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Polychlorinated Biphenyls: *In situ* Bioremediation from the Environment

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1. USAGE AND PHYSICO-CHEMICAL PROPERTIES OF POLYCHLORINATED BIPHNEYLS (PCBS)

Polychlorinated biphenyls (PCBs) were first synthesized in the early 1880s by Schmidt and Schultz (1881) and their commercial production began in 1929. Commercial PCB formulations were sold under a variety of trade names; for example, in the United States and the Great Britain, Aroclor was the most common trade name for PCBs. PCB mixtures were named according to their chlorine content. Core structure and predominant PCBs that exist in the environment are shown in Figure 1. The phenyl rings may have a variable number of chlorines, from 1-10, in 209 possible combinations. The letters (o), (m), and (p) indicate ortho, meta, and para substitutions for chlorines. The numbers indicate position of chlorines (Figure 1). For instance, Aroclor 1254 contains 54% chlorine by weight, and Aroclor 1260 contains 60%. The PCB mixture formulations were different depending on the country of origin, and were produced in Germany (Clofen), France (Phenoclor and Pyralene), Japan (Kanechlor), Italy (Fenclor), Russia (Sovol) and Czechoslavakia (Delor). PCB mixtures were produced for a variety of uses such as hydraulic and health transfer fluids in electrical transformers and capacitors, lubricating and cutting oils, and as additives in plastics, paints, carbonless copy paper, printing inks, adhesives and sealants. Millions of tons of PCBs were produced worldwide (around 700,000 tons in USA) before they were banned in the US after two major poisoning incidents in Japan in 1968 and ten years later in Taiwan (Kodavanti and Ward, 2005). In the US alone, nearly 382,000 tons of PCBs have been estimated to have contaminated the environment thus far (Hopf et al., 2009; Topfer, 1998).

Man-made organochlorine compounds such as PCBs possess unique properties that render them highly persistent in the global environment causing chronic toxicity to wildlife and humans. The physico-chemical properties of PCBs vary widely and depend on the number and positions of chlorine atoms in the biphenyl rings. Generally, water solubility, vapor pressure and biodegradability decrease with increasing number of chlorine atoms, while lipophilicity and adsorption capacity show a reverse trend.

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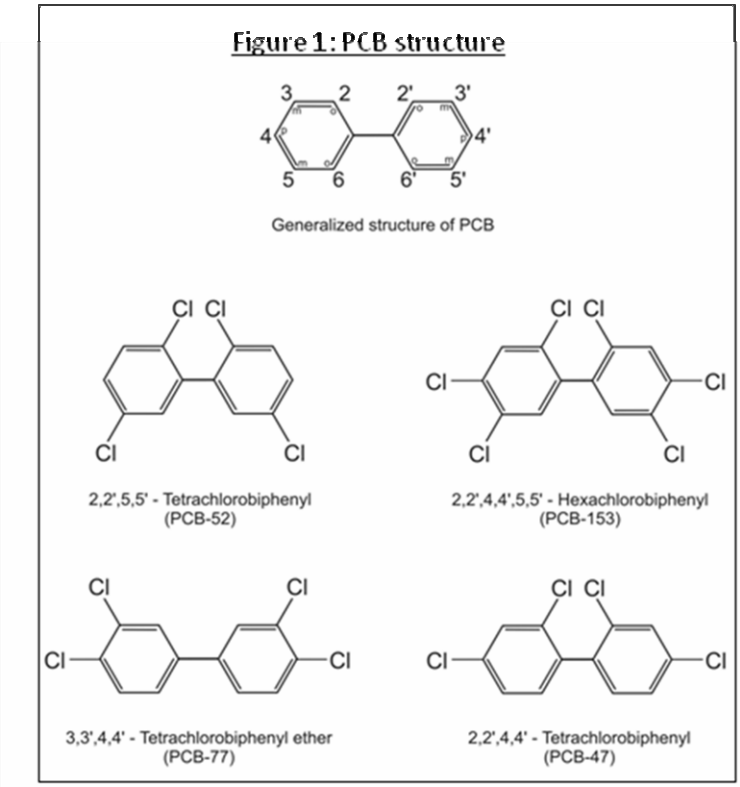


Figure 1. Core structure and predominant polychlorinated biphenyls (PCBs) in the environment. The phenyl rings may have a variable number of chlorines, from 1-10, in 209 possible combinations. The letters (o), (m), and (p) indicate ortho, meta, and para substitutions for chlorines. The numbers indicate position of chlorines.

2. ENVIRONMENTAL CONTAMINATION AND HUMAN EXPOSURE TO PCBS

Despite being banned from production since 1977, PCBs are continually being released into the environment via accidental spillage, storm water induced overflows, water runoff, leaching from uncontrolled landfills and hazardous waste sites, incomplete incineration of certain wastes, leakage from old discarded equipment, and illegal dumping of wastes or parts containing the chemical (Silberhorn et al., 1990). Once in the environment, PCBs cycle between water, soil, and air, and are in fact found everywhere in the environment from all the depths of the temperate oceans to the aerial and polar regions (Loganathan and Kannan, 1994). These compounds are particularly concentrated in surface soils and in lacustrine, riverine and estuarine sediments due to the location of their release and their strong adsorption to organic materials, which serves as a reservoir for continual release into the biosphere (Carpenter, 1998; McFarland and Clarke, 1989; Weber et al., 2008). Their solubility in organic solvents, oils, and fats makes them easily absorbed into virtually any organisms, from invertebrates to mammals. While the physical and chemical properties of each congener may vary according to the number and position of the substituted chlorine atoms, PCBs are generally colorless,

oily liquids with a high dielectric constant, heat transfer coefficient, soluble in hydrocarbons, and least soluble in water. In ecosystems, PCBs have low volatility and are chemically inert substances that fail to undergo significant oxidation, reduction, addition, elimination or electrophilic substitution reactions, except under extreme conditions or as catalyzed by a few species of aerobic and anaerobic microorganisms (Hardy, 2002; Huang et al., 2004; Wang et al., 2003).

The primary route of human exposure to PCBs was through consumption of contaminated foods such as dairy products, meat, and fresh water fish (Kannan et al., 1997). High levels of PCBs are found in human adipose tissue, blood, and milk (Loganathan et al., 1997).

3. BIOMAGNIFICATION AND HEALTH EFFECTS OF PCBS

PCBs, owing to their recalcitrant property of resisting bacterial and chemical breakdown, are readily adsorbed from water into the lipid depots of plankton, thereby enter the aquatic food chain. Bioaccumulation of PCBs in plankton leads to their biomagnification via various trophic levels including, fish, reptiles, birds, aquatic and terrestrial mammals including humans. Toxic effects of PCBs in birds varied from reproductive impairment, which is characterized by high embryonic and chick mortality, edema, growth retardation, developmental abnormalities (bill deformity, club feet, defective feathering, pericardial, peritoneal edema, liver enlargement and liver necrosis) and other deformities. Congenital deformities attributed to PCB contaminations were found in gulls, terns, herons and cormorants. Endocrine disruption and Ah-receptor (arylhdrocarbon hydroxylase enzyme) mediated toxicity were attributed to exposure to PCBs (Giesy and Kannan, 1998). PCB exposures have been associated with low birth weight and learning- and behavioral- deficits in children of women who consumed PCB-contaminated fish. Further, coplanar PCBs (chlorine substitutions in para and meta positions in the biphenyl molecule), elicits dioxin-like toxicity via AhR-mediated mechanism, which can lead to the development of cancer. However, non-dioxin like PCBs seems to exert neurotoxicity through effects on thyroid hormones and intracellular signaling processes (Faroon and Olson, 2000; Kodavanti and Tilson, 1997; Lein et al., 2007; Tilson et al., 1998).

Biomagnification of PCBs and their accumulation in species such as fish, consumed by humans, is well known and the transport mechanisms relevant to the aquatic environment are well understood (Beyer and Biziuk, 2009; Letcher et al., 2009). PCBs pose significant hazards and have been shown to be toxic to aquatic fauna causing tumor formation, reproductive failure, developmental defects, immune system deficiencies, and neurological problems (Ohe et al., 2004; Reijnders, 1994). Similar deleterious effects are also suspected or demonstrated to occur in humans (Carpenter, 2006; Domingo, 2006; Engel et al., 2007; Korrick and Sagiv, 2008; Tilson and Kodavanti, 1998). While the exposure-response relationship in terms of human cancer is still unclear, the U.S. Environmental Protection Agency (EPA) has classified PCBs as probable human carcinogens and some studies have shown exposure related increases in digestive system cancers and malignant melanomas (Knerr and Schrenk, 2006). Studies on humans exposed to PCBs have also shown reduced reproductive capacity (sperm motility, fetal development) (Younglai et al., 2007), immune deficiencies (Tryphonas, 1994), dermatological changes (chloracne, pigmentation changes) (Kitchin et al., 1994), and neurological abnormalities (in exposed fetuses/infants) (Hertz-Picciotto et al., 2005; Jacobson and Jacobson, 2002; Korrick and Sagiv, 2008). Moreover, because of biomagnification in aquatic organisms, many species now contain significant levels of PCBs resulting in significant concern about the long term, deleterious effects to the general population.

4. BIOREMEDIATION OF PCBS

Bioremediation is a process of using microorganisms to degrade contaminants in place with the goal of converting harmless chemicals as end products. Most often *in situ* bioremediation (ISB) is applied to the degradation of contaminants in saturated soils and groundwater, although bioremediation in the unsaturated zone can occur (Abraham et al., 2002; Bedard, 2008; Haglund, 2007). The technology was developed as a less costly, more effective alternative to the standard pump-and-treat methods used to clean up aquifers and soils contaminated with chlorinated solvents, fuel hydrocarbons, and toxic metals. ISB has advantages such as complete destruction of the contaminant(s), lower risk to site workers, and lower equipment/operating costs. ISB can be categorized by metabolism or by the degree of human intervention. The organisms degrading these contaminants can be divided into aerobic and anaerobic. The type of organism for an ISB system will depend on the contaminants of concern and their location. Some contaminants (e.g., fuel hydrocarbons) are degraded via an aerobic pathway (Field and Sierra-Alvarez, 2008a; Martin et al., 1999), some anaerobically (e.g., carbon tetrachloride) (Magar et al., 2005), and some contaminants can be biodegraded under either aerobic or anaerobic conditions (e.g., trichloroethene and PCBs) (Teuten et al., 2009). Accelerated ISB is where substrate or nutrients are added to stimulate the growth of a target consortium of bacteria. Usually the target bacteria are indigenous; however enriched cultures of bacteria (from other sites) that are highly efficient at degrading a particular contaminant can be introduced. Accelerated ISB is used where it is desired to increase the rate of contaminant biotransformation, which may be limited by lack of required nutrients. The type of amendment required depends on the target metabolism for the contaminant of interest. Aerobic ISB may only require the addition of oxygen (Adebusoye et al., 2008b; Field and Sierra-Alvarez, 2008a; Pieper, 2005), while anaerobic ISB often requires the addition of both an electron donor (e.g., lactate, benzoate) (Abraham et al., 2005; Baba and Katayama, 2007; Martinez et al., 2007; Stratford et al., 1996) as well as an electron acceptor (e.g., nitrate, sulfate) (Lambo and Patel, 2006; Rysavy et al., 2005; Wu et al., 2002).

4.1 Advantages of in situ bioremediation (ISB)

Accelerated ISB can provide total volumetric treatment, thereby removing both dissolved and sorbed contaminants. ISB often costs less than other remedial options. Contaminants may be completely transformed to innocuous substances (e.g., carbon dioxide, water, and ethane). The time required to treat subsurface pollution using ISB can often be faster than pump-and-treat processes alone (Sayler et al., 1995).

4.2 Limitations of ISB

Depending on the site, some contaminants may not be completely transformed to innocuous products. If biotransformation halts at an intermediate step, the intermediate may be more toxic and/or mobile than the parent compound. Some contaminants cannot be biodegraded (i.e., they are recalcitrant). When inappropriately applied, injection wells may become clogged from profuse microbial growth resulting from the addition of nutrients, electron donor, and/or acceptor. Accelerated ISB is difficult to implement in low-permeability aquifers because advective transport of nutrients is limited. Heavy metals and toxic concentrations of organic compounds may inhibit activity of indigenous microorganisms. ISB usually requires an acclimated population of microorganisms which may not develop for recent spills or for recalcitrant compounds (Braeckevelt et al., 2007; Spanoghe et al., 2004).

4.3 Microorganisms in Bioremediation of PCBs

Bioremediation of PCB contaminated soil is a difficult task due to the structure and level of chlorination of the compounds and occurs primarily by co-metabolic means (Abramowicz, 1995; Fedi et al., 2005; Han et al., 1995; Natarajan et al., 1999; Xu et al., 2010). Only a few microorganisms have been used to aerobically reduce the toxicity and quantity of PCBs (Bedard, 2008; Bedard et al., 2006; Lee and He, 2010; Luo et al., 2008; Robrock et al., 2009; Xu et al., 2010) . There is also a bias in the process in that, PCB degrading microorganisms tend to breakdown the less chlorinated congeners faster than the highly chlorinated ones (Bokvajova et al., 1994) .

Two distinct classes of bacteria have now been identified that biodegrade PCBs by different mechanisms (Evans et al., 1996; Field and Sierra-Alvarez, 2008b; Furukawa, 2000; Natarajan et al., 1999). These two PCB-degradative systems are divided into aerobic and anaerobic bacteria. The aerobes attack PCBs oxidatively, breaking apart the aromatic rings and substantially diminishing their toxicity (Furukawa, 2000). Anaerobes, on the other hand, leave the biphenyl rings intact while removing the chlorines (Tiedje et al., 1993). These two naturally occurring processes can be complementary, and a two step treatment may permit the biological destruction of nearly all of the PCB mixtures commonly used.

PCBs, being one of the most widely distributed classes of chlorinated chemicals in the environment; bioremediation seems to be a promising approach for cleanup of large areas of PCB-contaminated environments. However, the multitude of PCB congeners, their low bioavailability and high toxicity, and the choice of organism (Chavez et al., 2006) are important factors that affect the cleanup progression. The genetic organization of biphenyl catabolic genes has been elucidated in various groups of microorganisms, their structures have been analyzed with respect to their evolutionary relationships, and new information on mobile elements has become available (Capodicasa et al., 2009; Pieper, 2005). Key enzymes, specifically biphenyl 2,3-dioxygenases, have been intensively characterized, structure/sequence relationships have been determined and enzymes optimized for PCB transformation (Pieper, 2005). However, due to the complex metabolic network responsible for PCB degradation, optimizing degradation by single bacterial species is necessarily limited. As PCBs are usually not mineralized by biphenyl-degrading organisms, and co-metabolism can result in the formation of toxic metabolites, the degradation of chlorobenzoates has received special attention (Martinez et al., 2007). A broad set of bacterial strategies to degrade chlorobenzoates has recently been elucidated, including new pathways for the degradation of chlorocatechols as central intermediates of various chloroaromatic catabolic pathways. To optimize PCB degradation in the environment beyond these metabolic limitations, enhancing degradation in the rhizosphere has been suggested (Pieper, 2005), in addition to the application of surfactants to overcome bioavailability barriers.

Since 1973, an increasing number of microorganisms that degrade PCBs have been isolated and characterized (Adebusoye et al., 2008a; Bedard, 2008; Bedard et al., 2006; Bedard et al., 2005; Bedard et al., 2007; Bedard et al., 1987b; Di Toro et al., 2006; Fish and Principe, 1994; Furukawa, 2000; Levin et al., 2007; Oh et al., 2008; Pieper, 2005; Seeger et al., 1999; Ye et al., 1992). The major biodegradation pathways of PCBs in these microorganisms have also been established. In general, there are four specific enzymes, biphenyl dioxygenase, dihydrodiol dehydrogenase, 2, 3-dihydroxybiphenyl dioxygenase and 2-hydroxyl-6-oxo-6-phenylhexa-2, 4-dienoic acid hydrolase that are sequentially involved in the oxidative degradation of PCBs into chlorobenzoates and 2-hydroxypenta-2, 4-dienoate (Furukawa and Miyazaki, 1986; Taira et al., 1988).

Numerous soil bacteria break down PCBs via the dioxygenase pathway. Some of the identified organisms belong to the genera: *Pseudomonas* (Adebusoye et al., 2008c; Barton and Crawford, 1988), *Achromobacter* (Ahmed and Focht, 1973), *Acinetobacter, (Furukawa et al., 1983) Alcaligenes* (Bedard et al., 1987a), *Arthrobacter* (Gilbert and Crowley, 1997), *Corynebacterium* (Bedard et al., 1987a), *Rhodococcu* (Asturias and Timmis, 1993) , *Burkholderia* (Rehmann and Daugulis, 2008). In general, the more highly chlorinated the PCB is, the fewer species that are able to degrade it aerobically. Consequently, the possible complete biodegradation of the PCBs have been intensively pursued, though this goal has yet to be achieved. To utilize these microorganisms optimally, improvement of the PCB-degradation pathway is required, because the PCB-degradation pathways exhibit narrow substrate specificities.

PCB degradation rates can be increased when biphenyl itself is added to contaminated soils (Fava, 1996). Biphenyl enhances degradation because the microorganisms that degrade the biphenyl also co-metabolize the more recalcitrant PCBs. Certain organic compounds, including salicylic acid, also stimulate PCB degradation by microorganisms in some environments (Borrione et al., 2008; Singer et al., 2000). However, the usefulness of these amendments for improving PCB removal by microorganisms from diverse habitats has not been extensively explored.

Current efforts to remove PCBs and other hydrocarbon contaminants from aquatic sediments through bioremediation have been disappointing, thus far. When most effectively applied, they have involved dredging of contaminated sediments, and off-site treatment.

5. PCB DESORPTION

Previous studies have suggested that cyclodextrins accelerate removal of hydrophobic compounds through direct collisional interactions with the surface adsorbed guest molecule (Edwards and Shamsi, 2000; Edwards and Shamsi, 2002; Lein et al., 2007). Cyclodextrins are a group of homologous cyclic oligosaccharides produced from enzymatic breakdown of starch. They are classified as alpha, beta, and gamma cylcodextrins and consist of six, seven, or eight glucose rings respectively. In each of these groups there can be many different modifications of the molecule particularly at the 2 and 6 position hydroxyl group, or even as with randomly-methylated-beta-cyclodextrin can contain a mixture of cyclodextrins with methyl groups attached at varying positions. While water soluble, the inner hydrophobic cavity has the ability to form inclusion complexes with a wide variety of guest molecules (Li et al., 1992). Our molecular modeling studies show that cyclodextrin can accommodate PCB in its inner core. The alpha group with six carbons was predicted to be too small to sequester the Aroclor, while the gamma group with eight carbons was predicted to be too large. The beta group was chosen because it seemed the correct size for the encapsulation of the Aroclor 1254 (Figure 2).

Geometric factors rather than chemical factors seem to be the only requirement for this process to occur. If the guest molecule is too small, it will easily pass through the hollow cylindrical center of the cyclodextrin, and conversely of the guest molecule is too large it will not fit within the cavity and therefore not bind effectively.

Over the past decade there have been a series of investigations using cyclodextrins that test their binding capacity and found that they enhance the bioavailability and biodegradation of hydrophobic organic pollutants such as PCBs. One such study conducted by Fava et al. found in particular randomly-methylated-beta-cyclodextrin (RMBCD) improves the aerobic biodegradation of PCBs in soil contaminated with PCB containing transformer oil (Fava et al., 2003). However their studies did not address which cyclodextrins may bind the best to PCBs or to what affinity. We performed a visual

clarity experiment to show that randomly methylated beta-cyclodextrin can clear Aroclor 1254 in solution Figure 3. While there are several microbial, physical, and chemical methods available for disposal and remediation of PCBs they are all relatively inefficient and expensive. As relatively inert and extremely safe molecules, cyclodextrins seem to be the best answer for this persisting environmental problem if developed properly.

Figure 2: Molecular modeling - Aroclor inside a methyl beta-cyclodextrin molecule

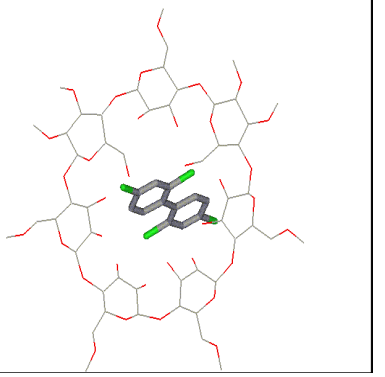
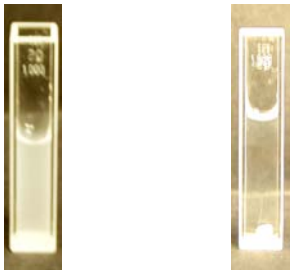


Figure 2. Molecular modeling of pcb core structure inside a methyl beta-cyclodextrin molecule. This modeling clearly shows that cyclodextrin can accommodate pcb molecule in its inner core.

Figure 3: PCB clearing by Cyclodextrin- Visual clarity experiment



Before addition of RMBCD
2mL of 0.75mM AROCLOR

After addition of RMBCD
2mL of 0.75mM AROCLOR
+ 400uL of 300mM RMBCYCLODEXTRIN

Figure 3. Clearing of PCB mixture, aroclor 1254 (aroclor) by randomly-methylated-beta-cyclodextrin (RMBCD) in a cuvette. This is a visual clarity experiment demonstrating the clearance of aroclor 1254 emulsion in water by RMBCD.

The question that we are faced with is whether cyclodextrins increase the rate of desorption of PCBs from sediments or simply increases their solubility in water. While cyclodextrins have been used to solubilize PCBs in contaminated soils, the key step that ultimately limits bioavailability is slow. Previous work with various hydrocarbon contaminants has shown that small portions can be removed using surfactants (Edwards and Shamsi, 2000; Edwards and Shamsi, 2002; Forgacs and Demnerova, 1996), but that significant amounts remain bound that exhibit extremely slow desorption rates. These fractions increase with the age of the contaminated soil or sediment samples (Fava et al., 2003). The other question is if cyclodextrins actually improve bioavailability of PCB's to bacteria bound to a matrix. Biodegradation in a sediment/water system can be described by the following equation $dC/dt = -B_f*k_b*C$ where C is the aqueous concentration of contaminant, B_f is the bioavailability factor; k_b is the first order biodegradation rate constant (for irreversible reactions). Thus increasing B_f, k_b or C will enhance removal of aqueous contaminant. It can be shown that $B_f = 1/1+R_{s/w}*dS/dC$. Where $R_{s/w}$ is the sediment/water m/V ratio and dS/dC is the changes of sediment bound and aqueous phase contaminant. Under conditions where desorption of the contaminant from a sediment is very slow and limits it biodegradation, $Bf = 1/1+K_d*R_{s/w}(k_b/k_m*K_d*R_{s/w})$, where k_m is the mass transfer coefficient. The relationship of time to biodegradation and removal from the aqueous phase of PCB, to the mass transfer coefficient k_m (h^{-1}) is modeled in Figure 4. Here we used literature K_d, k_m and k_b values and related to $t_{1/2}$ to k_m. $t_{1/2}$ is calculated from the relationship $t_{1/2} = Ln2/B_f*k_b$ where B_f and k_b were based on endogenous aerobic soil organisms (Cho et al., 2009). The relevant k_m for PCBs is likely to be < 0.02 h^{-1} (Hope, 2008), which is in the range of previously measured desorption rates for PCBs from contaminated soil samples (Konwick et al., 2006). Thus, cyclodextrin-

enhanced desorption could theoretically increase PCB biodegradation rates considerably. Indeed, positive effects of cyclodextrin on the extents of PCB degradation have been reported in soil reactor systems with aerobic bacterial mixtures, where enhanced desorption was suggested as the underlying mechanism (Fava et al., 2000).

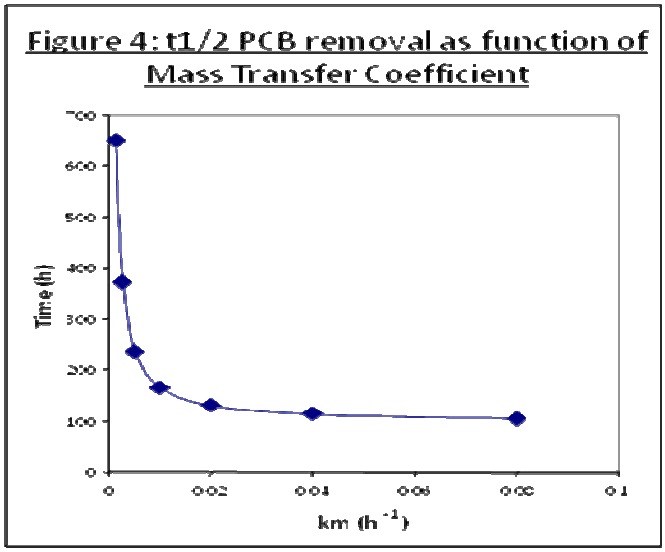


Figure 4. Model demonstrating the relationship of time to biodegradation and removal from the aqueous phase of PCB, to the mass transfer coefficient k_m (h^{-1}).

Now, we want to address the conditions where PCB-degrading aerobic bacteria thrive on simple artificial matrices. Previous remediation work has involved injection of significant biomasses of microorganisms which degrade PCBs in soils (Bedard, 2008; Francova et al., 2004; Mackova et al., 2009). This older method releases huge numbers of otherwise scarce species of bacteria into the environment, thereby disturbing the local ecosystem. Moreover, it is difficult to monitor their condition beyond measuring the continued degradation of the PCBs themselves. Concerns are further heightened when genetically modified organisms are used. Interestingly, after growth with biphenyl, many bacteria can oxidize PCBs (Chavez et al., 2004). It has been shown that PCBs follow the same catabolic pathway as biphenyl and use the same enzymes. Biphenyl dioxygenase plays a critical role in PCB degradation by catalyzing the first step. Its activity varies depending on the number of chlorines and their positions on the aromatic ring (Seeger et al., 1995). On the other hand, CMB1 bacteria has a strong ability to degrade di-*para*-chlorine-substituted PCBs (Williams et al., 1997). CMB1 degrades a spectrum of PCB congeners consistent with the expected substrates for a 2, 3-dioxygenase. There are a few comprehensive studies that evaluate the conditions affecting the growth and PCB degrading capacity of these bacteria (Adebusoye et al., 2008c; Fava et al., 2003; Rehmann and Daugulis, 2008; Rysavy et al., 2005).

6. CONCLUSIONS

Current efforts to remove PCBs and other hydrocarbon contaminants from marine sediments through bioremediation have not reached the full potential. The *in situ* methods are relatively ineffective,

requiring the dredging of contaminated sediments, which damages the marine ecosystem's integrity and requires extraction or sequestration of toxins once the sediment is deposited on land. Bioremediation technologies should be developed that would prove effective yet relatively low cost and low maintenance that will have a minimal impact on the ecosystem. Some of the limitations in the development and usage of bioremediation technologies are: (1) Under what conditions can aerobic PCB-degrading bacteria thrive on simple artificial matrices in field conditions?; (2) Do cyclodextrins increase the rate of desorption of PCBs from sediments or simply increase their solubility in water?; (3) Do cyclodextrins improve bioavailability of PCBs to bacteria bound to a matrix in field conditions? These are the limitations that can be verified with experimental results before they are considered as disadvantages and hence efforts have to be made to understand the basic principles underlying the bioremediation process before applying these principles to field use.

FOOTNOTE

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