hydrocarbons". Appl. Microbiol. Biotechnol. Vol. 60, 361-365 (2002).

Kim, I.S., Foght, J.M., Gray, M.R. "Selective Transport and Accumulation of Alkanes by *Rhodococcus erythropolis* S+14He", Biotechnol. Bioengin. Vol 80, 650-659 (2002).

He, J., Ritalahti, K.M., Aiello, M.R., L ffler, F.E., "Complete Detoxificaiton of Vinyl Chloride by an Anaerobic Enrichment Culture and Identification of the Reductively Dechlorinating Population as a *Dehalococcoides* Species", Appl. Environ. Microbiol. Vol. 69, No. 2, 996-1003, (2003).

Kelley, I., Cerniglia, C.E. "Degradation of a Mixture of High-Molecular-Weight Polycyclic Aromatic Hydrocarbons by a *Mycobacterium* Strain PYR-1", J. SOIL. CONTAM., Vol. 4, No. 1, 77-91 (1995).

Kropp, K.G., Andersson, J.T., Fedorak, P.M. " Biotransformations of three dimethyldibenzothiophenes by pure and mixed bacterial cultures" Environ. Sci. Technol., Vol. 31, No. 5, 1547-1554 (1997).

Kropp, K.G., Fedorak, P.M. "A Review of the Occurrence, Toxicity, and Biodegradation of Condensed Thiophenes Found in Petroleum", Can. J. Microbiol., Vol. 44, No. 7, 605-622 (1998).

Kropp, K.G., Gonfialves, J.A., Andersson, J.T., Fedorak, P.M. " Microbially Mediated Formation of Benzonaphthothiophenes From Benzo[b]thiophenes", Appl. Environ. Microbiol., Vol. 60, No. 10, 3624-3621 (1994).

Limbert, E.S.B., Betts, W.B. "Kinetics of Bio-oxidation of a Medium Comprising Phenol and a Mixture of Organic Contaminants" Appl. Microbiol. Biotech., Vol. 43, No. 1, 165-170 (1995).

Limbert, E.S.B., Betts, W.B. "Influences of Substrate Chemistry and Microbial Metabolic Diversity on the Bioremediation of Xenobiotic Contamination. The Genetic Engineer & Biotechnologist, Vol. 16, No. 3, 159-180 (1996).

Monticello, D.J. "Riding the Fossil Fuel Biodesulfurization Wave" Chemtech., Vol. 28, No. 7, 38-45 (1998).

Nikaido, H. "Prevention of drug access to bacterial targets: role of permeability barriers and active efflux". Science Vol 264, 382-388 (1994).

Pollard, S.J.T., Hrudey, S.E., Fedorak, P.M. "Bioremediation of Petroleum- and Creosote-Contaminated Soils: A Review of Constraints", Waste Manage. Res., Vol. 12, 173-194 (1994).

Ramos, J.L., Duque, E., P. Godoy, P., Segura, A. "Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E". J. Bacteriol. Vol 180**,** 3323-3329 (1998).

Samata, S.K., Sing, O.V., Jain, R.K. "Polycyclic Aromatic Hydrocarbons: Environmental Pollution and Bioremediation", Trends Biotechnol., Vol. 20, No. 6, 243-248 (2002).

Seymour, D.T., Verbeek, A.G., Hrudey, S.E., Fedorak, P.M. "Acute Toxicity and Aqueous Solubility of Some Condensed Thiophenes and Their Microbial Metabolites", Environ. Toxicol. Chem. Vol. 16, No.4, 658-665 (1997).

Stein, W.D. "The movement of molecules across cell membranes." Academic Press, New York (1967).

Stelmack, P.L., Gray, M.R., Pickard, M.A. "Bacterial adhesion to soil contaminants in the presence of surfactants". Appl. Environ. Microbiol. Vol 65, 163-168 (1999).

Environmental Fate Database, Syracuse Research Inc., Retrieved March 2003, http://esc.syrres.com/efdb.htm (2003).

University of Minnesota Biocatalysis/Biodegradation Database "Trichloroethylene Pathway Map", University of Minnesota, Retrieved Febuary 28, 2003, http://umbbd.ahc.umn.edu/tce/tce_map.html, (2003).

Vogel, T.M., Griddle, C.S., McCarty, P.L. "Transformations of Halogenated Aliphatic Compounds" Environ. Sci. Technol., Vol. 21, No. 8, 722-736 (1987).

Volkering, F., Breure, A.M., Rulkens, W.H. "Microbiological aspects of surfactant use for biological soil remediation" Biodegradation Vol 8, 401-417 (1999).

Ye, D., Siddiqi, M.A., Maccubbin, A.E., Kumar, S., Sikka, H.C. "Degradation of Polynuclear Aromatic Hydrocarbons by *Sphingomonas paucimobilis*", Environ. Sci. Technol., Vol. 30, No. 1, 136-142 (1996).