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Evaluation of Bacterial Strategies to Degrade Non-desorbable Naphthalene in Natural and Artificial sorbents

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EVALUATION OF BACTERIAL STRATEGIES TO DEGRADE NON-DESORBABLE NAPHTHALENE IN NATURAL AND ARTIFICIAL SORBENTS

By

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ABSTRACT

EVALUATION OF BACTERIAL STRATEGIES TO DEGRADE NON-DESORBABLE NAPHTHALENE IN NATURAL AND ARTIFICIAL SORBENT

By

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Recently, several studies demonstrated that bacteria might be able to degrade sorbed contaminants without prior desorption in water. Mechanisms including extra-cellular biomaterial production, chemotaxis/motility, and adhesion were hypothesized to be responsible for the enhanced bioavailability of these compounds. However, experimental evidence to confirm this bacterial potential in the soil system has not been verified. In this study we evaluated the role of the selected bacterial phenotypes in naphthalene degradation in a sorbent-slurry system. The rate and extent of sorbed-phase naphthalene was determined in the sorbent-slurry systems inoculated separately with the wild-type *Psuedomonas putida* G7 and the mutant strains (with one or other missing phenotypes), and the results were compared for evaluating the role of these phenotypes.

The experimental results showed that wild-type *P. putida* G7 degraded sorbed naphthalene at a rate and extent greater than what can be removed from both Tenax beads and serial extraction methods. Activity of extra-cellular enzyme was ruled out during the biodegradation study since the experiments with bacterial filtrates did not show any degradation of observable free naphthalene during the incubation period. The possible effect of biomaterial on the sorption/desorption parameters was also observed to be negligible. The amount of the biomaterial produced during the bio-availability studies was small enough to have caused any significant effect on mass transfer parameters.

This study demonstrates that the effect of chemotaxis/motility phenotypes could not be observed in natural soils under both stirred and quiescent conditions. In a stirred system, the mixing had masked the role of chemotaxis/motility due to maintenance of isotropic concentration and mixing-mediated adhesion of cells to sorbent particles. Under quiescent conditions, wild-type *P. putida* G7 and mutant strains were not able to degrade the non-desorbable fraction of naphthalene because of pore size exclusion and retardation of bacterial cell in natural soil. However, the role of chemotaxis/motility was apparent in the system containing activated carbon, where wild-type degraded the non-desorbable naphthalene fraction at a greater rate and extent as compared with the system containing mutant strains. The difference in uptake rates between wild-type and mutant strains was due to the presence of a significant concentration gradient at the soil/water interface as a result of a large pool of non-desorbable naphthalene fraction in the activated carbon.

This study also attempts to examine factors affecting bacterial attachment that, consequently, enhances bio-degradation. The wild type *P. putida* G7 and the mutant strains, deficient in adhesion, were observed to have equally accessed non-desorbable fraction of naphthalene and degraded the fraction at an equal rate and extent. Three orders of decrease in molar concentration of the buffer medium did not affect the rate and extent of sorbed phase naphthalene degradation by *P. putida* G7. Important attachment phenotypes of *P. putida* G7 did not play any significant role in enhanced bioavailability of sorbed phase naphthalene. The results of this study suggest that factors promoting bacterial attachment (i.e. removal of exo-polysaccharides, presence of flagella and reduction of buffer medium concentration) do not contribute to biodegradation of non-desorbable fraction of soil-sorbed naphthalene in a mixed soil-slurry system.

DEDICATION

To my courageous mother, Bacha Bibi

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CHAPTER 1. INTRODUCTION

INTRODUCTION

Recalcitrant organic compounds such as polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the biosphere due to anthropogenic activities. PAHs are a class of very stable organic molecules with a fused-ring chemical structure. Some of these compounds, with intermediate to high molecular weight e.g. bezop[a]pyene and bez[a]anthracene, are suspected carcinogen or mutagen, and are recognized by the USEPA as priority pollutants (keith and Telliard 1979). The main source of these compounds is fossil fuels, and these compounds may enter into the environment via point (e.g., automobile and refinery exhausts) or non-point(e.g., oil spill and urban run-off) sources. PAHs persist in the natural environment because of their hydrophobic nature and high molecular weight, resulting in low water solubility and low volatility. As a result, these compounds selectively partition into soil or sediment that in turn serves as a long-term source of PAHs contamination to ground and surface water.

Several treatment options such as pump and treat, air-sparging, air-stripping, and activated carbon adsorption have been used to remove the organic contaminants. However, these technologies failed to deliver due to the associated high cost to remediate large volumes of contaminated sites, incomplete removal of contaminants to meet regulatory standards, and large waste by-products that need to be disposed of (EPA. 1995). Bio-remediation has emerged over the last two decades as a favored and attractive alternative, which has the potential to successfully clean up the contaminated site without having the disadvantages associated with traditional physiochemical methods (Head

1998). The initial success of bio-remediation was, however, limited to easily biodegradable, low molecular weight or volatile organic compounds and soon it was realized that several issues have to be addressed before this technology can successfully be applied to soils containing recalcitrant pollutants such as PAHs (Mueller et al. 1996; Wilson and Jones 1993)

Two major factors are attributed to incomplete remediation of PAHs contaminated sites. First, high molecular weight PAHs (for e.g., PAHs with five or more fused rings like benzo-pyrene) are recalcitrant to biodegradation; and, only low to intermediate molecular weight PAHs degrading bacteria have been isolated from contaminated sites (Heitkamp et al. 1987; Mueller et al. 1996). Second, due to the hydrophobic nature of these compounds they have a high tendency to sorb to soil and sediment's matrix. As a result, sorbed molecules may commonly be unavailable for the direct uptake of soil bacteria. For sorbed molecules to be available for bacterial uptake, they have to diffuse out within the nano- and micropores to soil-water interface and, finally, diffuse through the aqueous phase to the bacterium.

In dealing with biodegradable compounds, the success of bioremediation, therefore, depends on the interplay of two steps 1) the pollutant release rate in the soil matrix and 2) the bacterial uptake rate. These two steps occur sequentially, and the overall bioremediation rate can be controlled and limited by any one of these two steps. The term bioavailability is often used to describe the rate of bio-remediation, and is defined as a ratio of rate of transfer of compound from the soil to the rate of uptake by the bacteria. Bioremediation of soils contaminated with HOCs is often limited by mass transfer from soil matrix to bacterial membrane and is referred to as limted bio-

availability. Several laboratory and field studies have demonstrated that slow release of biodegradable PAHs from soil matrix to aqueous phase is often the rate limiting step in complete biodegradation of these compounds (Beck et al. 1995; Luthy et al. 1994).

Bio-availability of PAHs has been identified as one of the major research priorities for bioremediation technologies (US-EPA 1991). Recent research has suggested that even unsuccessful bioremediation of high molecular weight PAHs is mainly due to low bio-availability of these compounds, rather than compounds being recalcitrant to biodegradation (Mihelcic et al. 1993). In the last two decades, extensive research has been conducted to identify factors affecting bio-availability of recalcitrant organic compounds to microbial degradation, and a review of the literature was recently published (Baveye 1999). These studies concluded that bioavailability essentially depends on interaction between the soil matrix, the contaminant, and the micro-organism; further, these interactions are in turn dependent on composition and structure of soil matrix, physico-chemical nature and residence time of contaminant, and the type and state of the micro-organisms.

Until recently, release of sorbed molecules from soil matrix to solution phase has been considered a pre-requisite for complete bio-remediation (Alvarez-Cohen and Speitel 2001; Ogram et al. 1985; Scow and Alexander 1992). Biodegradation in soils and sediments was considered de-sorption or diffusion rate limited. It was assumed that de-sorption rate (release rate from soil matrix) depends only on the interaction between composition/structure of soil matrix and the physiochemical nature and residence time of the contaminant. Whereas considerable effort has been undertaken to study the characteristics of soil-chemical interactions in soil, little was known about the pollutant

mass transfer-enhancing strategies of the bacteria. The role of bacterial phenotypes (physiology, adaptation etc) on rate of de-sorption or direct uptake of sorbed phase has been neglected or ignored. The bio-remediation technology is still based on this widespread and common paradigm and, consequently, bioremediation cleanup endpoint is based on a high concentration of PAHs that is sequestered in soil matrix and hence un-available for degradation.

Recent studies have challenged this paradigm based on the evidences that bacteria may be able to degrade sorbed contaminant without prior desorption in water (Bestiaens et al. 2000; Calvillo and Alexander 1996; Guerin and Boyd 1992; Tang et al. 1998). These previous studies suggested that while some bacteria can only uptake contaminant in an aqueous phase, others might be capable of accessing contaminant in the sorbed phase as well. Thus the researchers who conducted theses studies concluded that there exists two physiologically distinct strains of PAH degrading bacteria: one able to degrade PAHs in solution only, and the other capable of degrading PAHs sorbed to soil. This line of evidence clearly shows that bioavailability of a contaminant also depends on the type of PAHs degrading bacteria in the system. This new find has important ramifications for the interpretation of contaminant bioavailability and remediation endpoints that are still based on kinetic studies of isolate capable of degrading PAHs in solution, only.

All this led to a concept of enhanced bioavailability, which is based on the fact that bacteria can also access directly to the sorbed phase, and thus the degradation rate of contaminant can exceed those predicated from aqueous phase contaminant only. Mechanisms responsible for the enhanced bio-availability of these compounds in soil systems is still not clear (Dean et al. 2001; Harms and Zehnder 1995). Recent studies

hypothesized that specific physiological characteristics of the PAH degrading bacteria might contribute to enhance bioavailability. The mechanisms promoting enhanced bio-availability include: (1) production of extra-cellular biomaterials (enzymes and polymers) that either directly degrade inaccessible substrate or facilitate enhanced desorption of organic chemicals (Dean et al. 2001; Liu 2001; Mata-Sandoval et al. 2000; Vandyke et al. 1993); (2) Chemotaxis trait that guides bacteria to swim using flagella to locate substrate concentration in soil; (3) reduction of the distance between cells and substrate by means of adhesion-promoting bacterial-surface or structures such as flagella and lipo-polysaccharide (Bastiaens et al. 2000; Harms and Zehnder 1995).

The first mechanism stipulates that the biomaterial produces by bacterial enhances mobilization and release of soil bound organic hydrophobic contaminants (Dean et al., 2001; Liu, 2001; Mata-Sandoval et al., 2000; Schippers et al., 2000; Vandyke et al., 1993). The enhancement was attributed mainly to increases in apparent solubility of contaminants due to dissolved biomaterial. The water soluble biomaterials bind contaminant in solution, decreasing the solution concentration of the free, unbound contaminant molecule. This would have resulted in an increase in driving force for desorption of sorbed contaminant. It was expected that due to better solubalization properties of excreted biomaterial, an increase in biavailabilty of sorbed organic contaminants would result.

The second mechanism suggests that bacteria with chemotactic and motile capability actively seek target chemo-attractants. Chemotaxis ability may bring motile bacteria into close physical contact with soil-sorbed chemoattractants when soluble substrates are depleted. Several bacterial strains have been reported to be chemotactic

towards environmental pollutants such as benzene, toluene, trichloroethylene, biphenyl, naphthalene, *p*-nitrophenol and carbontetrachloride (Grimm and Harwood, 1997b; Parales et al., 2000; Witt et al., 1999; Samanta et al., 2000). Chemotactic *Pseudomonas putida* G7 can degrade naphthalene more rapidly than a mutant deficient in chemotaxis towards naphthalene and a motility-deficient in capillary tube (Marx and Aitken, 2000). It has been shown that flagella-mediated motility is important for the cells to make contact with a surface (Lawrence et al., 1987; O'Toole and Kolter, 1998b). Motility facilitates attachment by bringing the cell close to the surface to overcome repulsive electrostatic forces between the bacterium and the surface. Although it has been studied intensively in liquid media, there is evidence that chemotaxis may guide bacteria to seek localized concentration gradient in soil matrix (Wall and Kaiser, 1999).

The third mechanism suggests that bacteria with a strong tendency for adhesion in the soil may have a selective advantage to access sorbed compounds (Harms and Zehnder, 1995; Calvillo and Alexander, 1996; Tang et al., 1998). Surfaces containing sorbed chemicals create a microenvironment (e.g., a steeper concentration gradient and a shorter diffusion distance) that is different from the surrounding bulk liquid. A higher chemical concentration near the sorbent surface may render the chemical more readily available to attached bacteria than to bacteria present in the aqueous phase. Bacterial characteristics contributing to cell adhesive properties include cell surface hydrophobicity and charge, the presence of extra-cellular polymers, and the presence of cellular appendages (i.e., flagella and pili). For instance, members of *Coryneform* bacteria exhibit increasing cell surface hydrophobicity due to the presence and increasing chain length of mycolic acids in their cell wall, which in turn is related to increased adhesion to Teflon

surfaces (Bendinger et al. 1993). Special cell surface structures (e.g., pili or extracellular polymers) help form strong links between cell and solid surface as well as micro-colonies or biofilm development. Type IV pili (thin filaments that extend from the pole of a bacterial cell) found in some soil bacteria play an important role in microcolony formation (cell to cell interaction) and mediate surface-associated movement known as social gliding and twitching motility (O'Toole and Kolter 1998b; Wall and Kaiser 1999).

This study was undertaken to research the role of specific physiological traits of bacteria on enhance bioavailability of soil-sorbed organic contaminant. All of the identified mechanisms were tested in a systematic way in stirred soil-slurry reactors using a representative PAH, naphthalene. A wild-type naphthalene degrading bacteria (*Pseudomonas Putida* G7) along with its mutants strains (non-chemotectic, non-motile, and non-adhesive) were used for studying these mechanisms. The pure wild type *P. putida* G7 has been reported previously to uptake sorbed phase naphthalene directly in soil-slurry. Furthermore, it has been characterized as being motile and, chemotectic strain.

HYPOTHESES

The underlying hypotheses for this research work are as follows:

1) *Pseudomonas putida* G7 (PpG7) uptakes naphthalene intra-cellularly and does not produce extra-cellular catabolic enzyme in response to substrate limiting condition. PpG7 also excretes biomaterial in suspending solution that affect sorption/desorption coefficient of naphthalene to soil and hence affect bioavailability of naphthalene.

2) Chemotaxis and motililty are important traits that enable wild-type *P. putida* G7 to access localized concentration of pollutant in soil. Alternatively, chemotaxis and motility

deficient mutants of *P. putida* G7 cannot utilize sorbed naphthalene at the same rate as the wild-type.

3) Attachment phenotype enables wild-type *P. putida* G7 to access localized and strongly sorbed concentration of pollutant at the particle-water interface. A wild-type *P. putida* G7 degrade sorbed-phase molecules at greater rate than its adhesion-deficient mutant.

To address these hypotheses, this research work involved generating, isolating, and characterizing mutants with one of the missing phenotypes (motility, chemotaxi, adhesion). Sorption isotherms and single and series dilution desorption experiments were conducted to evaluate distribution coefficient, de-sorption rate, and amount of non-desorbable naphthalene. The initial degradation rate was measured for wild type as well as for all mutants in soil extract solution. All the studies were carried out assuming that initial degradation kinetics of all mutants remained same as that of wild type strain. Bioavailability experiments involved first establishing sorption equilibrium, inoculating the systems with bacteria, and measuring naphthalene concentrations in both sorbed and dissolved phases over a period of time. Rate of naphthalene degradation by each culture in stirred batch systems were fitted with non-linear regression model (DBM model) to determine sorbed phase degradation rate coefficients. Bio-availability data were compared for each type of organism for extent and rate of sorbed phase degradation to evaluate role of each phenotype.

The outcome of this research is expected to demonstrate the role of microorganism's physiological traits in enhancing bio-availability of sorbed contaminants in contaminated soil. The finding of this research work will help to improve

bioremediation technologies by incorporating microorganism equipped with one or more characteristics that are needed for microorganism to have direct access to sorbed contaminants. Consequently, the research finding may be instrumental in understanding enhance bioavailability, which play a major role in clean-up time, cost and end-points of the bio-remediation efforts.

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CHAPTER 2. LITERATURE REVIEW

INTRODUCTION

Bioremediation of soil contaminated with polycyclic aromatic hydrocarbons (PAHs) and other hydrophobic organic compounds (HOCs) is believed to be limited by slow transfer of these compounds from the soil matrix to the aqueous phase (Harms and Bosma 1997; Ogram et al. 1985; US-EPA 1991). Many organic compounds that are easily biodegradable in solution have been found to persist when sorbed to a soil or sediment matrix. The (bio) availability of sorbed compound to bacteria via the aqueous phase is, therefore, considered a limiting factor for successful bio-remediation. Bioavailability is considered a dynamic process dependent on the interplay between physicochemical and microbial processes (Luthy et al. 1994; Pignatello 1999). Any change in either one of these processes will affect bioavailability and, hence, the rate and extent of bioremediation.

The physicochemical processes include sorption, desorption, intra-aggregate or intra-particle diffusion, and sequestration. These processes, in turn, are dependent on the characteristics of the chemical, the soil matrix, and the residence time of the chemical. Whereas considerable effort has been undertaken to study the characteristics of soil-chemical interactions in soil, little was known about the uptake or mass transfer-enhancing strategies of the bacteria. However, the roles of bacterial phenotypes (physiology, adaptation, etc.) on the rate of desorption or direct uptake of the sorbed phase is rapidly gaining attention.

Evidence supporting the role of specific phenotypes (chemotaxis, mobility, adhesion, ability to produce bio-surfactants, etc.) is slowly accumulating(Guerin and

Boyd 1993; Harms and Zehnder 1995; Tang et al. 1998). The bioavailability is likely to be a function of both physiological properties of bacteria (chemotaxis, motility, and attachment) as well as soil and contaminant processes (sorption, desorption, etc.). Any change in either microbial physiological properties or sorption-desorption characteristics is expected to affect the contaminant availability to the microbial uptake. It is expected that bioavailability of the sorbed phase naphthalene varies with strains having different physiological properties as well as with sorbents with different sorption/desorption properties. In the following paragraphs, a brief account of important physio-chemical and biological processes in controlling bioavailability will be discussed.

AFFECT OF SORPTION/DESORPTION KINETICS ON BIOAVAILABLITY

For solute to be available for microbial uptake, it has to be accessible by being present either in the aqueous phase (dissolved) or on the surface of the solid phase (partition). Limited bioavailability can occur when organic compounds are either poorly water-soluble, strongly sorbed to a sorbent, or spatially separated from the microorganism (Mihelcic et al. 1993). The pollutants become inaccessible to microorganisms when they are absorbed into natural organic matter or adsorbed into the walls of submicron-sized pores inside soil particles. The adsorbed compound can only be taken up by a bacterium, if it diffuses out/desorbed of the pores of sorbent particles to the solid-water interface and is then partitioned into the water phase. These general observations support the theory that desorption and sorption processes are the limiting factors during bioremediation of hydrophobic organic compounds in a contaminated soil-water system. Therefore, it is important to include terms for sorption and desorption in the bioavailability model; ignoring the kinetics of these processes may lead to false

predictions about bioavailability of compounds. In the following paragraph a brief overview of sorption and desorption, their mechanism, and a conceptual model will be provided.

Sorption Process

The process of sorption involves partitioning of hydrophobic compounds, initially dissolved in water phase only, between water phase and the sorbent and results in a considerable removal of the organic compound from the water phase. Sorption processes have been considered to be of two types: partition and absorption (Chiou 2002). While, the partition is a surface phenomenon in which the solute accumulates at the surface or interface between water and sorbent phase; the absorption, on other hand, refers to contaminant sorption to the wall of pores inside soil particles. Sorption in soil and sediments is mainly attributed to the soil organic matter content. Sorption of HOCs has been shown to increase with hydro-phobicity of the chemical and the content of soil organic matter. The process of sorption is considered, since early 1980, as a reasonably fast process on a practical time scale ranging from less than a minute to a few days (Pignatello and Xing 1996a). When sorption is fast, and completely reversible, a linear isotherm equation can adequately describe the process as follow:

$$Cs = K_d \cdot C_e \qquad [1]$$

Where C_s and C_c represent the concentration of the compound in water and the solid phase, respectively; and K_d is the soil-water partition co-efficient. An example of a system that shows linear isotherm is the partition of HOCs into natural Organic matter of soil (also called soft SOM).

Numerous laboratory and field studies in the early 1990s noted that sorptive equilibrium may take weeks to months depending on the compound, and ideal linear relationships no longer remain valid (Ball and Roberts 1991; Young and Weber 1995). The simplest equilibrium model could not account for the non-ideal sorption behavior of HOCs as observed in heterogeneous soils or soils containing hard SOM (kerogen –like SOM e.g., soot). The sorption is, therefore, most commonly represented by the Frendulich equation as given by Equation 2:

$$Cs = K_f \cdot C_e^{\,n} \qquad [2]$$

Where n is an empirical constant indicating non-linearity of the isotherm, and K_f is the Frendulich capacity factor.

The long-term non-equilibrium sorption shows a bi-phasic pattern in which there is an initial rapid sorption portion followed by a much slower component of sorption. Several mechanisms have been proposed for non-equilibrium sorption, including: 1) intra-particulate diffusion (pore-filling), 2) the presence of a small amount of high-surface area carbonaceous material (HSACM) , and 3) the distributed reactivity model or dual mode sorption model. The first mechanism assumes that organic chemical, as taken up rapidly due to sorption by external sorption sites, can move slowly into internal sites and is retarded by very slow diffusion into tortuous pores of either an organic matrix or micro-pores of the mineral domain of soil (Wu and Gschwend 1986). This mechanism is supported by evidence that larger particles had a slow sorption rate, and dis-aggregation of larger particles into smaller particles improved the sorption rate (by reducing diffusive path the length).

The second proposed mechanism stipulates the presence of small amounts of high-affinity adsorption sites (e.g., charcoal like materials) besides significant amounts of SOM in the soil. The sorption on charcoal like material is largely an adsorption process (competitive) and gives non-linearity to the overall isotherm shape (Chen and Wagenet 1995; Chiou et al. 1998; Chiou et al. 1979). The distributed reactivity model (Weber and Huang 1996) hypothesizes that soil organic matter consists of two sorption domains: a rubbery or soft carbon domain (domain I) and a glassy or hard carbon domain (domain II), both domains contributing to the overall sorption process. Domain I exhibits a fast, reversible, and linear isotherm; domain II, on other hand, shows a non-linear, and adsorption (competitive) type of sorption. The overall sorption of a representative PAH is the sum of the fast and slower components that takes the following mathematical form:

$$C_s = f_s \cdot K_{oc} \cdot C_w + f_h \cdot K_f \cdot Cw^n \qquad [3]$$

Where f_s and f_h represent the weight fraction of rubbery or soft carbon and glassy or hard carbon, respectively.

Desorption Process

The desorption process is the opposite of the sorption process in which sorbed phase molecules diffuse out from surfacial and internal sites of the soil particles into the soil water. Desorption results in the removal of the chemicals from the solid phase and the concentration of the chemicals in the soil water. Desorption of HOCs from soils and sediments have also been observed to take place in two stages: a rapid stage followed by a stage of much slower release. Also, a number of studies have shown that a fraction of sorbed contaminants is difficult to remove, and this fraction has been called the

irreversible fraction (Kan et al. 1997; Khan 1991; Pignatello and Xing 1996a; Pignatello and Xing 1996b). Several researchers observed that fractions of slower release and irreversible components of desorption increase with the increasing contact time of organic compounds in soils; this phenomenon is known as the aging of organic compounds (Huang and Weber 1997; White et al. 1997). The most common mechanisms responsible for sequestration are the entrapment of HOCs within SOM matrices, the formation of HOCs-soil organic matter (SOM) complexes, and chemical degradation.

Several models have been developed to describe the kinetics of de-sorption including the two-site non-equilibrium model, the intra-aggregrate diffusion model, and the stochastic-Gamma model. None of them, however, incorporate the irreversible component of the desorption. In this study, I used a three-site desorption model (Park et al. 2001) to analyze the desorption data for rate of release of selected compound. The underlying assumptions for the models are : 1) the overall desorption sites consist of fast, slow, and irreversible components 2) sorption is a relatively fast process and approximated by a linear isotherm 3) the rate of non-equilibrium release rate is proportional to the difference between the concentration in liquid and non-equilibrium sorbent sites. The overall desorption site is theoretically represented by:

$$S = S_{eq} + S_{neq} + S_{nd} \qquad [4]$$

Where S(μg/Kg) is the total sorbed phase concentration, and S_{eq}, S_{neq}, and S_{nd} (μg/Kg) represent respectively equilibrium, kinetic, and irreversible site fractions of desorption. The equilibrium process is assumed very fast and the corresponding site (Seq) can be represented by a linear partitioning model of

$$S_{eq} = f_{eq} \cdot K_d \cdot C \qquad [5]$$

Where C (μg/L) represents the liquid-phase concentration, f_{eq} is the equilibrium site fraction, and K_d(L/Kg) is the sorption distribution co-efficient. The rate of contaminant release from non-equilibrium site is assumed to follow the following first-order equation:

$$J_n = dS_{neq}/dt = \alpha (S_{neq} - f_{neq} K_d C) \quad [6]$$

Where f_{neq} is non-equilibrium site fraction, and α (min^{-1}) is the desorption rate coefficient. The term K_d is determined from sorption isotherm. Desorption site fractions f_{eq}, f_{neq} and desorption rate α are estimated by fitting non-linear regression equation to desorption data, with the constraint that sum of all site fractions is equal to unity. The non-desorbable fraction is, however, determined from the plateau of desorption data profile.

INFLUENCE OF MICROBIAL KINETICS ON BIOAVAILABILITY

In soil systems, the degradation of organic compounds may be monitored by measuring with time either the parent compound or the product formation (such as CO_2). An understanding of the kinetics of degradation is important to predict (through mathematical models) the persistence of organic substances in the environment. The kinetics of degradation defines the relationship between the concentration of a compound of interest and the rate at which microorganism can degrade it (Grady et al. 1996). The natural environment contains different amounts of organic compounds, and as a result there exist different microbial population with defined kinetic properties. Several

different models have been proposed to describe the microbial degradation in soils, and the most commonly used models are described as follows:

Bio-degradation by Growing Micro-organisms

The empirical Monod model is commonly used to describe microbial growth kinetics under nutrient limited conditions. The concentration of the substrate is assumed to be in excess to support microbial growth. For pure culture, the Monod equation can be written as

$$\mu = (\mu_{max} \cdot S)/(K_s + S) \qquad [7]$$

Where μ is the specific growth rate, μ_{max} is the maximum specific growth rate, S is the organic substrate concentration, and K_s is the half-saturation constant or affinity constant and is taken as the substrate concentration at which the rate of growth is half the maximum growth rate. The two kinetic constants (μ_{max}, K_s) of the Monod equation have been widely used to differentiate between two groups of microbes: 1) the slow grower or oligotroph which exhibits an extremely low affinity constant value K_s, and 2) the fast grower or copiotroph which displays a high growth rate and a very high K_s value. The oligotroph, with low maintenance requirement as compared to copiotrophes, can potentially degrade contaminants to low endpoints in a diffusion-limited microenvironment. Several other growth models (e.g., logarithmic, Logistic, etc.) were proposed that may be applicable under different substrate concentration and bacterial K_s value.

Biodegradation by Non-growing Microorganisms

If the substrate concentration is low (rate limiting chemical) as compared to the cell density, the microbial growth assumed is either very small or negligible. Under this condition, the degradation kinetic is best described with the Michelis-Menten equation. According to this equation, the flux of the chemical through the cell membrane R_{bio} is

$$R_{bio} = (V_{max} \cdot C) / (K_m + C) \qquad [8]$$

Where V_{max} is the maximum flux that a cell can get, C is the chemical concentration and K_m is the Michael-Menten constant (half-saturation constant or substrate affinity) which is the chemical concentration corresponding to ½ V_{max}. Integrating Equation 8, and re-arranging its term, results in the following equation:

$$t = [-(K_m \cdot \ln(C/Co) + C-C_o)/V_{max}] M_o \qquad [9]$$

Where C_o is the initial substrate concentration, M_o is the microbial population density, and t is the time. The two parameters K_m, and V_{max} can be obtained by fitting the substrate depletion data with the integrated Michalis-Menten Equation (9)

When the concentration of the compound being degraded by non-growing bacteria is low, relative to the biological activity ($C<<K_m$), the flux through the cell membrane follows the first order kinetics, which is written as:

$$R_{bio} = k_l \cdot C \qquad [10]$$

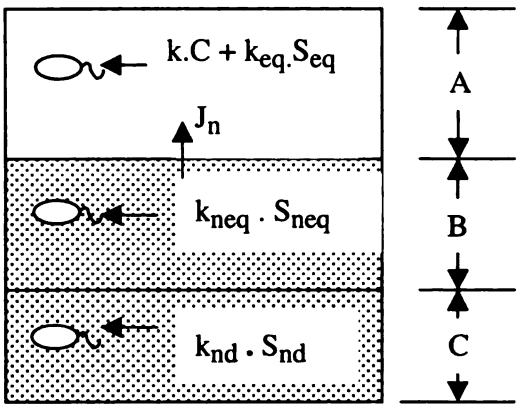
Upon integrating and re-arranging equation 2) takes the form $\ln(C/C_o) = -k_l \cdot t$, and can be solved graphically to determine the rate constant k_l. The first order expression

is often used to describe biodegradation of contaminants, which is low in concentration due to mass transfer limitation, in the soil (Bosma et al. 1997).

Bioavailability Models

In a slurry bio-reactor, the intracellular degradation of HOCs depends on their availability to the degrading organisms. Traditionally, it has been assumed that sorption reduces bioavailability and only readily desorbed and aqueous-phase substrate are available to microorgansms. However, there are some studies that suggest sorbed compounds are available to microorganisms without prior desorption (Guerin and Byod 1992 and others). Recently, Park et al. (2001) demonstrated that naphthalene degrading bacteria (*Pseudomonas putida* G7) could access to soil-sorbed naphthalene and, consequently, the observed degradation rate in soil slurries exceeded those predicted from the independently determined rate of de-sorption. In light of the above, several approaches can be taken to model bioavailablity depending on whether the sorbed phase is available or unavailable to the microorganism. In this study, it has been assumed that the selected microorganism (*P. putida* G7) can have direct access to the sorbed phase substrate.

The bioavailability model to be used incorporated a first-order three-site desorption model, linear sorption, and biodegradation. The graphical representation of the model is shown in Figure 2-1.



A = Dissolved and Equilibrium site

B = Non-Equilibrium site

C = Irreversible phase

Jn = mass flux from non-equilibrium site

Figure 2-1. Description of Desorption-Biodegradation model (DBM)

The model is based on the following assumptions: a) the desorption parameters as measured in abiotic condition are applicable in the bio-availability assays; b) degradation rates of the dissolved naphthalene remain the same in the presence or absence of the sorbent c) both the attached and suspended cells can utilize the dissolved naphthalene equally. The overall degradation of naphthalene in a batch slurry reactor using mass balance on naphthalene can be written as:

$$-(V \cdot dC/dt + m \cdot dS/dt) = V \cdot R_{bio} + m \cdot (k_{eq} \cdot S_{eq} + k_{neq} \cdot S_{neq} + k_{nd} \cdot S_{nd}) \quad [11]$$

Where m (kg) is the total mass of the soil, V(L) represents the volume of the liquid, and k_{eq}, k_{neq}, and k_{nd} are the first-order biodegradation coefficients of sorbed naphthalene in equilibrium, non-equilibrium, and non-desorption sites, respectively. The term R_{bio} is the rate of biodegradation of naphthalene in the liquid phase only and is defined in Equation-8 in the above section. To track the sorbed-phase naphthalene concentration in non-equilibrium and irreversible sites, the following mass balance equations are required:

$$dS_{neq}/dt = J_n - k_{neq} \cdot S_{neq} \quad [12]$$

$$dS_{nd}/dt = -k_{nd} \cdot S_{nd} \quad [13]$$

The different rate constants (k_{eq}, k_{neq}, and k_{nd}) are determined by a nonlinear regression analysis of the bio-availability data.

BACTERIAL PHYSIOLOGICAL CONSIDERATIONS

Recently, several studies reported evidence that there exist significant diversity in the abilities of bacteria to degrade soil-sorbed HOCs (Guerin and Boyd 1993, Tang et al. 1998, Calvillo and Alexander 1996). The possible physiological explanation for such diversity includes the production of biomaterial, variation in attachment to solid surfaces, and chemotaxis/motility traits. For example, two naphthalene-degrading bacteria (*Alcaligenes sp.* NP-Alk and *Pseudomonas putida* ATCC 17484) have been shown to possess differential capabilities to degrade soil-sorbed naphthalene (Guerin and Boyd 1993). It was hypothesized that the attachment capability of isolate ATCC 17484 to solid surfaces caused enhanced degradation due to 1) the exposure of cells to a steeper intra-sorbent concentration and, 2) cells act as competitive sorption media to a soil-sorbed

naphthalene. Other studies have shown the potential of chemotaxis in enhancing the biodegradation of organic compounds such as naphthalene (Marx and Aitken 1999), carbon tetrachloride (Witt ME 1999), BTEX compounds (Parales et al. 2000), and pesticides (Hawkins 2002) in laboratory scale microcosms. The following paragraphs deal with a brief description of important bacterial strategies to enhance the degradation of toxic compounds in a soil water system.

Bacterial Chemotaxis and Enhanced Bioavailability

Bacterial chemo-taxis is defined as the movement of motile cells toward (in case of attractants such as nutrient sources), or away from (in case of repellent such as toxic compounds) the region of elevated chemical concentration gradient in the media (Harwood et al. 1994). Movement toward the chemo-attractant is called positive chemotaxis, whereas movement away from the chemo-repellent is termed as negative chemotaxis. In the absence of a chemical gradient, the bacteria moves in a random fashion with no directionally biased movement. The random movement consists of a run, a brief forward movement, followed by a tumble, when the cell stops and bounces in a random direction. After each tumble, the bacteria re-orients randomly for the next run. Thus bacteria move randomly in an environment by means of runs and tumbles without any net directional movement. In the presence of a chemical gradient, these random movements become biased. The run length increases in proportion to increasing chemical concentration and is accompanied by less frequent. The net result of this behavior moves the bacteria in the direction of higher concentration of the attractant (Parales et al. 2000).

The mechanism controlling bacterial movement involves a signal transudation system, which transmits signals to the cell about the presence of concentration gradient. The signal transudation system consists of two sets of sensory proteins: 1) sensor protein located in the membrane and 2) a cytoplasmic protein. The sensor protein has a very high affinity for the attractant or repellants. As the concentration of attractants or repellants increases over a period of time, more and more binding of these molecules on sensor protein takes place. This binding sets in play a series of interactions with the cytoplasmic protein that eventually affects flageller rotation to increase or decrease the bacteria run length.

Chemotaxis may play an important role in enhancing bio-availability and , consequently, accelerates biodegradation. Chemotaxis allows cells to move in the direction of higher concentration, and thus bringing them in contact with the chemicals to be degraded. In soil contaminated with hydrophobic compounds, chemotaxis may enable microbes to access zones of concentrated chemical for degradation that would otherwise be inaccessible due to mass transfer limitations, low solubility or sequestration of chemicals to or inside a solid matrix. This is all the more important in heterogeneous soils, where there exist large variations in sorbed concentrations across different regions, and chemotaxis may help cells locate contaminants in these different regions (Jain 2002).

Most prokaryotes are motile, and many are chemotactic to various toxic organic compounds such as PAHs. *Pseudomonas putida* G7 and *Pseudomonas sp.* NCIB 9816-4 have recently been shown to be chemotactic to naphthalene. The chemotactic response is due to catabolic plasmid present within these organisms. A mutant derivative of *P. putida* G7 (G7.C1) which is devoid of plasmid (NAH7) was shown to be non-chemotact to

naphthalene. It has also been shown that the chemoreceptor gene (NahY) encoded by the NAH7 plasmid is involved in naphthalene chemotaxis (Grimm and Harwood 1997; Grimm and Harwood 1999).

Limited studies have been carried out to demonstrate the potential role of chemotaxis in rapid biodegradation of organic compounds in aqueous and porous media. In studies involving an aqueous medium alone, the concentration gradient was established by supplying naphthalene either from a glass capillary tube (Marx and Aitken 2000) or a NPAL-water interface (Law and Aitken 2003). Both studies clearly demonstrated that naphthalene was degraded at a higher rate by the wild-type strain than either the non-motile strain or the non-chemotactic strain. It has been shown that the wild-type strain could overcome mass transfer limitations by accumulating in regions of higher concentration that led to a rapid biodegradation rate in both studies.

Experiments with a porous medium, packed in sand column, involved detecting changes in the bacterial density distribution with time in response to concentration gradients. The concentration gradient establishes as bacteria continue to uptake compound that was present at an initial constant concentration. The experiments with porous medium, however, either failed to observe chemotaxis (Barton and Ford 1995) or provided indirect evidences of chemotaxis (Pedit et al. 2002; Witt ME et al. 1999). The presence of the porous medium has been shown to cause significant reduction in the bacterial chemotactic and motility responses. Also, if the initial isotropic concentration of the compound exceeds, the saturation of the chemoreceptor would lead to the suppression of chemotactic responses (Barton and Ford 1995 and 1997).

Witt et al. (1999) demonstrated the movement of *Pseudomonas stutzeri* KC in columns packed with aquifer sediments. In a continuous flow column, the cells migrated through the column at a velocity exceeding the average linear velocity of flow. The enhanced movement was attributed to the creation of a nitrogen gradient as a result of the rapid consumption of nitrates by the bacteria. Experimental results with no flow column showed that the mean velocity of KC cells due to chemotactic movement towards increasing concentration of nitrates was 5 cm/day. However, the study was not able to distinguish between chemotactic and random motility.

Padit et al. (2001) clearly demonstrated the role of chemotaxis in increased biodegradation of naphthalene in a porous medium. They used an experimental system similar to Marx et al. (1999) with the exception that both the micro-capillary and the reservoir were filled with glass beads. It was shown that chemotaxis can occur in a porous medium; however, the presence of solids had significantly reduced the chemotaxis responses as compared to responses measured in a solid-free system.

Bacterial Adhesion

Adhesion is shown to be an important mechanism through which microorganisms are involved in the direct utilization of sorbed chemicals (Calvillo and Alexander 1996; Harms and Zehnder 1995). The phenomenon of adhesion suggests that microorganisms possess some special cell walls and/ or bonding properties that help them to adhere to hydrophobic surfaces containing sorbed chemicals. Bacteria, adhering to solid surfaces containing sorbed chemicals, experience a microenvironment that is different from the surrounding bulk liquid. The microenvironment, which lies within a thick static boundary

layer at the solid-water interface, provides a steep concentration gradient to bacteria that results in the degradation of the sorbed substrate. The role of adhesion in providing bacteria direct access to the sorbed phase organic pollutant is illustrated in Figure 2-2

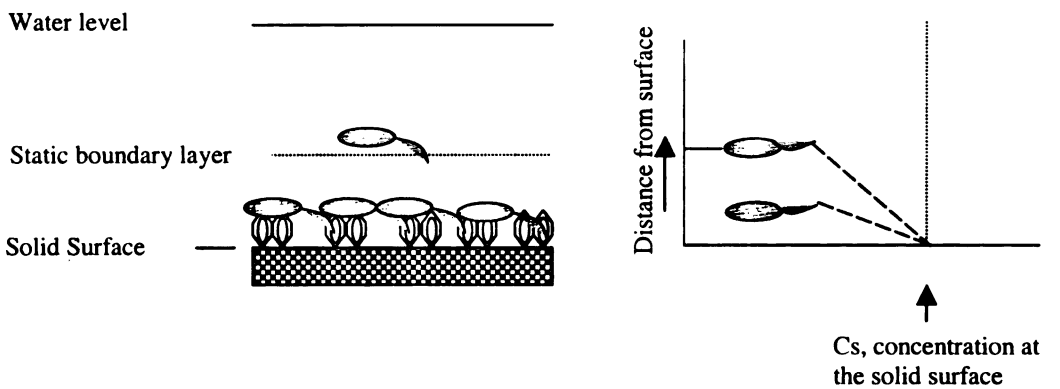


Figure 2-2. Adhesion as a bacterial strategy to directly access sorbed chemicals at solid

The attachment to the solid surface exposes bacteria to a steep concentration gradient by shortening the diffusion distance. To understand the mechanisms responsible for bacterial adhesion to solid surfaces, it is important to know the structure and properties of bacterial cell surfaces.

Structure of the Gram-negative Cell Surface

The bacterial cell surface is a three dimensional, multi-layered, and complex structure. The surface of most bacteria consists of three layers: the outer membrane with associated structures, the cell wall, and the inner cytoplasmic membrane. Of these three layers, the outer membrane is mainly responsible for cell surface properties. The schematic of the outer membrane along with its associated lipopolysaccharides layer

(LPS) is shown in Fig 2-3. The outer membrane is a coating of protein macromolecules that provides an additional layer on top of the cell wall. The outer membrane serves to protect the cell and also gives the cell shape and rigidity. It contains pores that facilitates the selective transport of water and nutrient molecules and avoid the entry of toxic substances. On top of the outer membrane there exist additional structures made up of

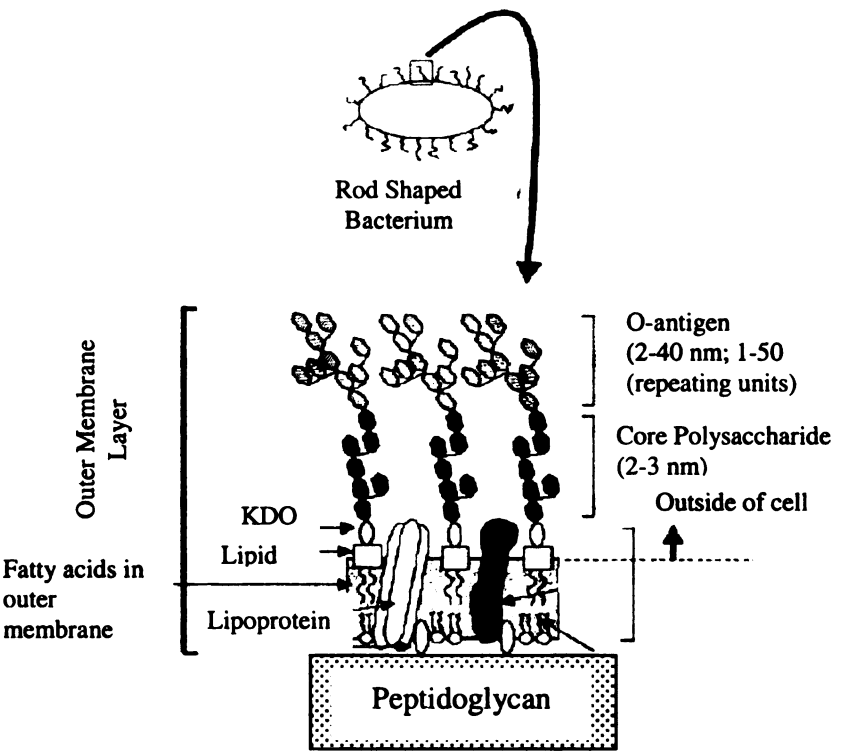


Figure 2-3: Schematic of the outer membrane of gram negative bacteria along with its associated LPS layer

protein and polysaccharides molecules; they form what is often called lipo-polysaccharide layer or simply LPS. The LPS is composed of three components and is anchored into the hydrophobic region of the outer membrane by its lipid-A component. The lipid-A component is then attached to a second component of LPS, called core

polysaccharide, which consists of a unique eight-carbon sugar called 2-keto-3-de-oxyoctonic acid (KDO). The core polysaccharide region of the LPS is negatively charged due to the presence of phosphates and carboxylic groups. The outer part of the LPS is the O-antigen, which consists of 15 to 20 repeating units of three to five sugars and can extend out from the cell surface upto 50 nm into the cell surroundings (Brock 2000).

A variety of different other structures can also be found on top of the outer membrane; they include flagellae, fimbriae, and fabrils. They are appendages of different length and diameters and are mainly proteinaceous in chemical composition. Many bacterial strains also produce polymeric substances that are referred to as extra-cellular polymeric substances (EPS). EPS may be secreted as a loose layer of carbohydrates called the slime layer or a more rigid layer of proteins known as the capsule (Fletcher 1985; van Loosdrecht et al. 1990).

The adhesion of bacteria to a solid surface has been described as a four-step process (van Loosdrecht et al. 1990). The first step involves the transport of bacteria toward the solid surface by one of three different modes: diffusion due to Brownian motion, advection due to liquid flow, or active movement of the cell due to chemotactic response to the concentration gradient using flagellated structures. The diffusion process is the slowest of all (at the rate of 40 um/hr) and occurs due to the random interaction of cells with surfaces. The advective transport is, on other hand, a very fast process depending on the velocity or mixing of water. Finally, bacteria respond to the existence of the chemical gradient in a heterogeneous medium and move with the help of flagella toward a region of higher concentration (van Loosdrecht et al. 1990).

Once bacteria come in contact with a solid surface, initial adhesion takes place. The initial adhesion may be reversible or irreversible depending on the mechanism involved. During a reversible adhesion, there is a continuous exchange between the free and adhered cells. The initial adhesion of bacteria can best be described by the DLVO (Derjaguin, Landau, Verwey, Overbeek) theory. The DLVO theory envisages adhesion as an interplay of long ranging electrostatic and van der Waals interactions; and initial adhesion occurs when attractive forces overcome repulsive forces. The main factors influencing these interactions are surface charges and hydro-phobicity. The electrostatic forces arise due to the fact that both the bacterial surface and solid surfaces are negatively charged. Therefore, when bacteria approach solid surfaces, they experience electric double layer repulsion. The intensity of the double layer repulsion depends on the concentration and valency of the ions in the culture medium. The electrolyte repulsion can be decreased or eliminated by increased electrolyte concentration of the culture medium (van Loosdrecht et al. 1989).

Figure 2-3 illustrates how the interaction of long ranging attractive and repulsive forces governs initial adhesion at various separation distances between bacterium and solid surface. When cells approach very close to surfaces, and this occurs at high ionic strength, the attractive forces become very strong, creating a primary minimum. In this situation, the primary attractive forces are hydrogen bonding and ion pair formation. As the bacterium moves slightly away from the surface, repulsive forces grow quickly and cause the adhesion to cease. As cells move further away from the surface, another shallow minimum exists called the secondary minimum. The reversible adhesion is thought to exist at the secondary minimum and both cells and surface are not in actual

contact at this distance of separation. The reversible part of initial adhesion is very weak, and cells can easily be removed from surfaces due to slight shear force (Rijnaarts et al. 1993; van Loosdrecht et al. 1989).

There are, however, experimental evidences that indicate that other factors such as outer membrane proteins, composition of LPS, flagella, and fambriae may be required for the initial adhesion to surfaces (DeFlaun and Fletcher 1999; Fletcher 1985). There are many examples of bacteria adhering to a solid surface due to hydrophobic interaction, thus overcoming any water barrier to bacteria attachment (Stenstrom 1989). After the initial adhesion, irreversible attachment of cells can occur due to the adsorption of polymer to surfaces. The LPS polymers are long enough to bridge the distance between the cells and surfaces, even if no DLVO attraction exists. In irreversible attachment, cells form mono-layer on the surface. During the final stages of adhesion, the attached cells grow in micro-colonies and ultimately form a multi-layer growth called bio-film (van Loosdrecht et al. 1990).

Mechanism of Adhesion

Mechanisms of attachment to inanimate surfaces is still controversial due to the wide variation of cell surface properties among bacteria and also because bacteria cell surfaces are dynamic in nature due to varying environmental conditions. It is well known that bacteria have different abilities to attach to surfaces. Even for a given population of bacteria, some cells attach, while others remain suspended, and there can be a significant difference in adhesion properties even among the clones of a single organism (Fletcher 1996).

The mechanism of bacterial adhesion is complex and unlikely to be explained by one or other property of the cell surface (Bos et al. 1999). There are a number of factors that contribute to the adhesiveness of the bacterial surface that include cell surface charge, bacterial hydrophobicity, the presence of extra-cellular polymers, and flagella (Deflaun 1999). Besides cell surface properties, bacterial adhesion is also influenced by the chemical composition of the solid surface and the liquid medium.

Numerous studies have demonstrated that cell adherence increases with more cell hydrophobicity (as measured by contact angle), and less negative charge (as measured by electropherotic mobility) (e.g., van loosdrecht et al 1987, Stenstrom, 1989). Mueller et al. (1992), found that bacteria with low hydrophobicity (*P. flouresences*) attached at far lower rate (five times) on to a glass surface than bacteriam with high cell hydrophobicity (*P.* aeruginosa). Bestiaens et al. (2000) isolated adherent PAH-degrading bacteria by an enrichment technique using hydrophobic membranes containing sorbed PAHs. The isolated bacterium was shown to be very hydrophobic and adherent to different surfaces. Microbial surface charge can also influence adhesion, but to a smaller degree as compared to cell hydrophibicity. For example, adhesion of the cells increased, when the surface charge of the cells was chemically modified from a negative to a positive charge (Klotz et al. 1985). Cells are defined to be hydrophobic when the measure of water contact angle is above 30. Also, the experiment using Gram-negative indicated that adhesion with polystyrene and rice roots becomes zero, when the value of zeta-potenial becomes more negative than –25 V (Bos et al 1999).

LPS and EPS have also been shown to play an important role in cell adhesions, and are especially useful for strengthening cell binding to surfaces (Rijnaarts, 1995;

Fletcher, 1996). However, the characteristics of specific polymers (e.g., compostion, size, charge, etc.) in turn play an important role in adhesion to the surfaces (Fletcher 1996). Razatos et al. (2000) used and compared interactions of the wild-type strain (with longer LPS chain) and its mutant (with truncated LPS chain) with glass surfaces. The authors reported that while the wild-type was able to adhere to the glass surface, the mutant was not able to adhere. They hypothesized that as the mutant lost its core polysaccharide component of LPS, the exposed negatively charged KDO molecules experienced a repulsive interaction with negatively charge glass surface. The wild-type on the other hand had experienced an attractive interaction due to the shielding of the negatively charged KDO by longer LPS molecules.

Deflaun et al.(1999) also investigated the role of cell surface-polymer in adhesion of *B. cepacia* G4 to sand surfaces. The authors isolated the adhesion-deficient mutant of *Burkholderia cepacia* G4 in a sand column assay and compared its adhesion phenotypes to that of the wild-type strain. The mutant was reported to have lost its adhesion phenotype due to the attenuated O-antigen on the LPS. The loss of O-antigen part of LPS caused a reduced shielding of the charged group near the membrane (such as the phosphate group in the core-lipid A region) and, consequently, there was a net repulsive interaction between the mutant and the negatively charged sand surfaces.

In some studies the truncation of the LPS, however, resulted in a more hydrophobic and, hence, more adherent mutant than that of the wild-type (Fletcher et al. 1996). The authors generated and isolated mutants of *pseudomonas fluorescens* that were altered in adhesion ability. The mutants were demonstrated to have increased adhesion to a variety of surfaces (quartz sand, hydrophobic polystrene) when compared to the

adhesion of the wild-type strain. The mutants showed an increased attachment to hydrophobic surfaces and a decreased attachment to the more hydrophilic sub-straturm. The authors attributed the enhanced adhesion property of the mutant to attenuation of the O-antigen on the LPS. The lack of the O-antigen exposed lipid moiety and, hence, rendered the cell surface more hydrophobic.

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CHAPTER 3. ENHANCE BIOAVAILABILITY OF SORBED PHASE NAPHTHALENE: A COMPARISON OF BIODEGRADATION AND DESORPTION RATES IN NATURAL SOIL

ABSTRACT

The removal of naphthalene in a soil-slurry system was studied using the bacterial strain *Pseudomonas putida* G7 (*Pp* G7), Tenax beads extraction, and serial extraction with a 0.1 M buffer solution alone. The overall removal of naphthalene by Tenax beads was observed to be greater than that obtained with serial desorption. At high concentration both Tenax and serial dilution showed similar trends for naphthalene removal rate. However, Tenax extracted more sorbed phase naphthalene than the serial dilution method. A comparison of the results of *Pp* G7 biodegradation and Tenax extraction showed that the rate and extent of naphthalene removal by biodegradation was at all times significantly greater than the corresponding Tenax extraction of sorbed phase. It seems that naphthalene does not have to be desorbed in to the aqueous phase for biodegradation by *Pp* G7. It appears that *Pp* G7 was able to overcome the mass-transfer limitation and directly take-up sorbed-phase naphthalene. The experimental results showed that *Pp* G7 degraded sorbed naphthalene at a rate and extent greater than what can be removed by both Tenax beads and serial extraction methods. Also, explored was the effect of excreted biomaterial on enhancing bioavailability. The activity of extra-cellular enzymes was eliminated during the biodegradation study, as the experiment with the bacterial filtrate did not result in any degradation of observable free naphthalene

during a 3-days incubation period. It was observed that the partition coefficient (K_d) of naphthalene to Colwood soil was reduced as the concentration of biomaterial increased. However, the reduction in K_d was observed to be statistically insignificant at a biomaterial concentration as obtained at naphthalene concentration less than 10 mg/L. It is, therefore, expected that any sorption experiment carried out with biomaterial obtained at less than 10 mg/L of naphthalene will not significantly affect the partition coefficient of naphthalene. All bioavailability experiments were carried out at 2 mg/L of naphthalene; thus, the possible effect of corresponding biomaterial on partition co-efficient (and consequently mass transfer) of naphthalene is highly unlikely.

INTRODUCTION

Sorbed phase HOCs have been reported to be available for biodegradation without prior desorption into the aqueous phase(Guerin and Boyd 1992; Guerin and Boyd 1993; Park et al. 2001; Tang et al. 1998). This finding has led to the concept of enhanced bio-availability, which has been defined as the availability of organic compounds for bacterial uptake at a rate greater than abiotic desorption rate from the sorbent. The evidence that non-desorbable fraction of sorbed phase PAHs can be available for bacterial uptake has significant implications for remediation endpoint and risk assessment. Enhanced bioavailability of soil-sorbed contaminants may render bioremediation a viable technology that will have the potential to decrease residual soil/sediment contaminant concentration to an environmentally acceptable endpoint that meets regulatory requirements.

The previous studies mostly put forward indirect evidence of enhanced bio-availability (Harms and Zehnder 1995; Park et al. 2001). They compared independently measured rate and extent of desorption with intrinsic activity of the test bacteria to define enhance bioavailability. For example, Harms and Zehnder (1995) reported that attached bacteria degraded 3-cholorodibenzofuran from porous Teflon beads at a rate greater than abiotic desorption rate that was measured by sequential replacement of the aqueous phase. No study has, however, measured desorption kinetics during bio-remediation treatment.

The approach of independently measuring abiotic desorption rate has some potential limitation in determining enhance bio-availability. Some investigators have assessed that rate and extent of desorption measured under abiotic condition does not represent rate of desorption in presence of degrading bacteria. The desorption rate changes due to utilization of contaminants by degrading bacteria in aqueous phase, leading to the desorption of more contaminant from the sorbent to the aqueous phase. Furthermore, water alone seems to be a poor choice of extractant for determining non-desorbable fraction of HOCs because: 1) HOCs are sparingly soluble in water and strongly sorbed and, 2) water cannot access labile fraction of the contaminant in sorbent matrix. This view has been has been supported by earlier researcher that concluded that desorption rate alone may under-predict biodegradation potential of bacteria (Cornelissen et al. 1997b).

Furthermore, previous experimental studies also examined role of bacteria and suggested that the utilization of non-desorbable PAHs requires bio-availability-enhancing strategies of the bacteria (Calvillo and Alexander 1996; Guerin and Boyd 1992). One of

the strategy that bacteria can employ to enhance bioavailability is to excrete compound that can either enhance desorption rate or degradation of sorbed phase naphthalene. These compounds are extra-cellular in nature and include catabolic enzymes and polysaccharides substances.

While the concept of enhance bioavailability is appealing, the actual mechanism responsible for the phenomenon is still not clear. The main objective of this study was to observe phenomenon of enhanced bio-availability by directly comparing abiotic desorption in presence of Tenax and biodegradation rates for naphthalene at various times during the treatment period. In the absence of a technique to measure desorption rate in the presence of bacteria, we used equivalent amount of Tenax beads to represent degrading bacteria. The Tenax beads have been shown to be a suitable adsorbant to assess desorption rate and extant during biodegradation treatment. When place into contact with a soil-water slurry, Tenax maximizes the diffusion gradient by acting as an infinite sink for desorbing chemicals. Thus, the contaminant mass transfer rate sensed by degrading bacteria cells could potentially be represented by those measured in the soil-slurry using Tenax beads. Possible role of extra-cellular biomaterial (such as enzymes and polymer) was also explored to explain enhance bioavailability. Two hypotheses were tested: 1) *Pp* G7 does not produces extra-cellular enzymes during uptake of naphthalene and, 2) PAHs adsorb to Extra-cellular polymeric substances (EPS) from soil phase and thereby become more available to the degrading bacteria.

MATERIAL AND METHODS

Sorbent

The three soils selected for this are CapacA, Colwood, and Houghton Muck with low, intermediate and high soil organic content. . The soil was prepared as described elsewhere (Park et al. 2001), and their characteristics are summarized in Table 3-1.

Chemicals and Culture Medium

Both ^{14}C-labeled and unlabeled naphthalene were used in this study. The ^{14}C-labelled naphthalene (sigma, 0.1 mCi, >98% pure) stock solution was prepared by supplementing it with unlabelled naphthalene and was used for sorption, desorption, serial dilution studies. The un-labelled naphthalene (sigma, > 98%) stock solutions were prepared in methanol and stored in 5 ml screw cap vials (capped with Teflon septa) at 4°C before use. All biological assays were carried out with unlabelled naphthalene stock solutions. Microbiological media were prepared as described previously. The experiments were carried out with chemotaxis buffer (pH =6.8) as described elsewhere (Harwood et al.)

Bacterial Strains

Wild-type *Pseudomonas putida* G7 was used in this study. Wild-type *Pp*G7 (ATCC 17485) was obtained from Dr. Caroline Harwood (University of Iowa) and has been reported previously to uptake dissolved and sorbed phase naphthalene in soil slurry (Park et al. 2001). Furthermore, it has been characterized as being motile, and chemotactic (Grimm and Harwood 1997; Grimm and Harwood 1999).

Table 3-1. Selected properties of soils to be used in this study

Soil	% O.C.	% Sand	% Silt	% Clay	PH	CEC [cmol(+)/kg]
Capac	3.3	55	24	21	6.8	24
Colwood	7.8	64	21	15	6.0	43
Houghton Muck	38.3	ND	ND	ND	5.1	156

ND: not determined yet.

O.C.: organic carbon content.

CEC: cation exchange capacity.

Sorption, Desorption, and Serial dilution Experiments

Sorption, desorption, and serial dilution experiments were carried out as described previously (Park et al. 2001). Briefly, all experiments were carried out with initial equilibrium period of two days. The ratio of sorbent to liquid was kept the same in all the experiments: 1:15 for Capac-A soil, 1:65 for Colwood soil, and 1:240 for muck. Sorption experiment was carried out at initial liquid-phase naphthalene concentrations, ranging from 0-2500 ug/l in triplicate for each type of soil. The desorption and sequential extraction experiments were both carried out at an initial naphthalene concentration of 2 mg/l. Desorption and sequential extraction experiments were initiated, at the end of 2 days sorption equilibrium period, by adding naphthalene free soil extract to make up the decanted volume. In all these experiments, vials were tumbled at 9 rpm in the dark. After mixing, each vial was centrifuged and supernatant sampled. The remaining supernatant was then decanted and replaced with methanol to extract soil-sorbed naphthalene. The concentration of naphthalene was then determined by liquid scintillation counting (LSC) and verified by high-pressure liquid chromatography (HPLC) for some samples.

Bioavailability and Biodegradation rate experiments

Bioavailability assay was adopted from the method described by Park et al. (2001). Briefly, soil extract controls and soil slurries were set up in 5-ml vials sealed by screw caps with Teflon-lined septa. The soil to water ratio was kept same as for sorption/desorption experiments. Unlabelled naphthalene was injected in all vials at initial liquid phase concentration of ~ 2000 ug/L. After 2 days of initial equilibrium period on tumbler, soil slurries and soil-free extracts (to determine degradation rate in liquid only) were inoculated with cells harvested at early stationary phase. Each vial

received bacterial cell suspension to a final cell density of ~ $5x10^6$ CFU/ml. At precise time intervals, selected vials were centrifuged and 1-ml of supernatant was transferred to a HPLC vial containing 0.01 ml of 10 N NaOH to stop biodegradation of naphthalene. The soil-sorbed naphthalene was then extracted by methanol after removing left over supernatant. Appropriate control vials were included, with no bacteria, to check for any abiotic losses.

Chemical Analysis

Naphthalene was analyzed by HPLC using a C_{18} reverse-phase column, with 80% acetonitrile/20% water mobile phase, and fluorescence detection (280 nm excitation, 340 nm emission). Radioactivity was determined by LSC. The analytical detection limits of naphthalene solution were 5 ppb and less than 1ppb for HPLC and LSC, respectively.

Extra-cellular Enzyme Activity Assay

Extra-cellular enzyme activity was measured, if there was any, by first growing bacteria (PpG7) in a mineral salt medium containing succinate (100 mg/L) for 36 hours and the cells were removed by filtration through 0.2 um Millipore filter. The culture filtrate (~ 2 mL) was then injected into each of the triplicate 20 ml screw-cap vials containing 16 mL of Chemotactic buffer (CB). The solution was then supplemented with Naphthalene at a final concentration of 1000 ug/L. The control vials did not receive filtrate and account for any abiotic losses due to volatilization and wall sorption. The vials were then mixed at 9 rpm and samples were removed at predetermined time intervals for HPLC analysis.

Extra-cellular Biomaterial Extraction

To obtain extra-cellular polymer, *Pseudomonas putida* G7 were first grown in mineral salt medium supplemented with different concentration of naphthalene solution (10-30 mg/L) in a 50 mL flask with stopper. The solution was aerated by placing magnetic bar in each flask. The loss of naphthalene by volatilization was compensated based on control flask. The growth of bacteria was monitored over the period of time by standard plating. After 4 days of incubation, naphthalene was degraded to a non-detectable level. The bacterial suspension was then filtered through a 0.22-μm Millipore filter to separate cells and dissolved biomaterial. The filtrate was supplemented with sodium azide at a final concentration of 400 mg/L to prevent any microbial activity. The filtrate was then stored at 4 °C before being used for sorption/desorption experiments. The organic content (TOC) of the filtrate was determined by the TOC analyzer.

Abiotic De-sorption Assay Using Tenax Beads

The abiotic rate of naphthalene release from soil slurries was measured using procedure as described elsewhere (Cornelissen et al. 1997a) The initial step for desorption assay, utilizing Tenax-TA beads, was same as in the sorption experiment. The solid-to-solution ratio was kept same as in the sorption experiment. Desorption was initiated by placing Tenax-TA beads (0.05 grams), enclosed in sealed dialysis bags (MWCO =13000-15000), in the sediment/water slurry bottles. A Teflon-lined cap was tightly screwed onto each vial that was then tumbled at 9 rpm. During the mixing, the Tenax beads absorb instantaneously any naphthalene that are present in liquid phase or released into the aqueous phase from the soil particles by desorption. At the predetermined time interval, selected bottles were removed and centrifuged for phase

separation. The dialysis bags, containing Tenax TA beads, were removed and liquid phase was sampled. The remaining supernatant was decanted and the sorbed phase was extracted with methanol. The concentration of naphthalene was determined by LSCE and verified by HPLC.

Rate of extraction and maximum sorptive capacity of Tenax beads were measured in separate experiment without any sediment. In this experiment, the CB volume and naphthalene concentration were kept comparable to those used in the desorption experiments. Extractions were carried out with 0.05 grams of Tenax for extended period of time. At predetermined time intervals, water phase was sampled for residual naphthalene concentrations. The extraction rate was determined from the plot of time vs. residual concentration by fitting first order kinetic equation to the data.

DATA ANALYSIS

Tenax Model

The Tenax data were analyzed according to Cornelissen et al. (1997). The model is described with one water compartment, and two sediment compartments. The amount of naphthalene left at a given time is determined by equation –1.

$$\frac{S_t}{S_o} = (F_{aq} e^{-k_{extr} \cdot t} + F_{slow} \cdot e^{-k_{slow} \cdot t} + F_{v\text{-}slow} \cdot e^{-k_{v\text{-}slow} t}) \qquad [1]$$

Where, S_t and S_o (μg) are the naphthalene contact time t (h) and at the beginning of the experiment, respectively; F_{aq}, F_{slow} and $F_{v\text{-}slow}$ are the fractions in the liquid phase, slow fraction in sediment, and very slow fraction in sediment, respectively; and k_{extr},

k_{slow}, and $\mathbf{k}_{v\text{-}slow}$ are the corresponding rate constant of liquid and sediment. The extraction rate (k_{extr}) in aqueous was determined in aqueous phase with out sediments.

Coupled Desorption-Biodegradation Model (DBM model)

The overall degradation of naphthalene in a batch slurry reactor using mass balance on naphthalene can be represented by equation-2.

$$-(V_l \cdot \frac{dC}{dt} + m \cdot \frac{dS}{dt}) = (R_{bio} \cdot V + m \cdot k_{nd} \cdot S_{nd}) \qquad [2]$$

Where m (kg) is the total mass of the soil, C (ug/L) represent naphthalene concentration in solution phase, S(μg/Kg) represent total sorbed-phase naphthalene, V (L) represents volume of the liquid, and k_{nd} is the first-order biodegradation coeffcient for non-desorbable fraction of total sorbed contaminant (S). R_{bio} is the liquid-phase biodegradation rate expression, which can be expressed by following equations:

$$R_{bio} = \frac{V_{max} \cdot C}{k_m + C} \quad \text{for Michaelis Menten kinetics,} \qquad [3]$$

$$R_{bio} = k_1 \cdot C \quad \text{for first-order kinetics} \qquad [4]$$

Where, V_{max} is the maximum flux that a cell can get and, K_m is the Michaelis-Menten constant, and k_1 first-order degradation rate co-efficient. The total sorbed phase (S) was assumed to consist of equilibrium (S_{eq}), non-equilibrium (S_{neq}) and non-desorbable(S_{nd}) sites. The equilibrium site represents fraction that release spontaneously during desorption experiment and is described by a linear partitioning model. The non-

equilibrium site fraction allows slower release of contaminant and can be represented by first order expression. The non-desorption site is defined by Park et al. (2001) to be containing contaminant that cannot be released to aqueous solution during the experimental desorption period. Mathematically, these three-sorbed phase fractions are described as follow:

$$S_{eq} = f_{eq} \cdot K_d \cdot C \quad [5]$$

$$S_{nd} = f_{nd} K_d Ce \quad [6]$$

$$dS_{neq}/dt = \alpha (f_{neq} K_d C - S_{neq}) \quad [7]$$

Where K_d is the sorption co-efficient, C_e is the liquid phase concentration in sorption equilibrium, t is desorption time in minutes, α is the first-order desorption rate coefficient (per minute). The three remaining terms f_{eq}, f_{neq}, and f_{nd} represent, respectively, equilibrium site fraction, non-equilibrium site fration, and non-desorption site fraction. The three site fractions were measured from desorption experiment: f_{nd} corresponds to the plateau of the desorption rate profile; and f_{eq}, f_{neq} and α were estimated by non-linear regression analysis of desorption data with the constraint that:

$$f_{eq}+f_{neq}+ f_{nd} =1 \quad [8]$$

RESULTS

Sorption and Desorption

Figure 3-1 shows sorption isotherms for naphthalene on the selected natural soils. The isotherms were found to be linear over the evaluated concentration range of

naphthalene. The sorption coefficients (K_d =slope of isotherms) ranged from 15 to 240 mL/g that increases as organic content of the soil increases (Table 2-2). Figures 2-3 shows naphthalene release during desorption and sequential extraction experiments, respectively. Whereas de-sorption experiment involved single extraction cycle, the sequential extraction experiment consist of seven extraction cycles. In both type of experiments, release of naphthalene from the soil was expressed in percent of the total sorbed naphthalene desorbed at the time of measurement.

The desorption rate profiles showed initial instantaneous release of naphthalene followed by a period of rate-limited de-sorption (Figure 2-2). The rate-limited de-sorption was significant within first 6 hrs; however, it becomes negligible beyond 6 hours. In all three soils, the maximum amount of desorbed naphthalene was observed to be less than 50%. The attainment of de-sorption plateau after 6 hrs is probably due to apparent de-sorption equilibrium. Further release of naphthalene was achieved by sequential extraction experiment (Figure 2-3). The subsequent steps with fresh naphthalene-free CB solution extracted more naphthalene, but amount of desorbed naphthalene decreases (exponentially) with each steps. This shows that repeated replacement with fresh CB solution cause greater total release than a single desorption experiment carried out for a longer period of time. At the end of seventh extraction cycle, no further release of naphthalene occurred indicating attainment of extent of desorption endpoint. The fraction of non-desorbable naphthalene was then determined by extracting soil with methanol at the end of last extraction cycle (Table 3-2).

Table 3-2. Parameters for sorption of naphthalene by the A horizon of selected soils

Soil	K_d	R^2	K_{oc}	Non-desorbable fraction, fnd
CapacA	15	0.96	457	0.21 ($6x10^{-3}$)
Colwood	68	0.98	872	0.19 ($2x10^{-3}$)
Muck	240	0.997	626	0.18 ($1x10^{-2}$)

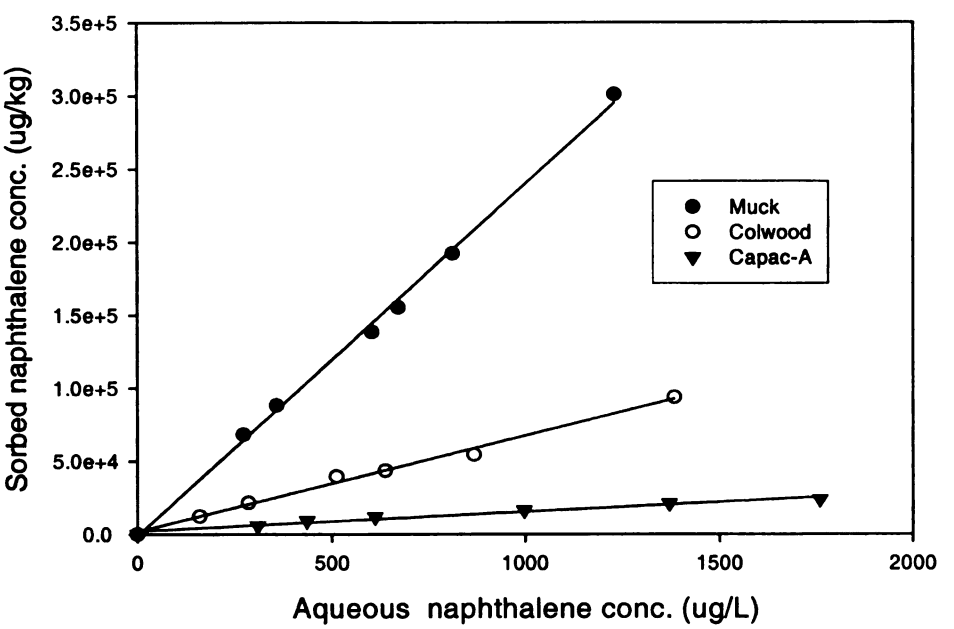

Figure 3-1. Sorption isotherm of naphthalene in natural sorbents

The three site desorption model (Park et al. 2001) was used to fit de-sorption data (Figure 3-4) and to estimate de-sorption rate coefficient (α) along with each site fractions (Table 3-3). The de-sorption data shown in Figure 3-4 were expressed in percent of the total amount expected assuming complete reversibility. In all the experiments, the overall naphthalene mass recovery was found to be 94-97%, indicating no degradation.

Removal of Naphthalne in Soil-slurry System by Tenax

In Figure 3-5, adsorptions of naphthalene to Tenax in presence and absence of soil are plotted as a function of time. The rate of extraction of naphthalene by Tenax in a dialysis bag, in absence of any soil, can be modeled as a first order kinetics; the value for k_{ext} was calculated as 0.97 /hr. The added amount of 0.05 gram of Tenax was sufficient to extract more than 97% of naphthalene in the system. For an equivalent system but in presence of soil, however, the rate and extent of naphthalene adsorption to Tenax was significantly retarded due to slow de-sorption of naphthalene from the soil. After a rapid removal of dissolved and desorbable naphthalene (as shown by initial steep curve), the adsorption of further naphthalene was mass transfer/desorpiton limited. The data were then fitted with two sediment compartments and a water compartment (Cornelissen et al. 1997) model and the rate constants for rapid and slow desorption (K_{rap} and K_{slow}) are presented in Table 3-4. The rate of removal naphthalene in muck soil was observed to be slower than the Capac-A soil. This was probably due to higher content of organic material in muck.

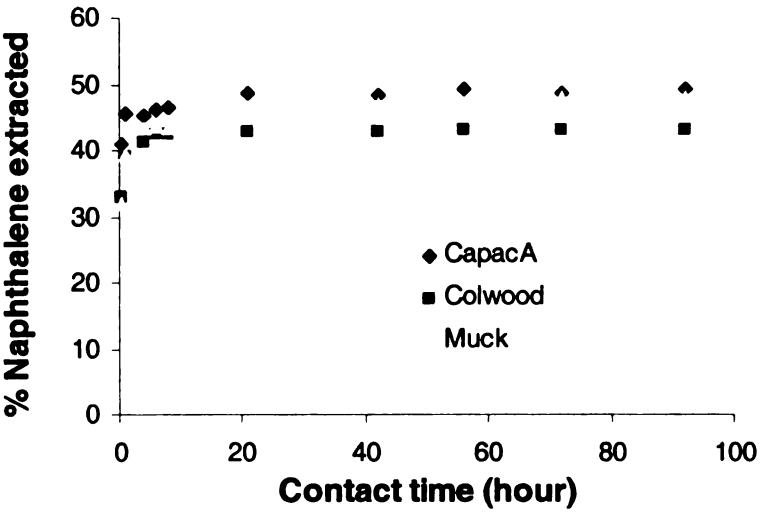

Figure 3-2. Kinetic disorption data for naphthalene from three soils

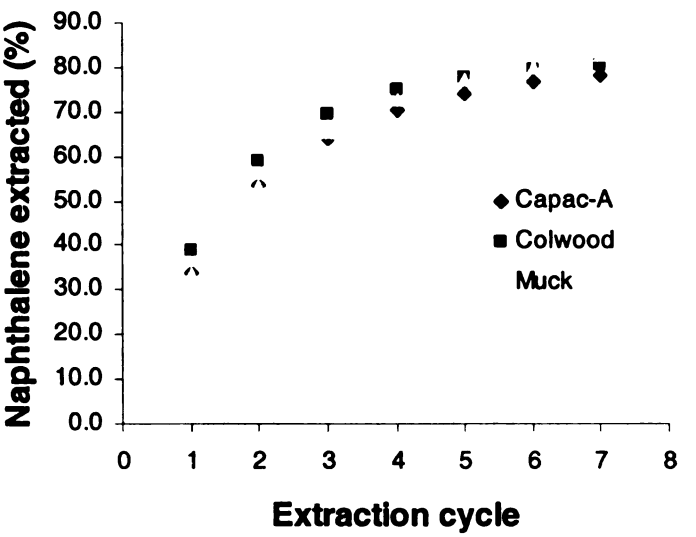

Figure 3-3. Sequential desorption data for the removal of Naphthalene from three soils

Table 3-3. Desorption parameters[a] evaluated by a three-site desorption model[b]

Soil	f_{eq}	f_{neq}	f_{nd}	α (min^{-1})	R^2
Capac	0.74(0.042)	0.1	0.16(0.009)	0.0025(0.0008)	0.94
Colwood	0.62(0.023)	0.23	0.15(0.010)	0.0016(0.0004)	0.94
Houghton Muck	0.58(0.034)	0.27	0.15(0.005)	0.005(0.0017)	0.97

[a] f_{eq} f_{neq} f_{nd}= equilibrium-, non-equilibrium-, non-desorption-site fractions; α= first order desorption rate coefficient for non-equilibrium sites.

[b]Numbers in parentheses are standard deviations of the evaluated values.

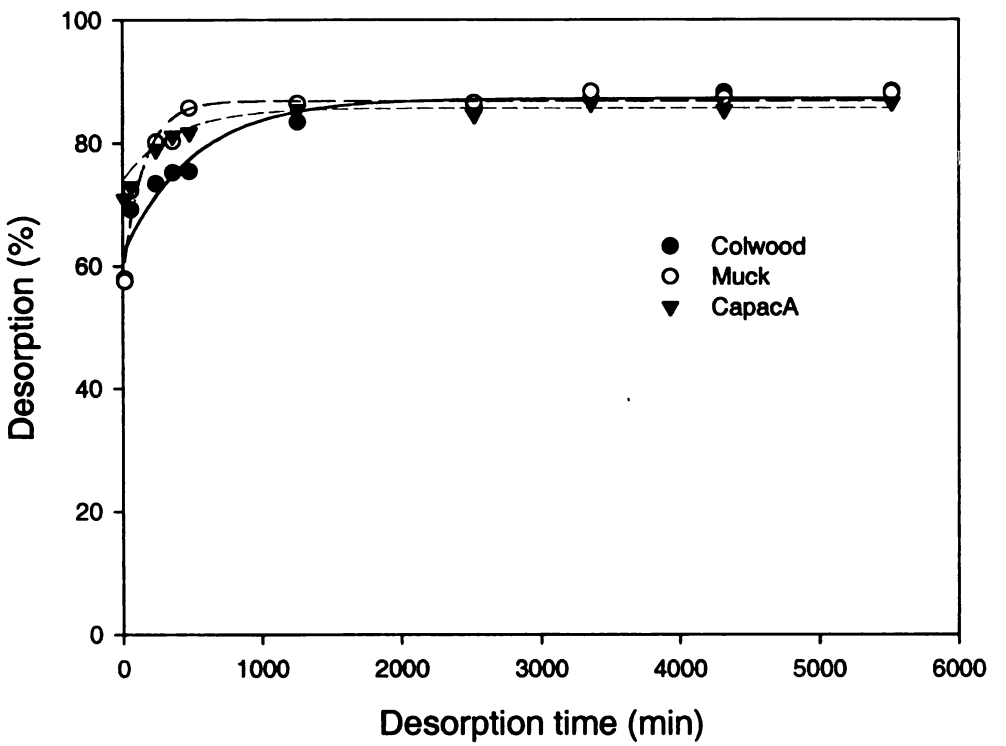

Figure 3-4. De-sorption of naphthalene from three natural soils

Biodegradation of Naphthalene by PpG7 in soil-slurry

Figure 3-6 shows removal of naphthalene in soil-slurry system by *Pp* G7 for Capac-A and Muck soil, respectively. *Pp* G7 degraded naphthalene in a biphasic curve. The first, rapid decline of naphthalene (~80%) was completed with in first 2 hours and was followed by a much slower decline. The rapid decline occurred in liquid phase, which had contained naphthalene either in dissolved phase or desorbable phase ; whereas, the slower decline was mostly occurred at solid-water interface containing non-desorbable fraction of naphthalene. The wild-type *Pp* G7 was able to decrease naphthalene to a level below the non-desorbable fraction as shown by the dashed horizontal line. The fitted model (DBM model), as represented by solid lines, reasonably described degradation of naphthalene in soil-slurry system; the estimated parameters are shown in Table 3-4.

Effect of Extra-cellular biomaterial on Bioavailability

Experiments were performed to determine role of extra-cellular biomaterial on enhancing bioavailability either by degradation of non-desorbable fraction due to extra-cellular enzymes activity or enhancement of rate and extent of de-sorption of naphthalene due to presence of solubalized bacterial polymers.

Extra-cellular Enzyme Activity

The data showing naphthalene concentration with and with out culture filtrate (from 0.2 μm filter) are shown in Table 3-5. Comparing data between samples receiving

Table 3-4. Rate efficient of naphthalene removal by PpG7 and Tenax in soil-slurry system

Soil	Mixed PpG7			Mixed- Tenax		
	K_m (ug/L)	K_s (ug/L-hr)	K_{nd} (1/hr)	K_{ext} (1/hr)	K_{rap} (1/hr)	K_{slow} (1/hr)
Capac-A	980	300	0.05 (0.0017)	0.7	0.3	0.02
H. Muck	950	300	0.054 (0.0016)	0.65	0.05	0.001

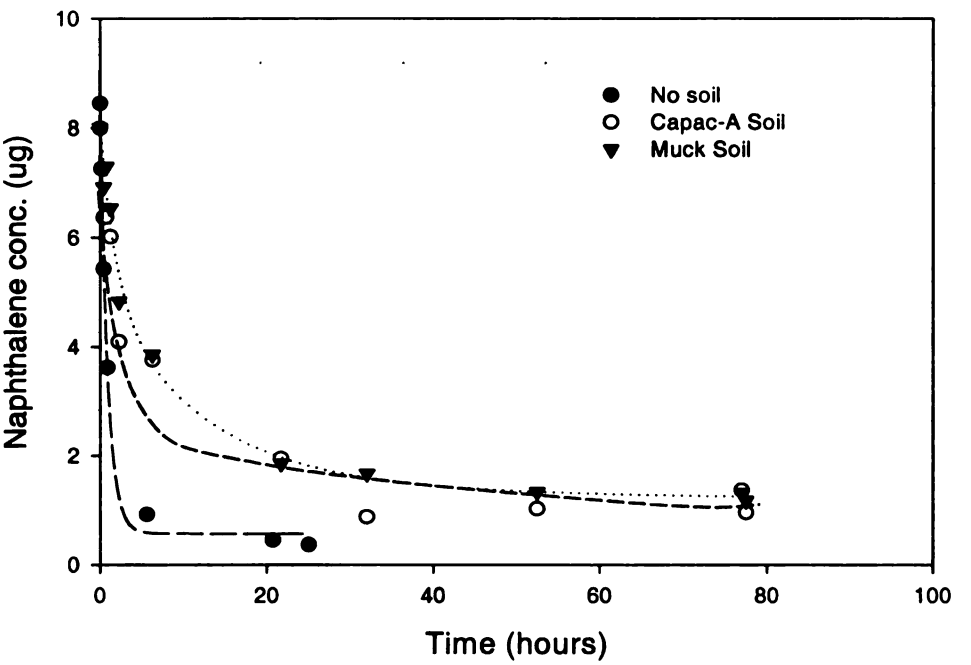

Figure 3-5. Extraction of Naphthalene by Tenax beads in presence and absence of soils

Table 3-5. Naphthalene concentration before and after addition of cell filtrate (0.2 um filter)

Sample no.	Initial Naphthalene conc. (ppb)	Control (ppb)	Filtrate addition (ppb)
1	1000	928	920
2	1000	918	901
3	1000	965	923
	Avg. ± std. Dev.	937±25	915±12

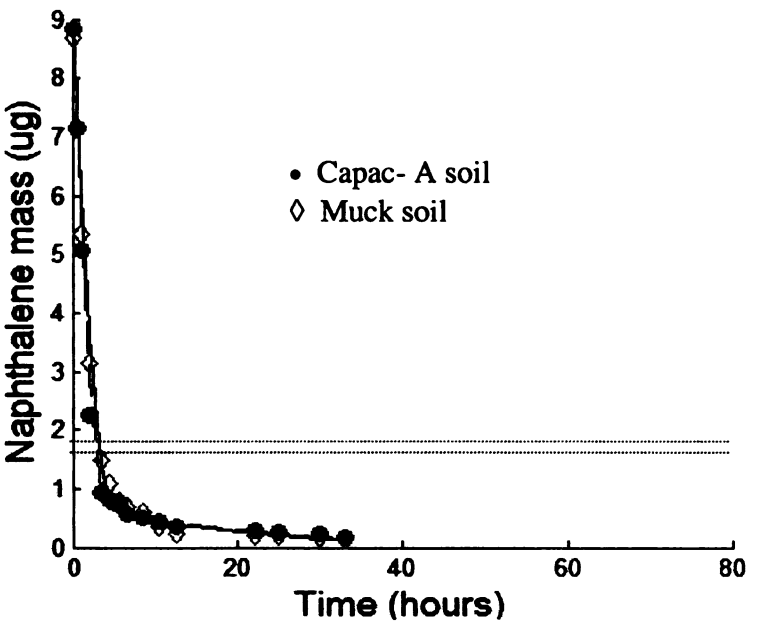

Figure 3-6. Degradation of naphthalene by PpG7 in mixed soil-slurry system

culture filtrate and control (with no filtrate) show no hydrolyzing activity of extra-cellular enzymes. Abiotic losses due to volatilization were observed in both samples and control, and were similar in amount.

Sorption/Desorption Experiment in presence of Excreted biomaterial

Figure 3-7 shows amount of excreted biomaterial in filtrate solution, obtained by growing *Pp* G7 at a range of naphthalene concentration (10-30 ppm) in CB solution. The quantity of biomaterial was measured by determining total organic carbon (TOC) of filtrate solution. It is evident from the data that as bacterial mass increase, in response to increasing naphthalene concentration, there is proportional increase in excreted biomaterial in CB solution.

The data obtained from the sorption experiments in presence of different quantities of biopolymer are shown in Figure 3-8. The data were fitted with linear isotherm for estimating distribution coefficient of naphthalene to soil (Kd) as shown in Table 3-6. The K_d value decreases as biomaterial concentration increases. The decrease in K_d value A trail de-sorption experiment was carried out in presence of biomaterial corresponding to 10 mg/L naphthalene. The presence of biomaterial did not alter the de-sorption kinetics of naphthalene in Colwood soil (data not shown). Since, our bio-availability experiments were carried out at 2 ppm, therefore, we did not see the need to further explore the effect of biomaterial on de-sorption and consequently bio-availability of sorbed phase naphthalene.

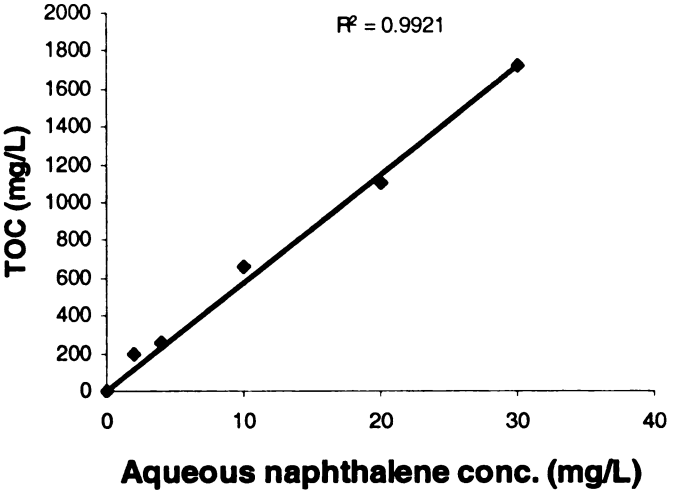

Figure 3-7. Production of Biomaterial (TOC) by PpG7 at different naphthalene concentration

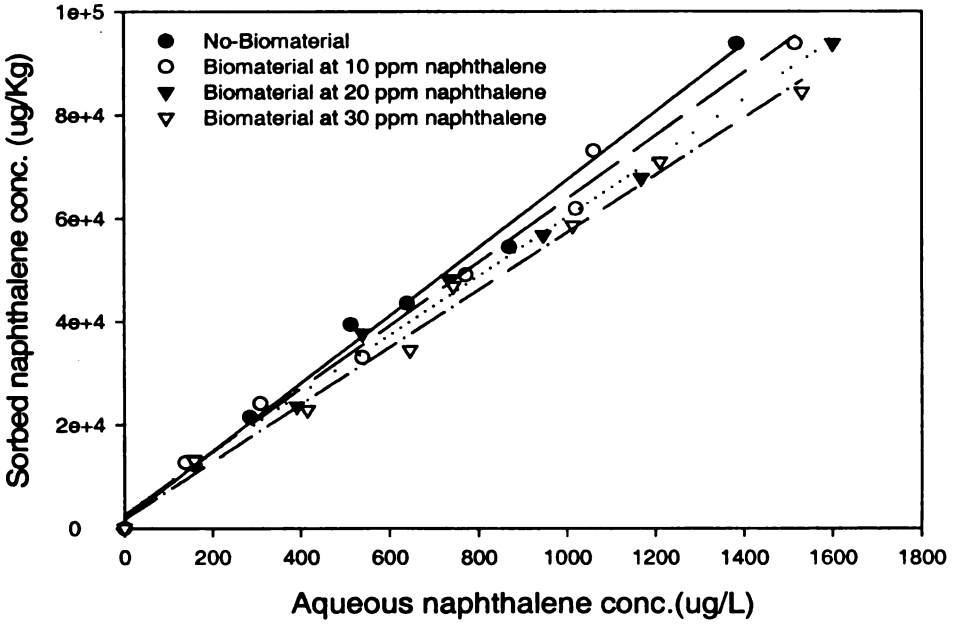

Figure 3-8. Decrease in partition co-efficient of naphthalene with increasing biomaterial in soil-slurry system

Table 3- 6. Distribution co-efficient of naphthalene to Colwood at different amount of biomaterial

Soil	Biomaterial at naphthalene concentration of (mg/L)	K_d (L/Kg)	R^2
Colwood	0	65.5	0.996
	10	61.5	0.995
	20	57.0	0.997
	30	55.5	0.993

Table 3-7. Non-desorbable fraction from Tenax and serial extraction methods

Soil	Non-exhaustive TA extraction method	Exhaustive Serial dilution method
	Non-desorbable fraction fnd	Non-desorbable fraction fnd
CapacA	0.12	0.21
Muck	0.14	0.18

DISCUSSION

Recently, several researchers have observed and reported enhanced bio-availability of soil-sorbed PAHs. Their studies suggested that biodegradation of sorbed PAHs is not exclusively dependent on abiotic mass transfer of the compound into the water phase. Several arguments supported the observations of enhance bio-availability: First, it has been shown that coupled biodegradation-desorption model (DBM) that incorporate degradation of non-desorbable fraction could only be successfully used to either curve-fit or predict the observed biodegradation data. Secondly, the observation that contaminant degradation kinetics exceeds independently measure desorption rate and extent has led to the conclusion that bacteria can uptake from sorbed phase, directly. In most of these studies, the de-sorpton rate was measured by sequential replacement of the aqueous phase. This study explores the validity of these observations by directly comparing biodegradation kinetics with abiotic extraction rate using Tenax and also to investigate role of bacterial extra-cellular biomaterial on enhance bioavailability.

Comparison of Biodegradation and Abiotic Removal rate in soil-slurry system

The main objective of this research was to compare kinetics of naphthalene removal in soil slurry system in presence of Tenax adsorbent as a third phase infinite sink, with biodegradation kinetics by *Pp* G7 performed in separate experiments. Tenax has been reported to be a better extractant for determining the size of the rapidly and slowly desorbable fractions of total sorbed phase HOCs as compared to using salt solution, alone. (Reid et al. 2000 a). Tenax has also been shown to be a suitable adsorbent for HOCs removal from liquid phase and can mimics the bio-degradation

capacity of PAH-degrading bacteria(Cornelissen et al. 1998; Theodora et al. 2003).When placed into contact with soil-water slurry, it tends to adsorb HOCs rapidly by acting as an infinite sink ($K_p = 10^4$ L/Kg). By adding fresh tenax to soil-slurry, a near zero naphthalene concentration was maintained that resulted in a complete extraction of desorbable fraction of sorbed naphthalene. Thus, it is expected that desorption with tenax represents biodegradation condition and is more representative of to evaluate the rate and extent of naphthalene removal from the soil.

Figures 3-6 and 3-7 show naphthalene removal by biodegradation and adsorption to Tenax beads in Capac-A and Muck soil-slurry systems, respectively. All the experiments began with equal mass of naphthalene in both liquid and sorbed phases at the end of the sorption period. During initial period of 1 hour, *Pp* G7 and Tenax bead removed the liquid phase naphthalene at approximately equal rates. After this initial period, the *Pp* G7 continued to degrade remaining naphthalene in sorbed phase at same rate until less than 5% of total naphthalene remained in soil after 8 hrs of treatment. The Tenax beads, on other hand, removed naphthalene at a slower rate in sorbed phase with biphasic pattern: an initial relatively rapid phase followed by much slower phase. The fraction of sorbed phase that is released rapidly (f_{eq}) was removed at a relatively faster rate (K_{rapid}), whereas slowly desorbing fraction (f_{neq}) was removed at much slower removal rate (K_{slow}). The overall removal of naphthalene by Tenax beads slightly exceeded total desorbable fraction, as determined by serial extraction method, and shown by horizontal dashed line.

Also plotted on Figures 3-9 and 3-10 results of serial de-sorption (dashed lines with asterisk symbols) with 0.1 M buffer solution. During serial de-sorption experiment, it was observed that desorption plateau during first 4 hours of each step (Figure 3-2). Therefore, an effective time of 4 hours was selected for each step during which de-sorption complete. Amount of naphthalene left at end of each step was taken and plotted against corresponding 4 hrs periods.

Both serial extraction and Tenax extraction shows similar trend. Either method showed presence of non-desorbable fraction of sorbed naphthalene. The amount of non-desorbable fraction was, however, greater in serial extraction than Tenax extraction (Table 3-7). The amount of non-desorbable fraction is often assumed to be non-available for biodegradation, and this underlies the importance of having method that accurately measure the fraction in solid media. The result shows that Tenax extraction provides a better estimate of desorbable fraction (and hence bioavailable) and can be used as a rapid and straightforward alternative to conventional serial extraction.

Comparison of the results of Tenax extraction and *Pp* G7 biodegradation shows that the rate and extent of naphthalene removal by bio-degradation was at all time significantly greater than the abiotic removal rate and extent in the sorbed phase. It seems that naphthalene do not have to be desorbed in the aqueous phase for bio-degradation by *Pp* G7. Apparently, *Pp* G7 were able to overcome the mass-transfer limitation by unknown mechanism and could able to directly uptake sorbed phase naphthalene.

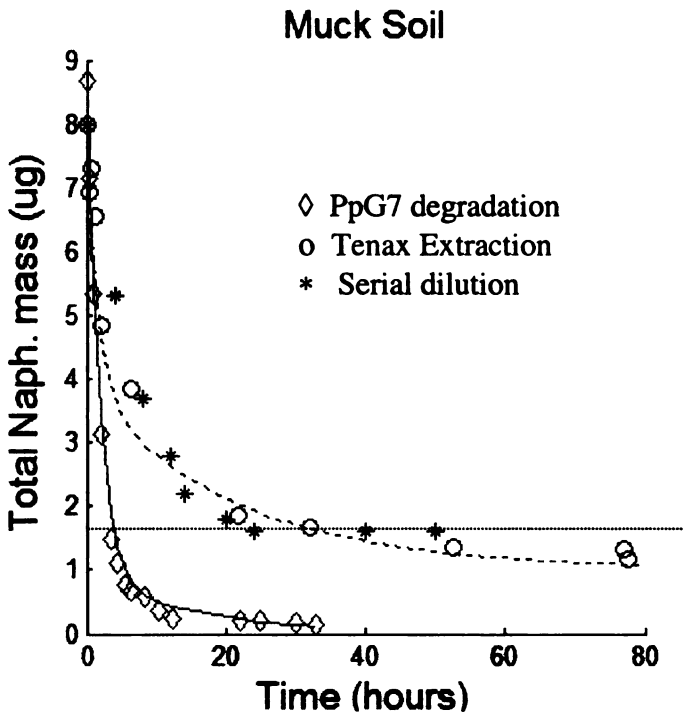

Figure 3-9. Removal of naphthalene by PpG7, Serial dilution, and Tenax beads in Muck soil

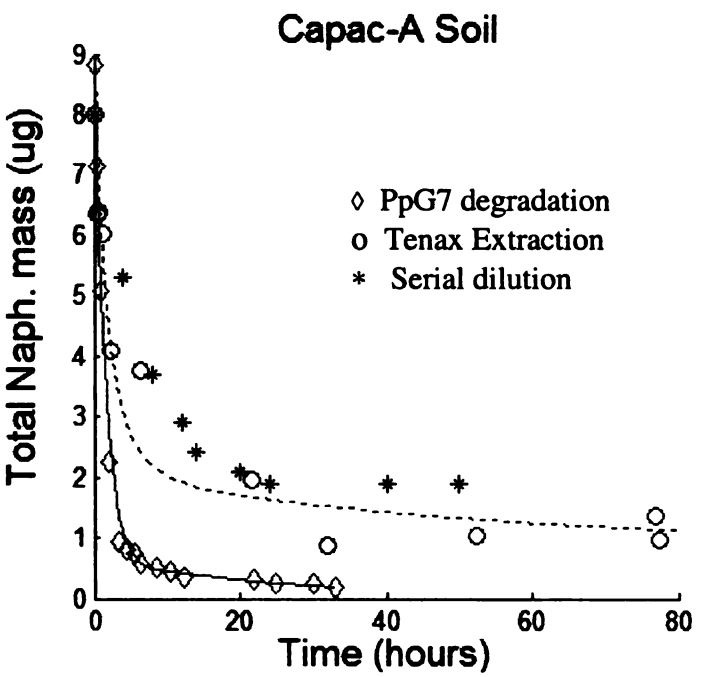

Figure 3-10. Removal of Naphthalene by PpG7, Serial dilution, and Tenax beads in Capac-A soil

Thus, this study lend credence to the concept of enhance bio-availablity, which has been observed previous works. Two mechanisms had often been cited for the observed enhance bio-availability: 1) direct uptake by bacteria and, 2) micro-organism mediated increase in mass transfer(Harms and Zehnder 1995; Rosenberg et al. 1989). Our tenax data support the former mechanism because tenax adsorb HOCs rapidly in soil-slurry system by acting as an infinite sink. Thus, tenax can represent all possible bacterial strategies (i.e. production of bio-surfactant or EPS) that can result in increase mass-transfer of sorbed phase naphthalene from soil to water.

It is plausible that when *Pp* G7 degrade dissolved concentration to zero; passive uptake by bacteria becomes negligible. Consequently, *Pp* G7 can uptake further naphthalene from non-desorabable pool by direct uptake of naphthalene at solid-water interface. Bacteria have been known to attach soil particles containing organic molecules and thus degrade these molecules directly.

Extra-cellular Biomaterial and Enhanced Bioavailability

Bacteria can employ a variety of strategies to access sorbed-phase HOCs. Among these, bacterial production of extra-cellular biomaterial (including enzymes and polymer) can influence bio-degradation of sorbed molecules either directly or indirectly. In this study, no catabolic/hydrolyzing activity of extra-cellular enzymes was observed. Naphthalene metabolism has been observed to be intracellular in Pseudomonas bacteria (Bugg et al. 2000; Gray and Bugg 2001; Grimm and Harwood 1999; Whitman et al. 1998). The activity of extra-cellular enzymes in response to pollutant uptake is still unknown. Micro-organism, mostly fungi, are reported to excrete extra-cellular enzymes

in response to large (complex polymer) and inaccessible substrate (very low solubility). The excreted enzymes acts upon large and complex molecules and convert them into smaller, and more soluble or diffusible compounds (Faber 1979). In soils, micro-organism including bacteria do not have direct access to contaminant molecules that becomes sorbed or entrapped with nano- and micro-pores (Alexander et al. 1995). Microorganism can, however, degrade these trapped molecules by excreting enzymes molecules, which are small enough to enter these inaccessible molecules.

Pseudomonas putida G7 is known to possess both extra-cellular polymeric substances (EPS) as well as lipopoly-saccharide (Kachlany et al. 2001). The bacteria (*Pp* G7) excrete these polymer under changing environmental condition (low C/N ratio, starvation condition, etc) (Neu and Marshall 1990). These polymers have high molecular weight and, consist of polysaccharides, proteins, lipo-polysaccharides, lipoproteins or complex mixture of these polymers. They are, however, less effective in lowering surface and interfacial tension in solvent, but can increase compound solubility due to unknown mechanism(Ron and Rosenberg 2002).

Presence of bacterial polymer in soil-slurry can influence the fate of naphthalene by affecting it sorption/desorption rate and extent. In this study, partition co-efficient (Kd) of naphthalene to colwood soil reduced from 6- 15% as biomaterial concentration increases. This enhancement corresponds to biomaterials obtained at naphthalene concentration of 10-30%. It is, therefore, expected that any sorption experiment carried out with biomaterial obtained at less than 10 ppm of naphthalene will insignificantly affect partition co-efficient of naphthalene. Furthermore, our all bioavailability experiments were carried out at 2 mg/L of naphthalene, the possible affect of naphthalene

on partition co-efficient is highly unlikely. This was further substantiated by desorption experiment carried out at biomaterial obtained at naphthalene concentration of 10 mg/L, where we did not see any change in rate or extent of naphthalene desorption.

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CHAPTER 4. AFFECT OF CHEMOTAXIS AND MOTILITY ON THE BIODEGRADATION OF NON-DESORBABLE NAPHTHALENE IN NATURAL AND SYNTHETIC SORBENT

ABSTRACT

The purpose of this study was to evaluate the role of chemotaxis/motility in soil-sorbed naphthalene degradation by wild-type *Pseudomonas putida* G7 in a sorbent-slurry system under stirred and quiescent conditions. Naphthalene degradation by the wild –type strain was compared to that of a non-motile strain and a mutant strain deficient in naphthalene chemotaxis. Any difference in naphthalene degradation rate and extent between wild-type and mutant strains was considered to come from chemotaxis/motility phenotypes. In natural soils (with low to high organic content), under both stirred and static conditions, the rate and extent of naphthalene degradation was observed to be similar for all the strains. Whereas in stirred system all the strains had an equal access to a non-desorbable fraction of naphthalene; in the static system, however, none of the tested strain had gained access to the non-desorbable fraction of naphthalene. The effect of mixing was clearly seen to have caused the difference of bacterial uptake in mixed and static systems. The well-mixed batch system is expected to have caused uniform concentration gradient, greater cell to soil particles interaction, and shorter transfer distances that contributing to the enhanced bioavailablity. In a well-mixed system, however, bacterial chemotaxis/motility is a much slower process as compared with other processes and therefore chemotaxis/moitility effects was not isolated. In a static system, however, numbers of sites available for bacterial attachment for direct uptake of sorbed

phase napthalene were substantially reduced due to the pore size exclusion and retardation (due to cell adosption to soild surfaces) of bacterial cells. The chemotactc/motility movements (and hence access to high concentration sites) were also impeded due to a lack of mixing in organic soils, bacterial size exclusion, and adsorption adhesion effects.

Experiments carried out with activated carbon, under stirred conditions, showed that both WT and mutant strains had accessed to the non-desorbable fraction of naphthalene. However, WT degraded non-desorbable phase naphthalene at a significantly greater rate as compared to its mutant strains. The difference of uptake rate between WT and mutant strains could be explained due to the presence of significant concentration gradient at soild/water interface as a result of large pool of non-desorbable naphthalene. The concentration gradient attracted greater number of WT cells, due to its chemotaxis/motility phenotypes, that resulted in a greater rate of non-desorbable naphthalene degradation as compared with system inoculated with mutant strains.

INTRODUCTION

Limited bio-availability of chemicals trapped in soil micro-pores pose great challenges the remediation of contaminated soil and sediments(Head 1998). Bioavailability of soil sorbed contaminants is considered to be an interplay between desorption/mass transfer and microbial processes. Any change in either mass transfer from the soil microenvironment to aqueous phase or bacterial characteristics can affect the bioavailability of contaminants. During the initial step of bio-degradation, the mass transfer increases as contaminant is removed from the aqueous phase. As rapid and slow

de-sorption sites are exhausted, the bio-degradation of a hydrophobic compound has been observed to be limited by the extremely slow rate of chemical transfer from non-desorbable compartments of desorption sites (Bosma et al. 1997; Rijnaarts et al. 1990). The mass transfer to a microorganism can be, however, improved if the degrading organism swims toward localized concentration gradient at the soil-water interface. It is, therefore, quite comprehensible that bacteria capable of sensing and responding to localized soil gradients can increase net mass transfer rates.

It has been recognized that bacteria can sense and respond to the presence of aromatic compounds in the environment (Grimm and Harwood 1997; Harwood et al. 1994). Bacterial chemotaxis and motility are two physiological functions that have been shown to confer advantages to microorganisms to increase the rate of degradation of aromatic hydrocarbon(Marx and Aitken 1999). Chemotaxis allows cells to move in direction of higher concentration, thus bringing them in contact with the chemicals to be degraded. In soil contaminated with hydrophobic compounds, chemotaxis may enable microbes to access zones of concentrated chemicals for degradation that would otherwise be inaccessible due to mass transfer limitations, low solubility, or sequestration of chemicals to or inside a solid matrix. This is all the more important in heterogeneous soils, where there exist large variations in sorbed concentrations across different regions and chemotaxis may help cells locate contaminants in these different regions.

Most prokaryotes are motile, and many are chemotactic to various toxic organic compounds such as PAHs (Jain 2002; Madigan et al. 2002). *Pseudomonas putida* G7 and *Pseudomonas sp.* NCIB 9816-4 are recently shown to be chemotactic to naphthalene (Grimm and Harwood 1997; Grimm and Harwood 1999). The chemotactic response is

due to catabolic plasmid present within these organisms. A mutant derivative of *P. putida* G7 (G7.C1) that is devoid of plasmid (NAH7) was shown to be non-chemotact to Naphthalene. It has also been shown that chemoreceptor gene (NahY) encoded by the NAH7 plasmid is involved in naphthalene chemotaxis.

Limited studies have been carried out to demonstrate the potential role of chemotaxis in rapid biodegradation of organic compounds in aqueous and porous media. In studies involving aqueous medium alone, the concentration gradient was established either by supplying naphthalene from a glass capillary tube (Marx and Aitken 2000) or NPAL-water interface (Law and Aitken 2003). Both studies clearly demonstrated that naphthalene was degraded at a higher rate by the wild-type strain than either non-motile strain or non-chemotatic strain. It has been shown that the wild-type strain could overcome the mass transfer limitation by accumulating in region of higher concentration that led to rapid biodegradation rate in both studies.

Experiments with porous medium involved detecting temporal changes in bacterial density distribution in packed sand column in response to concentration gradients. The concentration gradient established as bacteria continued to uptake a compound that was present at an initial constant concentration. However, the experiments with porous medium either failed to observe chemotaxis (Barton and Ford 1995), or provided indirect evidences of chemotaxis (Pedit et al. 2002; Witt ME et al. 1999). The presence of the porous medium has been shown to contribute significantly to the reduction in the bacterial chemotactic and motility responses compared to the measured in aqueous system. Also, if the initial isotropic concentration of the compound exceeds

saturation capacity of the chemoreceptor, it would lead to suppression of chemotactic responses (Barton and Ford 1995 and 1997).

Witt et al. (1999) demonstrated movement of *Pseudomonas stutzeri* KC in columns packed with aquifer sediments. In continuous flow columns, cells migrated through the column at a velocity exceeding the average linear velocity of flow. The enhanced movement was attributed to creation of a nitrogen gradient that resulted from the rapid consumption of nitrate by bacteria. Experimental results with no flow column showed that the mean velocity of KC cells due to chemotactic movement towards increasing concentration of nitrate was 5 cm/day. However, the study was not able to distinguish between chemotactic and random motility.

Padit et al.(2001) clearly demonstrated the role of chemotaxis in increase biodegradation of Naphthalene in porous medium. They used an experimental system similar to Marx et al.(1999) with the exception that both micro-capillary and reservoir were filled with glass beads. It was shown that chemotaxis can occur in a porous medium; however, the presence of solids had significantly reduced chemotaxis responses as compared to a solid-free system.

All of the previous studies related to bacterial chemotaxis and motility in biodegradation of organic compounds were carried out either in a homogenous solid (glass beads or clean sand) or liquid medium. Furthermore, in experiments with solid mediums, the rate of degradation by wild type was not compared to mutants deficient in chemotaxis and motility trait to clearly identify the role of the bacterial trait. In earlier studies with columns packed with porous medium, the loss of substrate was speculated to

be either due to chemotactic or random motility. The possible role of chemotaxis in the bioavailablity of organic compounds in a soil matrix has so far remained unexplored.

The aim of this study, therefore, was to explore the role of chemotaxis and motility on the rate and extent of biodegradation of both desorbable and non-desorbable fractions of soil-sorbed organic compound such as Naphthalene in natural and synthetic sorbents. The rate of sorbed phase naphthalene was monitored in both mixed and static slurry system with Wild-type *P. putida* G7 and two mutant strains, one of which was motile but not chemo-tactic toward naphthalene, and the other of which was non-motile. Comparing degradation rate and extent of the wild type to the mutant strains allowed us to distinguish the effect of chemotaxis and motility on biodegradation of sorbed phase naphthalene in the test systems.

MATERIAL AND METHODS

Geo-sorbents and Soil Extracts

The three sorbents selected for this study comes from A-horizon of agricultural soils found in Michigan. The selected soils spanned a range of organic matter content. These included the Houghton muck soil, which is essentially devoid of mineral matter; and two mineral soils of differing organic matter contents (Capac-A, and Colwood), whose mineralogy is predominately illite and vermiculite. The selected characteristics of these geo-sorbents are summarized in Table 4-1.

Before use, all the soil samples were air-dried, and passed through 2 mm sieve. The soil were then filled in several 5-ml screw cap bottles and sent them to University of Michigan Ford Nuclear reactor for γ-irradiation. The soil samples were sterilized by

gamma irradiation (5 Mrad) and remained sealed in 5-ml bottles until use. The soil extracts were prepared by mixing soil and chemotaxis buffer solution at a ratio of 1:15 for Capac-A, 1:60 for Colwood, and 1:220 for Houghton muck soils. The suspension were mixed, in sterile plastic bottles, and tumbled on an end-over-end shaker at 9 rpm. After 2 days of incubation, the suspensions were centrifuged for separating soils particles from the supernatant. The separated supernatants were then filtered through two layers of Whatman no.2 filter paper before use for desorption and bioavailablity experiments.

Chemical

Naphthalene was selected as the test contaminant because it has often been used as a model compound for polycyclic aromatic hydrocarbons (PAHs); this class of contaminant also poses a critical bioavailablity issue at numerous petroleum contamination sites. Both 14C-labeled and unlabeled naphthalene were used in this study. The 14C-labelled naphthalene (sigma, 0.1 mCi, >98% pure) stock solution was prepared by supplementing it with unlabelled naphthalene and was used for sorption, desorption, serial dilution studies. The un-labelled naphthalene (sigma, > 98%) stock solutions were prepared in methanol and stored in 5 ml screw cap vials (capped with Teflon septa) at 4°C before use. All biological assays were carried out with unlabelled naphthalene stock solutions.

Table 4-1. Selected properties of soils to be used in this study

Soil	% O.C.	% Sand	% Silt	% Clay	PH	CEC [cmol(+)/kg]
Capac	3.3	55	24	21	6.8	24
Colwood	7.8	64	21	15	6.0	43
Houghton Muck	38.3	ND	ND	ND	5.1	156

ND: not determined yet.
O.C.: organic carbon content.
CEC: cation exchange capacity.

Bacterial strain and culture preparation

Wild-type *Pseudomonas putida* G7 along with its two mutant strains were used in this study. Wild-type *Pp*G7 (ATCC 17485) and a mutant strain [*Pseudomonas putida* G7.C1(pHG100)], which is non-chemotactic to naphthalene, were obtained from Dr. Caroline Harwood (University of Iowa). The non-flagellated (non-motile) mutant strain of *P.putida* G7 was obtained from Dr. Michael Aitken (University of North Carolina); the non-motile strain was spontaneously generated and isolated as described elsewhere (Marx and Aitken 2000). The pure wild type, pseudomonas putida G7, has been reported previously to uptake sorbed phase naphthalene in soil slurry. Furthermore, it has been characterized as being motile, and chemotectic (Grimm and Harwood 1997; Grimm and Harwood 1999).

All four strains were stored at (-80 °C) in cryo-vials containing aliquots of overnight culture supplemented with 75 % glycerol. Bacterial culture was grown by lightly scraping a frozen stock with a sterile inoculating loop and, transferred it to tryptone broth. For non-chemotectic mutant the broth was supplemented with a final concentration of 50 ug/l of tetracycline (sigma) to select it for the desired mutants. The cultures were allowed to incubate at 25 °C overnight on a shaker. The overnight cells were then centrifuged for 2 min., and subsequently re-suspended in the chemotactic buffer. To obtain pure colonies, the washed cultures were streaked on minimal media plates, containing tetracycline for non-chemotactic mutants. Naphthalene was provided as sole carbon source by placing its crystal in the lids of the plate. The plates were incubated at room temperature for 3-4 days for colonies to grow. The seed cultures were then stored at 5°C.

For each bioassay, fresh bacterial inoculums was prepared by placing colonies from seed culture plates (using sterilized inoculating loop) into 250 ml of flask with 50 ml of mineral salt media (Harwood et al. 1994). The pH of the medium was kept at 7.0. Naphthalene was added as a concentrated stock solution (in methanol) to the sterilized salt media to a final concentration of 200 mg/L. The liquid cultures were incubated at room temperature with stirring and growth was monitored by measuring the absorbance at 600 nm. Cells in the early stationary phase were harvested by centrifugation, and re-suspended in CB solution(Harwood et al. 1994). This procedure was repeated three times to ensure the removal of any remaining naphthalene from the cell growth medium. The number of viable cells was determined by standard plate count. The CB contains 13.6 g/L of potassium phosphate and 7.4 mg/L of EDTA, and adjusted by 10 N NaOH to pH. Tetracycline was added at a final concentration of 50 μg/ml to the mineral-salt medium while growing non-cheomotactic mutants

Sorption, Desorption, and Serial dilution Experiments

Batch sorption experiments were performed for all the test soils. An aliquot of sterile soil and 4.2 ml of chemotactic buffer was taken in each of the series of 5-ml vials and spiked with a known amount of U-^{14}C-labeled radioactive naphthalene (7000 – 60000 dpm). The serum vials were sealed with Teflon-lined caps. The ratio of sorbent to liquid was kept the same as the ratio in the bio-availability assay; the solid-to-solution ratio was set as 1:15 for Capac-A soil, 1:65 for Colwood soil, and 1:240 for muck soil. Seven initial liquid-phase naphthalene concentrations (ranging from 0-2500) were used (in triplicate) for each type of soil. The headspace was less than 1ml in volume. Control

vials without sorbents were prepared in triplicate. Vials were tumbled at 9 rpm in the dark for 2 days. After mixing, each vial was centrifuged for 5 min at 1200 × g to separate liquid from the sorbent, and 1 ml of the liquid phase was sampled in duplicate. The concentration of naphthalene in the supernatant was determined by measurement of radioactivity using liquid scintillation counter (LSC). LSC results were verified by high performance liquid chromatograph (HPLC) for representative samples. Solid-phase concentrations were calculated by subtracting equilibrium liquid-phase concentrations from initial liquid phase concentration, and the results were confirmed by direct analysis of sorbed-phase naphthalene concentration in selected samples for each type of soil. The direct analysis entailed removal of supernatant from the vials and replacing it with methanol. The extracted amount of naphthalene in methanol was then determined by LSC and HPLC to verify the mass balance.

Desorption assays were conducted to measure desorption rates. The initial step for desorption assay was the same as in the sorption experiments utilizing batch soil slurries. The only two exceptions were that one 50 ml vial was used instead of 7, 5 ml vials for each soil, and the aqueous phase was spiked with an initial naphthalene concentration of 2 mg/l. Each 50-ml centrifuge-tube, containing 45 ml of chemotactic buffer, was spiked with initial naphthalene concentration of 2 mg/l. The solid-to-solution ratio remained the same as in the sorption experiment. The tubes were capped with Teflon-lined Minnert valves and screw-sealed. A control tube with no soil was included in the experiment to keep track of naphthalene losses due to volatilization and wall sorption, if any. The tubes were tumbled at 9 rpm for two days to reach apparent equilibrium, as determined in the sorption kinetics assay. At the end of sorption

equilibrium, each tube was centrifuged for 20 min at 1200g to separate soil, and the supernatant was sampled. The concentration of naphthalene in the supernatant was determined as mentioned in sorption assay. After sampling, the supernatant was decanted to the maximum extent possible. Preliminary studies showed that the residual supernatant was 0.27 ml with a standard deviation of 0.03 ml. Desorption was initiated by adding naphthalene free soil extract to make up the decanted volume. The vials were tumbled again at 9 rpm, and at pre-determined time intervals they were removed and centrifuged for liquid phase sampling. The concentration of naphthalene was determined by LSCE and verified by HPLC. After final desorption samples were taken, the remaining supernatant was decanted and the sorbed phase was extracted with methanol. The extracted naphthalene amount was used to verify mass balance. The desorption data obtained from this assay was fitted with a three-site desorption model to evaluate desorption parameters and site fractions. These parameters and site fraction information were utilized in the bioavailability model to quantitatively account for the effects of desorbed naphthalene on bioavailability.

Seriel-dilution desorption assay was conducted to measure the amount of non-desorbable naphthalene in soil samples. The measurement was verified by an air-sparging method. The serial dilution experiment was carried out in 5 ml sacrificial vials containing sorbent slurries (with the same sorbent:solution ratio as in the sorption study) being spiked with an initial naphthalene concentration of 2 mg/L in the solution phase. For each soil triplicate vials were prepared for each sampling period. The slurries were tumbled at 9 rpm for 2 days, the supernatant decanted, sampled and analyzed for naphthalene, and the bottles refilled with naphthalene-free liquid to the original volume.

This procedure was repeated for a total of 6 successive desorption periods. The non-desorbable naphthalene was calculated by difference, with confirmation of mass balance by solvent extraction of the sorbent following this series dilution procedure. The calculated non-desorbable naphthalene amount was compared to the estimated value by the three-site desorption model from desorption data.

The air-sparging method involved sample preparation similar to the serial dilution method as mentioned above. At the end of 2 days sorption equilibrium, the vials were connected to each other in a serial configuration via steel tubing (as shown in Figure 4-1). The first vial was connected to a nitrogen supply source and the last vial attached to a carbon trap through a steel tube. The continuous injection of nitrogen gas below the water surface of each vial effectively purged dissolved naphthalene into overhead space. The purging operation was stopped when dissolved concentration of naphthalene was reduced to a non-detectable limit. The vials were then taken out of the assembly, decanted, and the non-desorbable fraction was extracted with methanol.

Motility and chemotaxis assays.

To determine the motility of the naphthalene-degrading bacteria, cells were grown in stabs of semisolid media with tetrazolium salt (nutrient broth containing 0.4% agar) for 24 hours at 28°C. Motile bacteria were migrated from the line of inoculation to form diffuse red-turbidity in the surrounding medium; nonmotile bacteria grew only along the line of inoculation (Krieg and Gerhardt 1994; Leboffe and Pierce 1999). Chemotaxis was

tested with a soft agar swarm plate assay. For the swarm plate assay, cells were inoculated with a needle stabbing into the center of the minimal medium that contains naphthalene and is solidified with 0.4% Noble agar. Chemotactic bacteria migrated outward while consuming naphthalene in the medium.

Bioavailability assay

To evaluate the availability of sorbed naphthalene to bacteria, a bio-availability assay was carried out as described previously (Park et al. 2001). Briefly, the assay was carried out in a set-up of 5-ml vials sealed by screw caps with Teflon-lined septa. The soil to water ratio was kept the same as for sorption/desorption assays. Aliquot of naphthalene stock solution was injected in each vial at an initial liquid-phase concentration of ~2000 μg/L. The vials were tumbled on an end-over-end shaker at 9 rpm for 2 days in dark. Sterility of the soil slurries was checked by plating out the suspension from selected vials on nutrient agar plates. After 2 days, each vial received an aliquot of microbial cell suspension harvested during the early stationary phase, to initial biodegradation of naphthalene. The aliquot of cell suspension corresponded to a desired cell density of $5x10^6$ CFU/ml in serum vials. Inoculated vials were tumbled on the end-over-end shaker at 9 rpm, and at predetermined time intervals vials were removed and centrifuged at 3000 rpm for 5 minutes to separate the soil from solution. One milliliter of supernatant was transferred to a HPLC vial containing 0.01 ml of 10 N NaOH to stop biodegradation of naphthalene. The remaining supernatant from the vial was decanted and replace with an equal volume of methanol to extract remaining soil sorbed

naphthalene. Both supernatant and methanol extracted naphthalene concentration was measured by HPLC. In bio-availability experiment two types of control vials, without test bacteria, were used as a check for losses: one contained only buffer solution, and the other contained slurry solution.

Biodegradation rates were measured in sorbent-extract solutions to be representative of those expected in systems with sorbent present. A sorbent-extract solution was prepared following the method outlined in the previous section. Kinetic data was obtained using the same procedure as in the bioavailability assay, except that no sorbent will be present in the vials. Both sterile-CB and soil-extract controls were used to monitor for abiotic losses of naphthalene during the sorption and degradation periods.

DATA ANALYSIS: DESORPTION-BIOVAIABILITY MODEL (DBM)

A coupled biodegradation-desorption model (Figure 4-2) that incorporates degradation of a non-desorbable fraction was used to track total as well as liquid phase and sorbed phase naphthalene in a batch slurry reactor. The overall degradation of naphthalene in a batch slurry reactor using mass balance on naphthalene can be represented by equation 4-1.

$$-\left(V_l \cdot \frac{dC}{dt} + m \cdot \frac{dS}{dt}\right) = \left(R_{bio} \cdot V + m \cdot k_{nd} \cdot S_{nd}\right) \qquad [1]$$

where m(kg) is the total mass of the soil, C(μg/L) represent naphthalene concentration in solution phase, S(μg/Kg) represent total sorbed-phase naphthalene, V(L) represents volume of the liquid, and k_{nd} is the first-order biodegradation co-efficient for

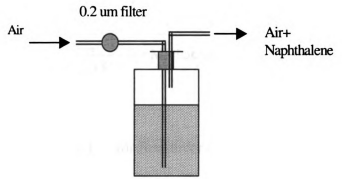

Figure 4-1. Configuration for Nitrogen-Sparging of Naphthalene in liquid phase

A = Dissolved and Equilibrium site

B = Non-Equilibrium site

C = Irreversible phase

Jn = mass flux from non-equilibrium

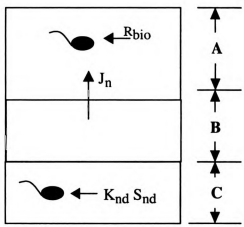

Figure 4-2. Different Compartments of Desorption-Bioavailability Model (DBM)

non-desorbable fraction(S_{nd}) of total sorbed contaminant (S). R_{bio} is the liquid-phase biodegradation rate expression, which can be expressed by following equations:

$$R_{bio} = \frac{V_{max} \cdot C}{K_m + C} \quad \text{for Michaelis Menten kinetics,} \qquad [2]$$

or

$$R_{bio} = K_1 \cdot C \quad \text{for first-order kinetics} \qquad [3]$$

Where, V_{max} is the maximum flux that a cell can obtain, K_m is the Michael-Menten constant, and K_1 the first-order degradation rate co-efficient. The non-desorbable fraction is tracked by following mass balance equation

$$\frac{dS_{nd}}{dt} = -k_{nd} S_{nd} \qquad [4]$$

The total sorbed phase (S) was assumed to consist of equilibrium (S_{eq}), non-equilibrium (S_{neq}) and non-desorbable (S_{nd}) sites. The equilibrium site represents the fraction that releases spontaneously during the desorption experiment and is described by a linear partitioning model. The non-equilibrium site fraction allows slower release of contaminant and can be represented by a first order expression. The non-desorption site is defined by Park et al. (2001) to be containing contaminant that cannot be released to aqueous solution during the experimental desorption period. Mathematically, these three-sorbed phase fractions are described as follows:

$$S_{eq} = f_{eq} \cdot K_d \cdot C \qquad [5]$$

$$S_{nd} = f_{nd} \cdot K_d \cdot C_e \qquad [6]$$

$$dS_{neq}/dt = \alpha \cdot (f_{neq} \cdot K_d \cdot C - S_{neq}) \qquad [7]$$

Where K_d is the sorption coefficient, C_e is the liquid phase concentration in sorption equilibrium, t is desorption time in minutes, α is the first-order desorption rate coefficient (per minute). The three remaining terms f_{eq}, f_{neq}, and fnd represent, respectively, equilibrium site fraction, non-equilibrium site fraction, and non-desorption site fraction. The three site fractions were measured from the desorption experiment: f_{nd} corresponds to the plateau of the desorption rate profile; and f_{eq}, f_{neq} and α were estimated by non-linear regression analysis of desorption data with the constraint that

$$f_{eq}+f_{neq}+ f_{nd} =1 \qquad [8]$$

RESULTS AND DISCUSSION

Sorption of Naphthalene

Figure 4-3 shows sorption isotherms for naphthalene on the selected natural soils. The isotherms on all the three soils were nearly linear over the evaluated concentration range, with the regression coefficient (n) ranging from 0.94 to 0.996. The sorption co-efficient (K_d = slope of isotherms) ranged from 15 to 240 ml/g (Table 4-2) that increases as organic content of the soil increases. To compare the sorption of natural sorbent with synthetic sorbent, activated carbon was tested using the same experimental procedure as used for natural soils. Sorption of naphthalene by granular activated carbon displayed a curvilinear isotherm, and the data were adequately fitted with a Freundlich's

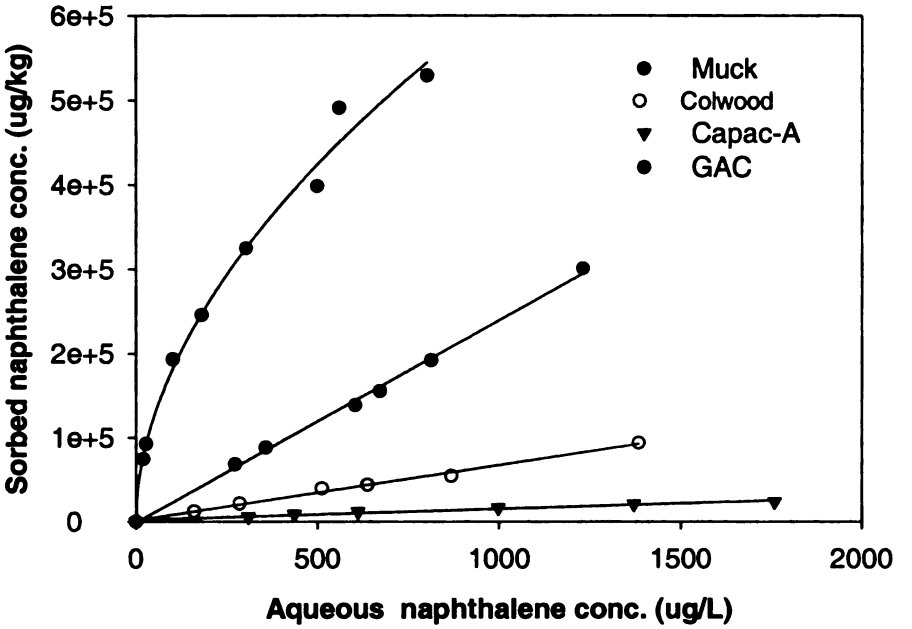

Figure 4-3. Sorption Isotherms of Naphthalene in natural soils and Granulated Activated carbon (GAC)

Table 4-2. Parameters for sorption of Naphthalene by the A horizon of selected soils and Granular Activated Carbon

	Freundlich equation			Linear equation		
Soil	K_F	n	R^2	K_d	R^2	K_{oc}
	$((\mu g/kg)/(\mu g/L)^n)$			(L/kg)		(L/kg)
Capac	--	--	--	15	0.94	457
Colwood	--	--	--	68	0.99	872
Houghton Muck	--	--	--	240	0.996	626
Granular Activated Carbon	15858	0.53	0.99	--	--	--

model (Figure 4-3). Values of the Freundlich's parameters (K_F, and n) are listed in Table-4-2. Mass balances in sorption equilibrium studies ranged from 93 to 102 percent of the initial naphthalene added to the aqueous phase of the system.

De-sorption of Naphthalene

De-sorption of naphthalene from all the selected sorbents was expressed in percent of the total sorbed naphthalene desorbed during the time of measurement (Figure 4-4). The three site desorption model was used to fit de-sorption data and to estimate desorption rate coefficient along with each site fraction (equilibrium, non-equilibrium, and non-desorbable) for all sorbents (Table 4-3). The three-site mathematical model fit well to all the sorbents, with R^2 values ranging from 0.92 to 0.96. The equilibrium site fraction, which desorbed instantaneously, was observed to decrease as the soil-carbon content increased ranging from 0.74 for low carbon soil (Capac-A) to 0.013 for activated carbon.

Desorption from non-equilibrium site fractions of all natural soil was very significant within the first 1000 minutes; however, further desorption beyond 1000 minutes was negligible. Desorption from the activated carbon was almost completed within the first 250 minutes. The desorption rate coefficient (α) is generally observed to increase as carbon content increases, ranging from 0.0016 for Colwood soil to 0.0075 for activated carbon. The extent of desorption is same for all natural soil (~85% within de-sorption time of 3 days); however, activated carbon desorbed only about 14% of the total sorbed naphthalene. A large fraction of the sorbed naphthalene was observed to be resistant to desorption in activated carbon, as compared to natural soil. This may be due to the surface adsorption of naphthalene on hard/black carbon of activated carbon

Table 4-3. Desorption parameters[a] evaluated by a three-site desorption model[b]

Soil	f_{eq}	f_{neq}	f_{nd}	α (min^{-1})	R^2
Capac	0.74(0.042)	0.1	0.16(0.009)	0.0025(0.0008)	0.94
Colwood	0.62(0.023)	0.23	0.15(0.010)	0.0016(0.0004)	0.94
Houghton Muck	0.58(0.034)	0.27	0.15(0.005)	0.005(0.0017)	0.97
Activated Carbon	0.013(0.004)	0.127	0.86 (0.01)	0.0079(0.0006)	0.98

[a] f_{eq} f_{neq} f_{nd}= equilibrium-, non-equilibrium-, non-desorption-site fractions; α= first order desorption rate coefficient for non-equilibrium sites.

[b]Numbers in parentheses are standard deviations of the evaluated values.

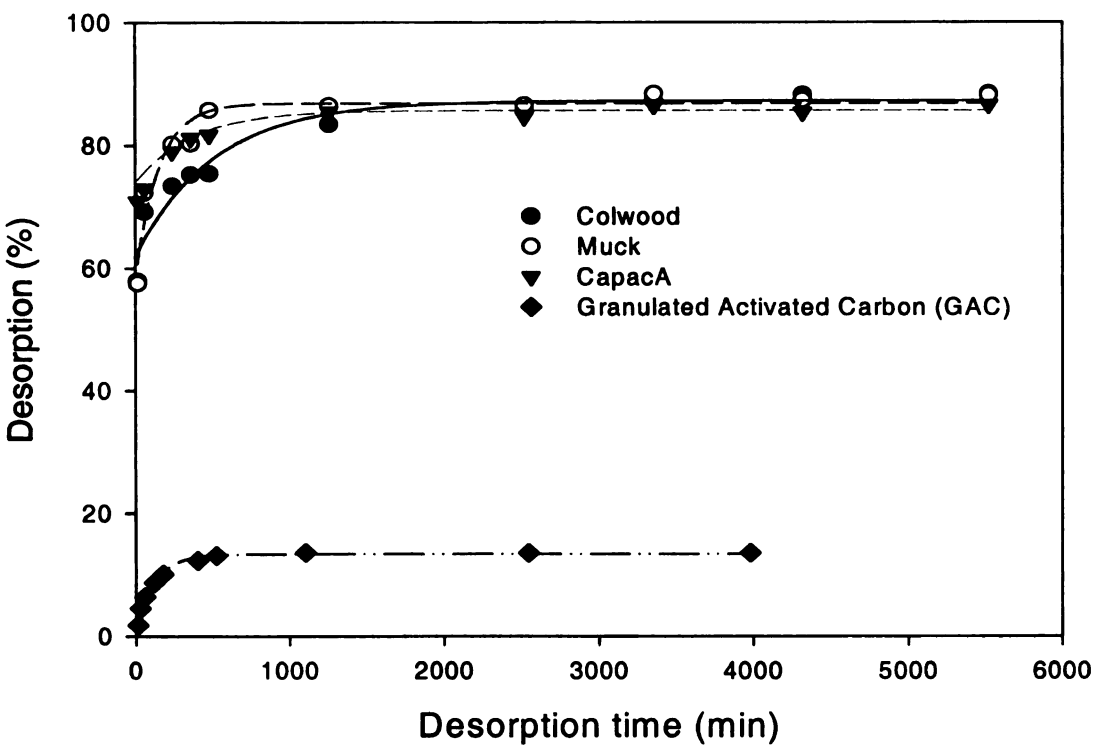

Figure 4-4. Desorption of Naphthalene from natural soils and Activated Carbon

as compared to natural soil. This may due to the surface adsorption of naphthalene on hard/black carbon of activated carbon. The surface adsorption is a competitive phenomenon and is expected to be stronger as compared with partitioning into natural soil organic matter.

Determination of Non-Desorbable fractions

The serial dilution and nitrogen-sparging experiments were carried out to determine non-desorbale fraction of naphthalene for all the sorbents. Table 4-4 presents a summary of the non-desorbable fraction for each of the sorbent using three different methods. Figure 4-5 shows desorption isotherms for all the sorbents using serial dilution experiment. The desorption isotherm for all the sorbents lie significantly above the sorption isotherm, indicating clear existence of non-desorption sites . The non-desorption site fraction was then calculated from the intercept of the desortion isotherm and the initial sorbed-phase concentration. The calculated values were further confirmed by extraction of the soil with methanol after the last desorption step. For activated carbon, the non-desorabable fraction was extracted sequentially in three steps. In the sparging experiment, the non-desorbable naphthatlene was extracted from sorbents when liquid phase napthalene in soil-water-slurry effectively reduced to a non-detectable concentration. The fraction was then calculated by dividing the mass of napthathlene in non-desorbable fraction to initial sorbed-phase.

For all sorbents, the serial dilution method showed greater non-desorbable fraction than nitrogen the sparging method. This difference could be explained by the fact that water alone cannot extract a non-labile fraction of naphthalene that has been shown

Table 4-4. Fraction of desorbable naphthalene by three methods in natural soil and and Activated Carbon.

Soil	Serial dilution	Sparging	Non-linear regression Analysis
	Non-desobable (fnd)	Non-desorbable (fnd)	Non-desorbable(fnd)
Capac-A	$0.21(6x10^{-3})$	$0.13(1.4x10^{-2})$	$0.16(9x10^{-3})$
Colwood	$0.19\ (2x10^{-3})$	$0.09(1x10^{-3})$	$0.15(1x10^{-2})$
Muck	$0.17(1x10^{-2})$	$0.1(7x10^{-3})$	$0.15(5x10^{-3})$
GAC	$0.69\ (1x10^{-2})$	$0.75(2x10^{-2})$	$0.86\ (1x10^{-2})$

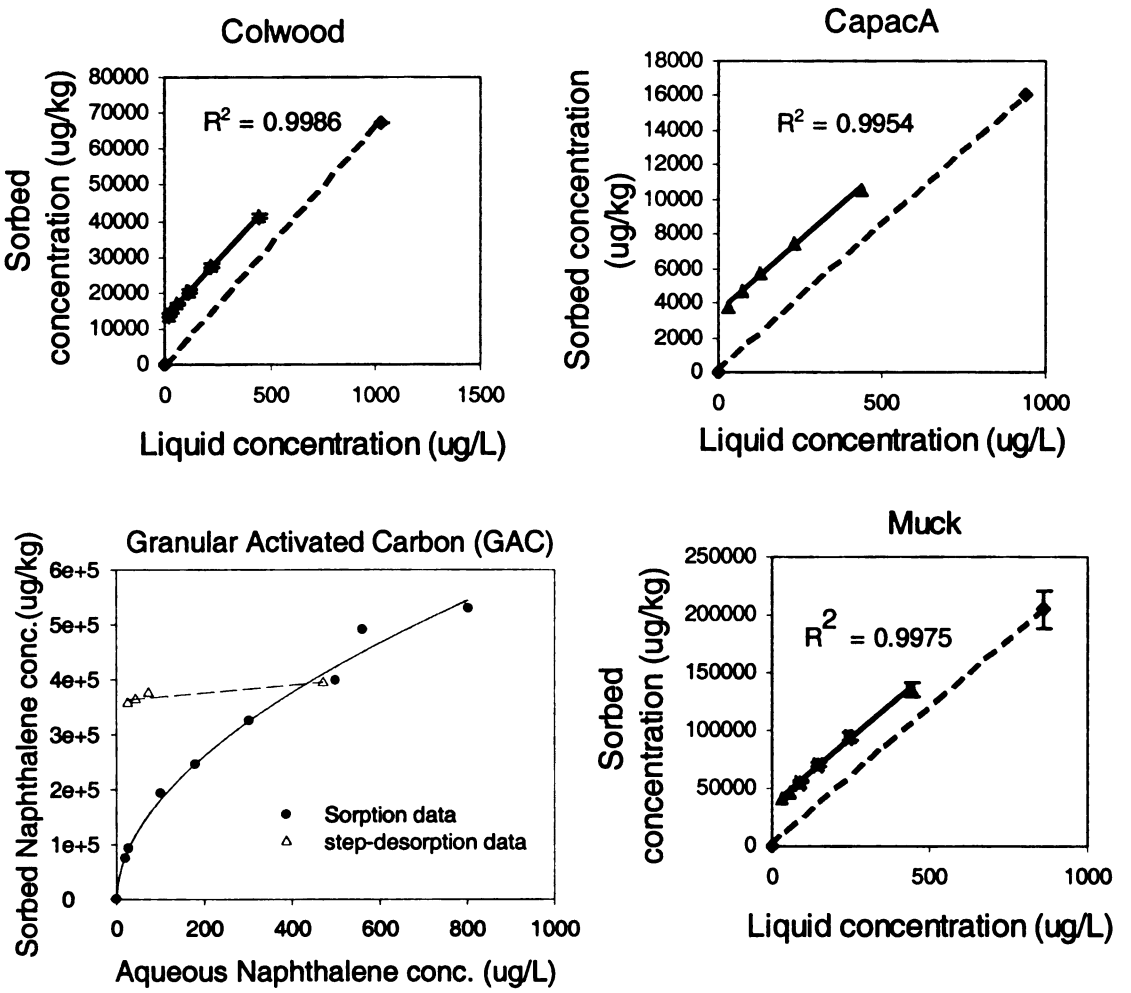

Figure 4-5. Serial dilution desorption in three natural soils and Granulated Activated carbon (GAC)

to be present in the solid phase. However, the sparged-nitrogen may access a larger pool of labile fraction of the naphthalene in the soil, perhaps representing a better method of determining this fraction. In all the abotic experiments, the HPLC analysis indicated that naphthalene was not transformed during the sorption and de-sorption periods. The total recovery of naphthalene was higher than 93% for all sorbents used.

Characterization of strains

To clearly observe the role of chemo-taxis and motility in naphthalene degradation, the wild type *Pp*G7 and its two mutant strains were first checked for these phenotypes. Each strain was first tested for chemotaxis and motility with a soft-agar swarm plate assay. Only wild-type cells had a positive chomotactic response to succinate and naphthalene. The wild type was observed to move outward from the center of plate as they metabolized the carbon source, forming a sharp ring of growth. Contrary to the wild-type, both non-chemotactic and non-motile mutants did not respond to the gradient of carbon source that was created as they metabolized the carbon source at the center of plate. All three strains were further observed under transmission electron microscope (TEM) for flagellated structures: the wild type and non-chemotactic strains possessed polar flagellated structure, while the non-motile strain was devoid of a flagellated structure (Fig 4-7). These two tests confirmed that the wild type possessed both motility and chemotactic phenotypes, whereas non-chemotactic and non-motile strains did not have the capability to sense and move due to missing flagella and chemo-receptor genes, respectively.

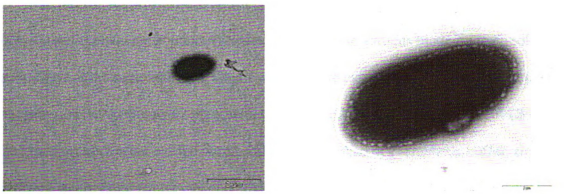

Figure 4-6. TEM for *Pseudomonas putida*-G7(wild-type) on Left, and Non-motile mutant on right

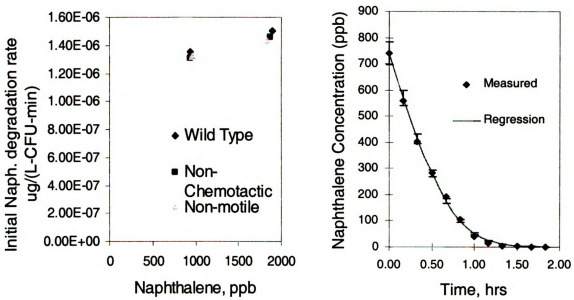

Figure 4-7. Naphthalene degradation of three strains in Liquid medium

Naphthalene Degradation Kinetics in Soil Extract

Initial studies with wild-type Pseudomonas putida G7, non-motile and non-chemotactic strains were conducted to determine if these variants biodegrade dissolved naphthalene at the same rate. The kinetic studies were carried out at initial naphthalene concentrations of 1 ppm and 2 ppm, with an initial cell concentration of ~$2x10^6$ CFU/ml. The kinetic studies were carried out in soil extract, instead of chemotaxis buffer, since dissolved soil organic matter can potentially affect naphthalene degradation kinetics. Figure 4-7 shows initial naphthalene degradation rates for each strain. The three strains showed similar rates of naphthalene degradation at both 1 ppm and 2 ppm of naphthalene under a well-mixed system. This confirms that all three strains possess the same enzyme system that is responsible for naphthalene degradation. Therefore, any difference in naphthalene degradation rate or extent among these strains in a soil-slurry system would be due to the respective phenotype (chemotaxis/motility). Also shown in Figure 4-7 is degradation of naphthalene by PpG7 over an extended period of time. The degradation of naphthalene in the soil-extract solution followed the Michaelis-Menten equation, and kinetic parameters were estimated by fitting the Michaelis-Menten equation to degradation data for three strains. The maximum specific utilization rate co-efficient, V_{max}, and the half-saturation constant, Km, were estimated as 500 ug/L-hr and 301 ug/L, respectively.

Bio-availability of Sorbed Naphthalene in Natural Soils Under Mixed Conditions

Bio-availability assays for wild-type, non-motile mutant, and non-chemotactic strains were performed using three natural soils with different sorption co-efficients. Data

from the bioavailability assays for all combination of bacteria and natural soils are shown in Figures 4-8 to 4-10. In all cases, liquid phase naphthalene degraded rapidly and become negligible within the first 4 hrs for all combinations of soil and bacterial strains. Sorbed phase fraction, on other hand, degraded in a biphasic manner. The initial steep curve represents rapid loss of desorbable naphthalene fraction followed by a much milder curve that represents loss of non-desorabable fraction. The degradation of the desorbable fraction was completed within the first 5 hours, coinciding with complete degradation of the liquid phase fraction. At the end of the desorbable fraction, the degradation of the non-desorabable fraction took place at a slower rate and last over an extended period of time (> 40 hrs). The later part of the sorbed phase degradation profile clearly indicates decreased bio-degradation rate due to the presence of the non-desorbable fraction of naphthalene.

Clearly, in all cases, the initial sorbed-phase naphthalene concentration decreased to levels below the non-desorbable concentration, as shown by the two horizontal lines. The upper dotted lines and lower dashed lines represent extent of non-desorbable fractions as obtained from serial dilution and Nitrogen-sparging experiments, respectively. It is to be noted that the inflection point of the sorbed phase degradation profile coincided with lower dashed lines, indicating that nitrogen-sparging method provided better estimates of the non-desorbable fraction. The decrease of sorbed phase naphthalene below the dashed lines, which represents a harsher extraction method, indicates that all strains were able to degrade and remove more sorbed phase naphthalene than could be accomplished in abiotic experiments. The bio-availability data, which included both liquid phase and sorbed phase data, were then fitted with the coupled

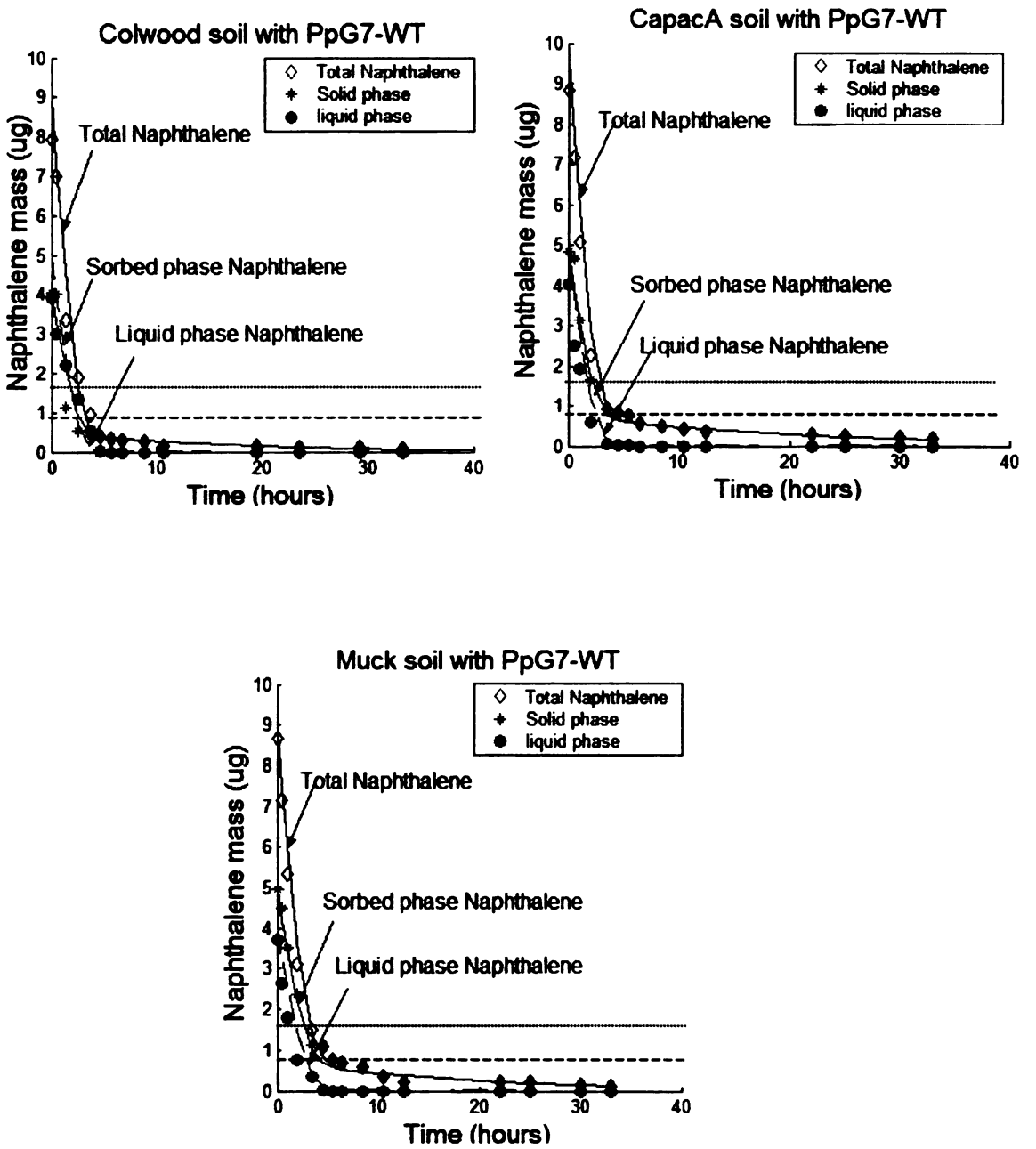

Figure 4-8. Naphthalene degradation due to PpG7-Wild type in three natural soils

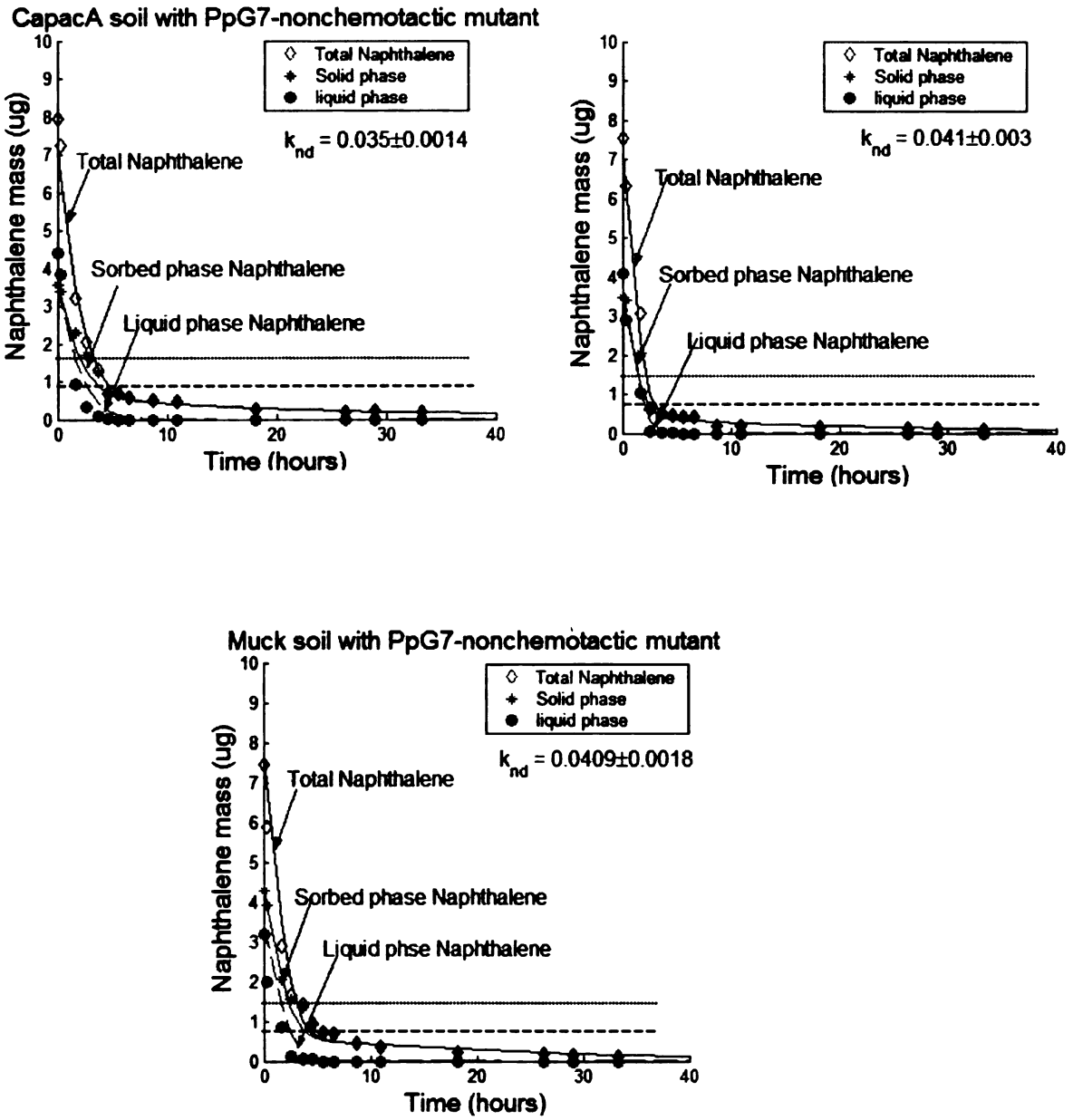

Figure 4-9. Naphthalene degradation by Pp-G7(Non-chemotactic mutant) in three natural soils.

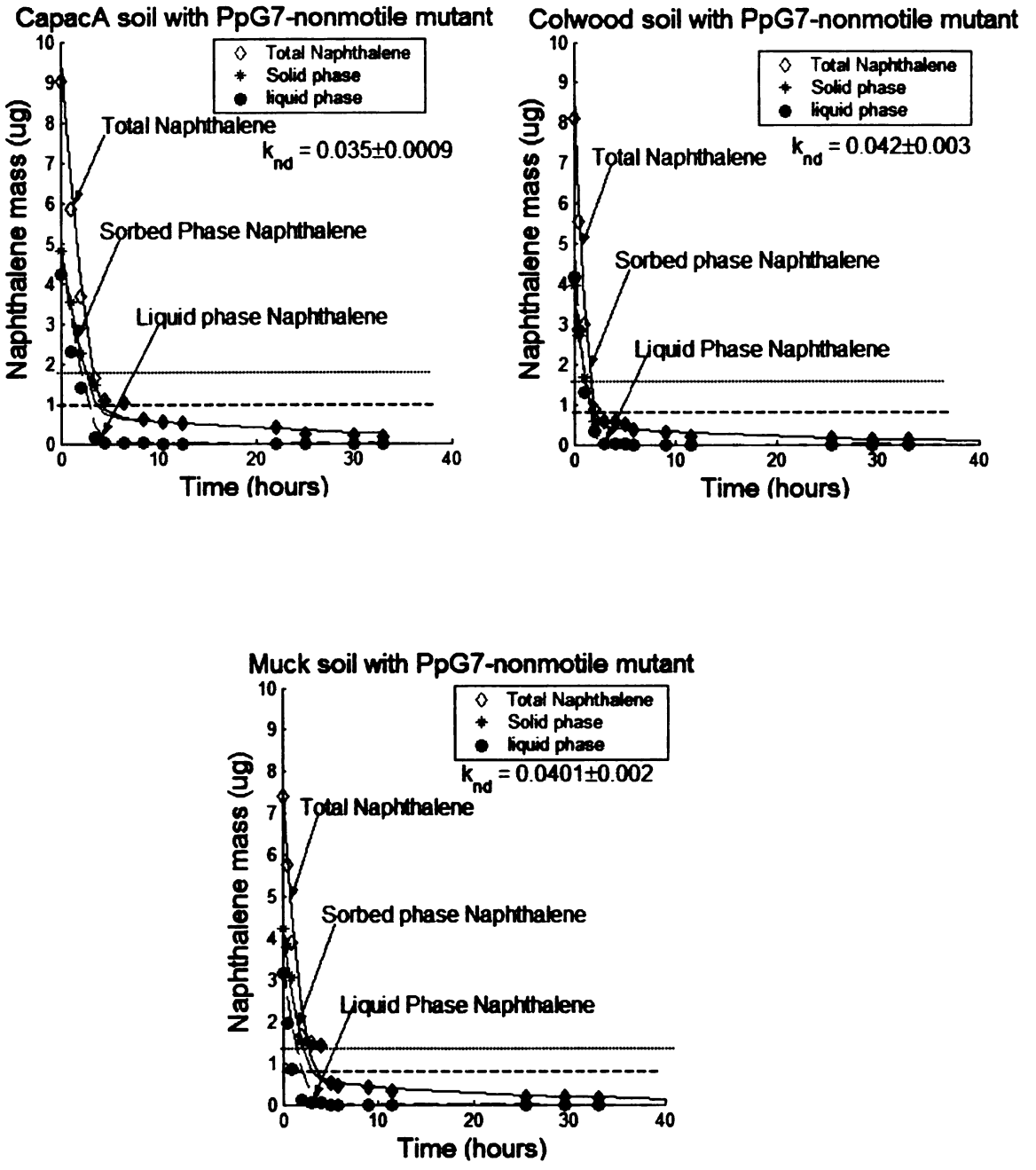

Figure 4-10. Naphthalene degradation by Pp-G7(Non-motile mutant) in three natural soils.

desorption-biodegradation model (DBM). The simplified DBM model provided a reasonable description of both liquid- and sorbed phase data for all combinations of strains and soils over the entire experimental period. The DBM model estimated two sets of degradation rate coefficient: one for easily degradable fraction (liquid and desorbable fraction) and other for difficult to degrade fraction (non-desorbable fraction). The liquid/desorbable fraction of naphthalene was assumed to be degraded following Michalis Menten kinetics with coefficient (K_m and V_{max}). The non-desorbable fraction was, on other hand, assumed to follow 1st order kinetics with coefficient represented by K_{nd}. These estimated degradation rate coefficients from all of the combinations are shown in Table 4-5.

Bio-availability of Sorbed Naphthalene in Natural Soil Under Static Condition

To illustrate more clearly the role of chemotaxis and motility in overcoming mass-transfer limitation, bioavailability experiments were repeated without mixing. All the other conditions were kept similar as in the mixed system, except the whole content was under quiescent conditions. The three strains were tested both in low organic (Capac-A) and high organic (Muck soil) soil-slurry systems. It was expected that only wild-type (which is motile and chemotactic) could access to localize zones of naphthalene, which is created due to lack of mixing, as well as to the non-desorbable/sequestrated fraction of sorbed naphthalene. Thus, comparing the rate and extent of wild type with two mutant strains would clearly demonstrate role of chemotaxis and motility phenotypes on biodegradation of soil-sorbed naphthalene.

Table 4-5. Bio-kinetic parameters[a] evaluated for three strains of *Pseudomonas putida* G7

Soil	Wild Type			Non-motile			Non-chemotactic		
	k_m	V_{max}	k_{nd}	k_m	V_{max}	k_{nd}	k_m	V_{max}	k_{nd}
	ug/L	1/hr	1/hr	(ug/L)	(1/hr)	(1/hr)	(ug/L)	(1/hr)	(1/hr)
Capac A	980	300	0.05 (0.02)	1050	325	0.035 (0.021)	890	260	0.035 (0.017)
Colwood	980	250	0.058 (0.03)	1080	280	0.041 (0.025)	1200	300	0.042 (0.022)
Muck	950	300	0.054 (0.02)	950	310	0.041 (0.026)	1050	280	0.04 (0.023)

[a] k_m *and* V_{max} are Michaelis-Menten degradation parameters for liquid and desorbable sorbed phases ; k_{nd} = first order degradation rate for non-desorbable fraction. Standard deviations are listed in parentheses.

Data from the bioavailability assays, under no-mix conditions, for all combinations of selected bacteria and natural soils are shown in Figures 4-11 and 4-12. In all cases, bacteria could only degrade naphthalene in liquid and desorbable fraction of the naphthalene; and, were unable to degrade mass-transfer limited and non-desorbable fractions of the sorbed phase. The degradation profiles for all three strains in each soil were observed to be similar, with initial rapid degradation phase (represented by the line with steeper slope) followed by no-degradation phase (represented by the horizontal line) due to mass transfer limitation of naphthalene from solid phase. None of the profile from all combinations dropped to a level below the non-desorbable naphthalene concentration, shown by horizontal dashed lines. At the end of the treatment period, the amount of residual naphthalene was observed to be greater in Capac A soil than Muck soil. This can be attributed to the fact that in the case of Muck soil, greater surface area was available for mass-transfer of naphthalene into the overlying water column as compared to Capac-A soil. Thus it can be expected that, under quiescent conditions, greater surface area would offer less resistance to mass transfer of naphthalene from the soil-water interface and hence increased degradation. The bioavailability data were then fitted with the DBM model to estimate the degradation rate coefficient for all combinations. By incorporating reduced desorption fraction, due to static conditions, DBM provides a reasonable description of total naphthalene degradation data for all combinations of strains and soils over the entire experimental period. The DBM model estimated only k_{eq}, which represent both liquid and desorbable fraction of the sorbed phase. The degradation of the non-desorbable fraction of the sorbed phase was estimated as zero. The estimated degradation rate coefficient (k_{eq}) from all combinations is shown in Table 4-6.

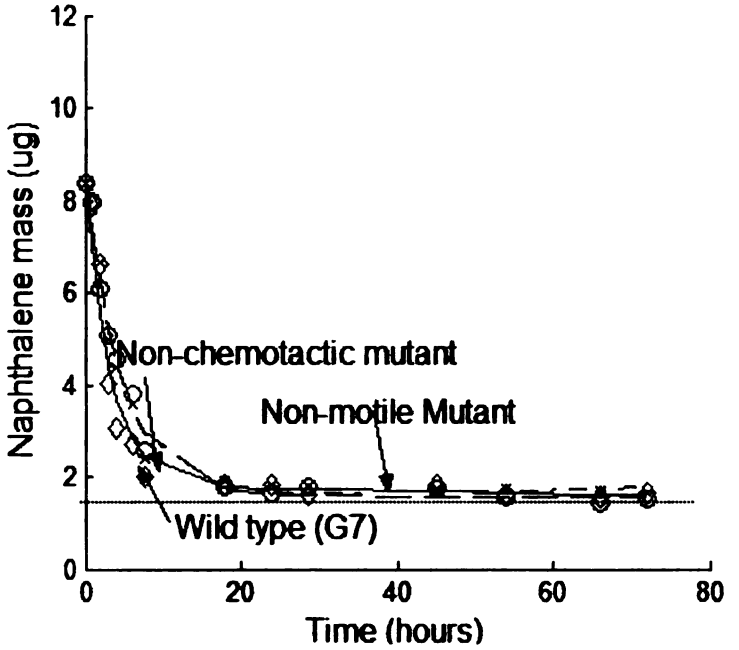

Figure 4-11. Naphthalene degradation by three strains in Muck soil under static condition

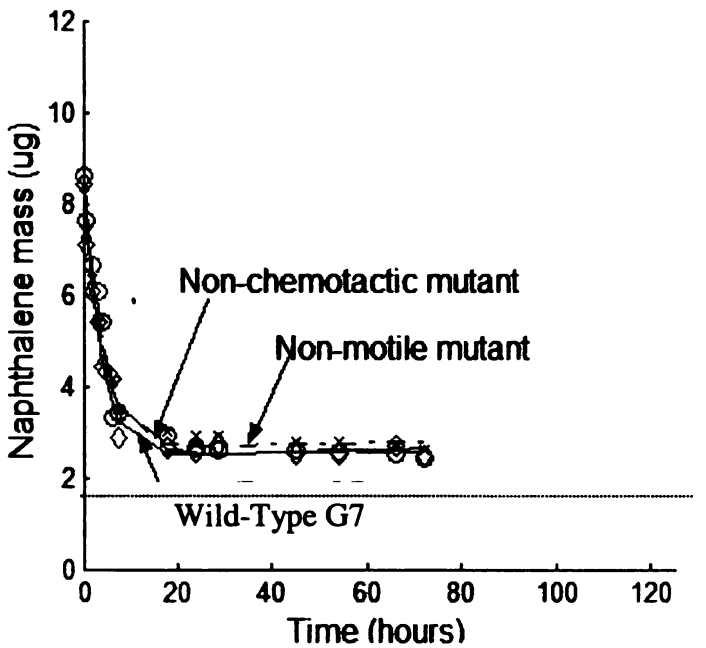

Figure 4-12. Naphthalene degradation by three strains in Capac-A soil under static condition

Bioavailability of sorbed naphthalene in Activated Carbon under mixed system

The bioavailability of naphthalene was also compared for wild type and non-motile strains in a mixed slurry system containing activated carbon. The objective of the experiment was to test whether *pseudomonas putida* G7 could differentially utilize GAC-sorbed naphthalene, and also to determine its accessibility to the non-desorbable pool of naphthalene due to its motility and chemotactic phenotypes.

The degradation rate data for both wild-type and non-motile strains in a mixed slurry system containing activated carbon is shown in Figure 4-13. The profile of naphthalene degradation for both strains indicates an initial rapid degradation followed by an extended period of slow degradation. The initial period of degradation, which mostly occurred in liquid and desorbable fraction, lasts for the first 3 hours. During the subsequent period, the degradation of naphthalene occurred in a non-desorbable pool of naphthalene at a relatively slower rate.

It is clear from Figure 4-13 that both strains could degrade non-desorabable fraction of naphthalene. This has been indicated by the profile of total napthalene mass dropped to a level below the non-desorbable fraction, shown by the dashed horizontal lines. However, wild-type strain could able to degrade non-desorbable fraction at a relatively greater rate, as indicated by slope of degradation profile (Figure 4-13, compare diamond symbols for wild-type and asterisk symbols for non-motile strain). For quantitative comparison, the data were fitted with the DBM model to estimate biokinetic parameters (Table 4-7).

Table 4-6. Estimated bio-kinetic co-efficient for liquid/desorbable fraction (Keq) for Wild-type and non-motile strains in CapacA and Muck soils under static conditions

Soil	Wild Type	Non-chemotactic mutant	Non-motile mutant
	Keq (h^{-1})	Keq(h^{-1})	Keq (h-1)
CapacA	0.50 ($6x10^{-3}$)	0.42 ($2.7x10^{-2}$)	0.41($4x10^{-2}$)
Muck	0.52 ($2x10^{-2}$)	0.352 ($1.57x10^{-2}$)	0.352 ($5x10^{-3}$)

Table 4-7. Estimated bio-kinetic co-efficients for desorbable phase (Keq) and non-desorbable phase (knd) for wild-type in Activated Carobon (GAC)

Wild Type			Non-motile mutant	
Keq (1/hr)	Knd (1/hr		Keq (1/hr)	Knd (1/hr)
1.6 (0.22)	0.004 (3.8×10^{-4})		1.48 (0.3)	0.0029 (5×10^{-4})

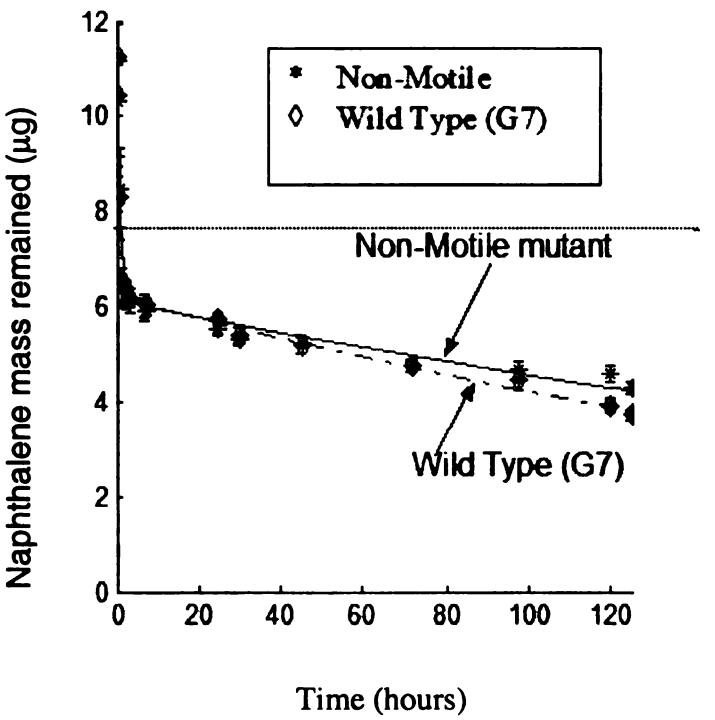

Figure 4-13. Degradation of Naphthalene by Wild type and non-motile strain in Activated carbon

The de-gradation coefficient of the non-desorbable fraction was determined to be greater for the wild type as compared to the non-motile strain, indicating a possible role of motility and chemotaxis phenotypes.

DISCUSSION

Chemotaxis/motility can provide a selective advantage to bacteria in an environment where bioavailability limits biodegradation of contaminants. In a soil-slurry system, containing hydrophobic compound, there always exists heterogeneity in contaminant distribution due to the presence of high-sorptive capacity constituents and a localized lack of mixing. Chemotaxis can guide bacteria to swim, using flagella, and bring them into a close physical contact with soil-sorbed contaminants when desorbable substrate is depleted. As bacteria accumulate in zones of concentrated chemical gradient, they degrade the contaminant at a faster rate. In addition, chemotaxis/motility might be required for the bacteria to move through inter/intra particle pore spaces in response to micro-scale chemical gradients. Thus even small increases in cell density surrounding chemical concentration gradients can lead to enhance bioavailablity as observed in earlier studies (Park et al 2001, Tang et al. 1998).

The role of chemotaxis/motility in enhanced PAH degradation has only recently been verified in porous media. For example, Pedit et al. (2002) reported an enhanced rate of naphthalene biodegradation from a capillary tube, filled with glass spheres, in systems inoculated with wild-type PpG7 as compared with systems inoculated with non-chemotactic mutant strains. The enhanced degradation was observed corresponding to increased number of chemotactic bacteria having moved into capillary tubes. Another

study found increased number of PAH degraders in rhizosphere, in response to PAHs, as compared with unplanted bulk soil controls. The author estimated chemotactic motion of the soil bacteria toward PAHs in the order of 1 mm/min (Ortega-Calvo et al. 2003).

In the present study, experiments were carried out in both natural and synthetic sorbents, with low to high non-desorbable fractions, to observe the effect of chemotaxis and motility on enhanced bio-availability under a mixed and quiescent experimental condition. In a mixed system containing natural soil, all the strains were observed to have similar access to a pool of both liquid/desorbable fraction as well as non-desorbable fraction of soil sorbed naphthalene (Figures 4-9 to 4-11). The evaluated degradation rate constants for all three strains were found to be similar at a 95% confidence interval for all three soils (as shown in Table 4-5). In a similar system containing GAC, the wild type degraded non-desorbable fraction at a significantly (at a 95% CI) greater rate as compared to two mutant strains. However, as in natural soil, both wild type and non-motile strains had equally degraded liquid/desorbable fraction (Table 4-7).

Several plausible explanations can be offered for different responses of strains in activated carbon and natural soils under mixed conditions. In both natural and GAC sorbents, biodegradation began with a constant concentration of naphthalene. As bacteria degrade naphthalene from the aqueous phase, the concentration changes from one isotropic concentration value to another constant value. The maintenance of isotropic naphthalene concentration in the liquid phase was due to uniform mixing of naphthalene that desorbed from the sorbed phase. In the absence of a concentration gradient, chemotactic movement did not occur, and hence both liquid/desorbable phase naphthalene was degraded equally by all strains in both natural and GAC. If there was a

concentration gradient at the solid-water interface, it was highly unlikely to attract bacteria due to the presence of a large amount of dissolved naphthalene. Thus, existence of an isotropic concentration condition during the rapid phase of degradation resulted in no detectable gradient and consequently no chemotactic movement.

After most of the liquid and readily desorbable fraction of naphthalene was degraded, there remained non-labile fractions of naphthalene in both natural and GAC. The non-labile fraction diffuses out naphthalene into surrounding liquid at a very low rate due to film resistance. For biodegradation to continue, the bacteria had to come closer to particle surfaces and directly uptake naphthalene from within the stagnant hydrodynamic boundary layer. Chemotaxis/motility would allow cells to move toward this higher concentration region located at water/solid interface. The results from the present study did not discern role of chemotaxis/motility, as all strains had gained equal access to the non-labile pool of naphthalene in both natural soil and GAC. This may also be attributed to the mixing affect as mixing increases the probability of random collision between soil particles and bacteria and hence greater chance of adhesion. Thus mixing had seemingly masked the role of chemotaxis and motility during initial phase of non-labile naphthalene degradation. Previous study with chemotaxis-mediated biodegradation illustrated the importance of relative rates of substrate mass transfer and bacterial movement. In a mixed or partially mixed system, bacterial chemotaxis and motility was shown to be a much slower process as compared with movement of naphthalene due to convection(Marx and Aitken 2000).

However, the role of chemotaxis/motility seems to become apparent at a longer incubation time in GAC (Figure 4-13). After initial mixing-mediated cell adhesion, more

cells become attracted from the liquid phase toward interface due to a chemical gradient. This resulted in a greater number of cells per unit area of the sorbent particles in a system inoculated with wild type as compared with non-motile strain. Consequently, the rate and extent of degradation of non-labile naphthalene was greater with PpG7 than corresponding non-motile strains. Similar observations were not observed in natural soil probably likely due to short incubation time and reduction of non-labile naphthalene to a threshold concentration, below which biodegradation stops.

To remove the effect of mixing in masking bacterial chemotaxis and motility, the experiments were repeated under quiescent condition. All the strains were introduced carefully at the surface of the water column located well above the thin layer of soil deposited at the bottom of the vials. It was expected that as bacteria degraded naphthalene in the liquid phase, a concentration gradient would be created in a vertical direction. In response to the naphthalene gradient, wild type PpG7 would migrate in large numbers from the liquid phase toward the sediment/water interface. The mutant strains, on other hand, either could not respond to gradient or might move downward in fewer numbers. It was also expected that the presence of a large number of wild type PpG7 and a higher concentration of naphthalene available at the water-soil interface would result in a greater rate and extent of Naphthalene degradation.

Furthermore, the porous structure of soils does not seems to hinder the movement of bacterial cells, since soil pores have sizes ranging from several to hundred of micrometers, which corresponds to the length of the straight-line segments of random bacterial motion (Zaval'skii and Voloshin 2003). Thus, it was expected that only bacteria with motility and chemotactic phenotypes would be able to move from the liquid-phase

into soil pores, as a result of large microscopic chemical gradients. The ability of wild type PpG7 to move within the porous spaces would further result in greater accessibility to sorbed phase naphthalene and hence increase degradation rate and extent. This role of chemotaxis in penetrating porous spaces has been well documented in earlier studies; the chemotactic migration was found to be faster in the porous medium than in the aqueous phase (Duffy et al. 2003). The faster movement was attributed due to large micro-scopic chemical gradients in porous medium.

However, the experimental results of this study did not support the expectation of the role of motility and chemotaxis in enhancing bioavailability under quiescent conditions. None of the strains, in either low or high K_d soil were able to access mass transfer limited/non-desorbable fraction of naphthalene. The degradation profiles for all the combinations were observed to be similar, with an initial rapid phase of degradation followed by a phase of almost negligible degradation as indicated by a horizontal line. This reduction can be attributed to changes in the mass transfer parameter as a result of biodegradation. As bacteria degrades naphthalene in solution phase, the mass transfer rate from the sorbed phase decreased gradually and finally diffusion appeared to control the movement of naphthalene from soil to overlying water. Both soils retained more naphthalene mass than non-desorbable fraction, clearly indicating degradation is limited by mass-transfer due to lack of mixing. Further evidence supporting this fact was the observation that the fraction of mass retained is observed to be greater for Capac-A soil than muck soil, due to its smaller surface area to total volume ratio. In conclusion, it appeared that chemotaxis and motility was not observed to have caused enhanced bioavailability of mass-transfer limited naphthalene.

Let us now consider the reasons for observing similar rates of naphthalene degradation for all tested strains, and absence of chemotaxis/motility phenotypes in overcoming mass transfer limited conditions in the quiescent system. Since the cells were introduced into the experimental system in a vertical direction, we may expect the potential effect of gravity on bacterial movement. The relative importance of gravity can be seen by comparing the terminal settling velocity and chemotactic velocity of injected cells. In the aqueous system wild type *P. putida* G7 respond to chemical gradient at a typical chemotactic velocity range from 20 to 40 μm/sec (Marx and Aitken, 2000 b), which is far greater than its corresponding terminal settling velocity of 0.02 μm/sec(Pedit et al. 2001). Therefore, in the experimental system, with a water column height of 6 cm above soil layer, the relative affect of gravity can be neglected on overall movement of bacterial cells due to chemotaxis/motility.

In the absence of a gravity effect, several plausible explanations can be offered for not observing chemotaxis and motility effects on naphthalene gradation in the quiescent system. If the initial concentration of the attractant is high enough to saturate bacteria's chemoreceptor, then the bacteria cannot sense the concentration gradient. For this study, the saturation of the chemoreceptors could have occurred, but this assumption cannot be validated since no data is available on dissociation constant for the PpG7 chemoreceptor.

The inaccessibility of WT strains to non-desorbable pool of naphthalene can be explained in terms of the complex and heterogeneous nature of the soil matrix. The soil used in this study contained various amounts of organic and clay matters as shown in Table-1. The presence of organic and clay content could have restricted bacterial movement due to: 1) bacterial size exclusion effects at the soil water interface, and 2)

adsorption-adhesion effect due to physio-chemical interaction between bacteria and soil particles. Earlier studies that observed chemotactic movement were mainly carried out in a saturated sand medium that contained continuous macro-pores with no clay or organic content. Even with clean sand matrix, the previous researcher (Barton and Ford 1995) observed marked reduction in chemotactic movement as compared to that measured in fluid system. The reduction in motility coefficient was shown to be a function of soil porosity and tortousity as given by equation-9:

$$\mu_{eff} = \frac{\varepsilon}{\tau} \qquad [9]$$

Where, μ_{eff} is motility in bulk water, and $\frac{\varepsilon}{\tau}$ represents the ratio of soil porosity to its turtosity. In a recent study, the chemotactic sensitivity coefficient, which represents key chemotaxis property, was determined to be reduced by 4 times in capillary assay in presence of glass beads (Marx and Aitken 1999). Thus, chemotactic sensitivity coefficient was greatly reduced in low porosity; high organic soils like Capac-A and muck soil, and resulted in low bioavailability of sorbed naphthalene in no-mix system.

CONCLUSIONS

In this study we evaluated the role of chemotaxis/motility on naphthalene degradation in sorbent-slurry system under stirred and quiescent conditions. The rate and extent of sorbed-phase naphthalene was monitored in the systems inoculated separately with the chemotactic wild-type strain and its mutant strains, and compared the results for

evaluating the role of chemotaxis/motility. In natural soils (with low to high organic content), under both stirred and static conditions, the rate and extent of naphthalene degradation was observed to be similar for all the strains. Whereas in stirred system all the strains had an equal access to a non-desorbable fraction of naphthalene; in the static system, however, none of the tested strain had gained access to the non-desorbable fraction of naphthalene.

Experiments carried out with activated carbon, under stirred conditions, showed that both WT and mutant strains had accessed to the non-desorbable fraction of naphthalene. However, WT degraded non-desorbable phase naphthalene at a significantly greater rate as compared to its mutant strains. The difference of uptake rate between WT and mutant strains could be explained due to the presence of significant concentration gradient at soild/water interface as a result of large pool of non-desorbable naphthalene. The concentration gradient attracted greater number of WT cells, due to its chemotaxis/motility phenotypes, that resulted in a greater rate of non-desorbable naphthalene degradation as compared with system inoculated with mutant strains.

This study suggest that in a heterogeneous soil containing medium to high content of clay and organic content, the role mixing is very important for successful bioremediation. In the absence of mixing both passive and active uptakes of naphthalene were retarded. Lack of mixing reduced effective diffusion of molecules in soils due to solid phases, dead-end pores and high tortousity of the system. Also, lack of mixing reduced the number of sites available for bacterial attachment which would result in the direct uptake due to pore sizes exclusion and retardation effect.

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CHAPTER 5. INFLUENCE OF BACTERIAL ADHESION AND ENHANCE BIOAVAILABILITY OF NAPHTHALENE IN SOIL

ABSTRACT

Bacterial attachment has the potential to increase the rate of degradation of organic contaminants, but its influence on degradation of non-desorbable organic contaminants in a soil-water system has not been examined. This study was an attempt to examine factors affecting bacterial attachment and consequently enhanced bio-degradation. The factors studied included molar concentration of buffer medium and the bacterial cell surface structure. Bio-availability experiments were carried out in a stirred batch soil-slurry system inoculated with: 1) wild-type *Pseudomonas putida G7* strain under variable molar concentration of buffer medium, and 2) different strains of *Pseudomonas putida* G7 (i.e. wild-type, non-motile mutant, and non-adhesive mutant), possessing variable attachment capabilities, under constant molar concentration of buffer medium. Three orders of decrease in molar concentration of buffer medium did not affect rate and extent of sorbed phase naphthalene degradation by *P.putida* G7. Important attachment phenotypes (e.g., expo-polysaccharides, flagella) of PpG7-WT were also observed to have played no significant role in enhanced bioavailability of sorbed phase naphthalene. The wild type *P.putida* G7 and the mutant strains were observed to have equally accessed non-desorbable fraction of naphthalene and degraded the fraction at an equal rate and extent. The results of this study suggest that factors promoting bacterial attachment (i.e. removal of exo-polysaccharides, presence of flagella and reduction of

buffer medium concentration) do not contribute to biodegradation of non-desorbable fraction of soil-sorbed naphthalene in soil-slurry system.

INTRODUCTION

Bacterial Attachment and Enhanced bioavailability

Recently, several researchers observed enhanced bioavailability of soil-sorbed PAHs (Calvillo and Alexander 1996; Guerin and Boyd 1992; Park et al. 2001; Tang et al. 1998). Their studies suggested that biodegradation of sorbed PAHs is not exclusively dependent on abiotic mass transfer of the compound into the water phase. Enhancement in observed bioavailablity was attributed either to the direct bacterial access to sorbed molecules or increase in abiotic mass transfer to the solution phase due to specific physiological traits of bacteria (e.g. bio-surfactant production, attachment etc). It has been demonstrated that different bacteria degrade sorbed PAHs at different rates, due to difference in cell physiological trait (Guerin and Boyd 1992). This finding was subsequently supported by several other studies(Tang et al. 1998, Calvillo and Alexander 1996).

Several studies have hypothesized the role of bacterial attachment in enhancing sorbed phase contaminant (Calvillo and Alexander 1996; Guerin and Boyd 1997; Harms and Zehnder 1995). In soils, the degradation of a non-desorbing compounds have been found to be essentially carried out by particle-bound bacteria only (Kirchman 1990; Kuhn 1987). (Fletcher 1986) observed that cells attached to solid surface utilized glucose at faster rate (by a factor of 5 or more) than un-attached cells. The author also examined role of type of solid surface (polythelene, glass, or polyvinylidene fluoride) on activities

of attached cells, and found that composition of solid surface had no affect on utilization of substrate. It was hypothesized that increase utilization of attached cells was either due to concentration of nutrient at surfaces due to adsorption or physiological modification of surface associated cells.

Guerin and Boyd (1992, 1997) used two bacterial species (*Pseudomonas putida* strain 17484 and *Alcaligenes sp.* Strain NP-Alk) to demonstrate different capabilities of bacterial species toward sorbed phase naphthalene in soil water system. Strain 17484 could degrade sorbed phase at the rate greater than de-sorption rate in all natural sorbents; strain NP-Alk, on other hand, was able to degrade aqueous phase naphthalene, only. The authors did not offer mechanism for the differential capabilities; however, they considered surface chemistry and geometry of cells as one of the major factor for the difference. The strain 17484 was shown to be more hydrophobic than strain NP-Alk, and consequently possess greater propensity to attach to soil particles. The attachment capability of strain 17484 was speculated to cause enhance degradation due to 1) exposure of cells to a steeper intra-sorbent concentration and, 2) cells acts as competitive sorption media to a soil-sorbed naphthalene.

Harms and Zehnder (1995) also studied role of bacterial properties in enhancing rate of degradation of 3-Chrlorodibenzofuran off the teflon granules. They observed that degradation rate exceeded de-sorption rate in a system containing bacteria, and that the degradation rate increased with increasing amount of cell attachment to the sorbent. The enhanced de-sorption rate was proposed to be due to specific affinity of the degrading bacteria and the tendency of the organisms to attach to the sorbent. The specific affinity depends on a type of bacteria, and is a measure of efficiency with which a bacterium

reduces the concentration of a substrate. The specific affinity increase as cells approaches closer to sorbent surface. As the cells attached to the sorbent surfaces, they exposed to the steeper concentration gradient, and consequently increase degradation rate.

Tang et al. (1998) also observed enhanced biodegradation rate of sorbed phase phenantherene as compared to abiotic desorption rate in a system containing poly-acrylic beads or sediments. The enhanced biodegradation was, however, observed, with the bacterium (P5-2) being obtained by enrichment on sorbed phenanthrene; and the bacterium that was obtained on non-sorbed phenanthrene could not able to cause enhance biodegradation rate. The authors hypothesized that direct utilization of sorbed chemicals was due to attachment of microorganism to solid surfaces.

Wick et al. (2002) investigated physiological response of *Mycobacterium* sp. LB501T to poorly water-soluble anthracene (which serves as a sole carbon source) crystal in a batch culture. To overcome low dissolution flux of anthracene, *Mycrobacterium* had attached to the solid crystal via extra-cellular polymeric substances (EPS). This indirect attachment of cell favors diffusive mass transfer and, consequently, dissolution of crystal. To further optimize attachment and consequently anthracene uptake, the *Mycobacterium* changes its cells-surface properties. Mycobacterium and related genera excrete mycolic acids in their cell wall, and these molecules are believed to stimulate attachment to hydrophobic surfaces.

Factor Affecting Bacterial Attachments

The mechanism of bacterial attachment to inanimate materials is complex and unlikely to be explained by one or other property of the cell surface (Bos et al. 1999).

There are number of factors that contribute to the attachment of bacteria to the surface that include cell surface charge, bacterial hydrophobicity, the presence of extra-cellular polymers and, flagella. Besides cell surface properties, bacterial adhesion is also influenced by chemical composition of solid surface and liquid medium (Bos et al. 1999).

Numerous studies interpreted bacterial adhesion in terms of hydrophobicity or surface free charge (Stenstrom 1989; van Loosdrecht et al. 1989). Both parameters influence cell adhesion to solid particles; however, the relative influence of high cell hydrophobicity is dominant as compared to surface charge on bacterial adhesion. (Mueller et al. 1992) found that bacteria with low hydrophobicity (*P. flouresences*) attached at far lower rate (five times) on to a glass surface than bacterium with high cell hydrophobicity (*P. aeruginosa*). Bestiaens et al. (2000) isolated adherent PAH-degrading bacteria by enrichment technique using hydrophobic membranes containing sorbed PAHs. The isolated bacterium was shown to be very hydrophobic and strongly adherent to different surfaces.

Microbial surface charge can also influence adhesion, but to a smaller degree as compared to cell hydrophibicity. For example, the contribution of surface charge to adhesion increases when it is chemically modified from negative to positive charge (Klotz et al. 1985). Surface charge also becomes more influential when bacterial cells displayed low degree of hydrophobicity (i.e., more hydrophilic), which allows the bacteria to adhere in the so-called secondary minimum.

Besides hydrophobicity and surface charge, surface components of Gram-negative bacteria such as lipo-polysaccharides (LPS) on the outer bacterial membrane, and more loosely associated layer called extra-cellular polymeric substances (EPS) have

also been shown to play important role in cell adhesions(Pringle et al. 1983; Rijnaarts et al. 1995; Williams and Fletcher 1996). The characteristics of these surface structures are still not well-known, and therefore their affects on bacterial attachment yields controversial results.

Razato (2000) used wild type strain (with longer LPS chain) and its mutant (with truncated LPS chain) to compare their interaction with glass surface. The author observed that while wild type was able to adhere to the glass surface, the mutant failed to adhere. They hypothesized that as mutant lost its core polysaccharide component of LPS, the exposed negatively charged KDO molecules experienced repulsive interaction with negatively charge glass surface. The Wild type, however, experienced attractive interaction due to shielding of negative charge KDO by longer LPS molecules.

DeFlaun and Fletcher (1999) also concluded role of cell surface polymer in adhesion of *B. cepacia* G4 to sand surfaces. The authors isolated adhesion-deficient mutant of *Burkholderia cepacia* G4 in sand column assay and compared its adhesion phenotypes with wild type strain. The mutant was reported to have lost adhesion phenotype due to attenuated O antigen on the LPS. The loss of O antigen part of LPS caused reduced shielding of charged group near the membrane (such as phosphate group in the core-lipid A region) and, consequently there was a net repulsive interaction between mutant and negatively charged sand surfaces.

In some studies the truncation of LPS, however, resulted in a more hydrophobic and, hence more adherent mutant than wild type (Williams and Fletcher 1996). The authors generated and isolated mutants of *Pseudomonas fluorescens* that were altered in adhesion ability. The mutants were demonstrated to have increased adhesion to a variety

of surfaces (quartz sand, hydrophobic polystrene) than wild type strain. The mutants showed increased attachment to hydro-phobic surfaces and decreased attachment to the more hydrophilic sub-straturm. The authors attributed enhanced adhesion property of mutant due to attenuation of O antigen on the LPS. The lack of O antigen exposed lipid moiety and hence rendered the cell surface more hydrophobic.

Objective of the Study

The objective of this study was to investigate the role of bacterial attachment in degradation of soil-sorbed naphthalene. Previous studies did not attempt to determine mechanism by which bacteria attached with soil particles for accessing the soil-sorbed contaminant molecules. In this study, a systematic approach was taken to examine factor affecting bacterial attachment and consequently rate and extent of naphthalene degradation in a batch soil-slurry system. The factors studied included molar concentration of buffer medium and the bacterial cell surface structure. Following hypotheses were tested for observed enhanced bioavailability of sorbed phase naphthalene in a soil-slurry system inoculated with *P. putida* G7:

1. Attachment of the *P.putida G7* cells with soil surfaces increases as the molar concentration of buffer medium increases
2. Wild type *P. putida* G7 adhere to soil particle more efficiently than mutant strains deficient in expo-polysaccharide layer (EPS) and flagellated structures

Bio-availability experiments were carried out in a stirred batch soil-slurry system inoculated with: 1) wild-type *Pseudomonas putida* G7 strain under variable molar concentration of buffer medium, and 2) different strains of *Pseudomonas putida* G7 (i.e.

wild-type, non-motile mutant, and non-adhesive mutant), possessing variable attachment capabilities, under constant molar concentration of buffer medium. Increase in molar concentration of buffer medium has been reported to decrease electrostatic repulsion between bacteria and surfaces and, hence increase in bacterial attachment (Rutter and Vincent 1984). Several researches have also demonstrated significance of EPS layer on adhesion of bacterial cells to solid. It was expected that mutant strain would have limited excess to sorbed phase naphthalene, due to its inability to attach with surface, as compared to wild type.

MATERIAL AND METHODS

Soil

The three soils selected for this are CapacA, Colwood, and muck with low, intermediate and high soil organic content. The soil was prepared as described elsewhere (Park et al. 2001), and their characteristics are summarized in Table 5-1.

Chemical and Media

Both 14C-labeled and unlabeled naphthalene were used in this study. The 14C-labelled naphthalene (sigma, 0.1 mCi, >98% pure) stock solution was prepared by supplementing it with unlabelled naphthalene and was used for sorption, desorption, serial dilution studies. The un-labelled naphthalene (sigma, > 98%) stock solutions were prepared in methanol and stored in 5 ml screw cap vials (capped with Teflon septa) at 4°C before use. All biological assays were carried out with unlabelled naphthalene stock solutions.

Table 5-1. Selected Properties of Soils used in the Study

Soil	% O.C.	% Sand	% Silt	% Clay	PH	CEC [cmol(+)/kg]
Capac	3.3	55	24	21	6.8	24
Colwood	7.8	64	21	15	6.0	43
Houghton Muck	38.3	ND	ND	ND	5.1	156

ND = not determined

Microbiological media were prepared as described previously. The experiments were carried out with chemotaxis buffer (pH =6.8) as described elsewhere (Harwood et al.)

Strains

Wild-type *Pseudomonas putida* G7 along with its two mutant strains were used in this study. Wild-type *Pp*G7 (ATCC 17485) and a mutant strain [*Pseudomonas putida* G7.C1(pHG100)], which is non-chemotactic to naphthalene, were obtained from Dr. Caroline Harwood (University of Iowa). The non-flagellated (non-motile) mutant strain of *P. putida* G7 was obtained from Dr. Michael Aitken (University of North Carolina); the non-motile strain was spontaneously generated and isolated as described elsewhere (Marx and Aitken 2000). The pure wild type, *Pseudomonas putida* G7, has been reported previously to uptake sorbed phase naphthalene in soil slurry. Furthermore, it has been characterized as being motile, and chemotactic (Grimm and Harwood 1997; Grimm and Harwood 1999).

Sorption/Desorption/Serial Dilution Experiments

Sorption, desorption, and serial dilution desorption experiments were carried out as described previously (park 2001). Briefly, all experiments were carried out with initial equilibrium period of two days. The ratio of sorbent to liquid was kept the same in all the experiments: 1:15 for Capac-A soil, 1:65 for Colwood soil, and 1:240 for muck. Sorption experiment was carried out at initial liquid-phase naphthalene concentrations, ranging from 0-2500 μg/L in triplicate for each type of soil. The desorption and serial dilution were both carried out at an initial naphthalene concentration of 2 mg/L. Desorption and serial dilution experiments were initiated at the end of 2 days sorption equilibrium period by adding naphthalene free soil extract to make up the decanted volume. In all these

experiments, vials were tumbled at 9 rpm in the dark. After mixing, each vial was centrifuged and supernatant sampled. The remaining supernatant was then decanted and replaced with methanol to extract soil-sorbed naphthalene. The concentration of naphthalene was then determined by liquid scintillation counting (LSC) and verified by high-pressure liquid chromatography (HPLC) for some samples.

Bioavailability and Biodegradation Rate Experiments

Bioavailability assay was adopted from the method described by Park et al. (2001). Briefly, soil extract controls and soil slurries were set up in 5-mL vials sealed by screw caps with Teflon-lined septa. The soil to water ratio was kept same as used for sorption/desorption experiments. Unlabelled naphthalene was injected in all vials at initial liquid phase concentration of ~ 2000 ug/L. After 2 days of initial equilibrium period on tumbler, soil slurries and soil-free extracts (to determine degradation rate in liquid only) were inoculated with cells harvested at early stationary phase. At precise time intervals, selected vials were centrifuged and 1-ml of supernatant was transferred to a HPLC vial containing 0.01 ml of 10 N NaOH to stop biodegradation of naphthalene. The soil-sorbed naphthalene was then extracted by methanol after removing left over supernatant. Appropriate control vials were included, with no bacteria, to check for any abiotic losses.

Chemical Analysis

Naphthalene was analyzed by HPLC using a C_{18} reverse-phase column, with 80% acetonitrile/20% water mobile phase, and fluorescence detection (280 nm excitation, 340 nm emission). Radioactivity was determined by LSC. The analytical detection limits of naphthalene solution were 5 mg/L and less than 1 mg/L for HPLC and LSC, respectively.

Isolation of Adhesion Deficient Mutant.

An adhesion-deficient mutant was generated by transposon mutagenesis using a standard procedure. *Escherichia coli* strain S17-1 (pUT) was used as the donor strain and *Pseduomonas putida* G7 serves as recipient strain. The donor and recipient strains were grown in Luria-Bertani broth (LB) to late log phase and then mixed at an appropriate ratio. The mixture was syringed out on a filter paper so that donor *E.coli* mate with *P. putida* G7. Filters were incubated for several hours generating approx. 7000 mutants per mating. The filters were then transferred to LB broth in 50 ml tubes for incubation at 30 ^{o}C for 30 min. After incubation the tubes were vortexed, and the bacterial suspension was plated on a minimal medium supplemented with Kenamycin (50 μg/mL) and naphthalene. The resulting transposon mutants that grew on the selective plates were then screened for to identify mutant defective in the production of exo-polysaccharides.

Screen for EPS deficient mutants

Calcoflour white was used to identify the defective mutants. Mutants were streaked on LB plates containing 75 μg/mL of Calcoflour; After 48 hrs of incubation, the florescence of the cells was observed under long wave UV light source and compared with the wild type strain. Calcoflour binding by wild type cells was clearly visible, while no Calcoflour binding was detected by Exo^{-} mutant strains. The adhesion deficiency was further verified by the soil adhesion assays as described elsewhere (Williams and Fletcher 1996).

Screen for adhesion-deficient mutants

Several potential mutants, as isolated by Calcoflour screening process, were first grown in LB to the logarithmic phase. Equal amount of each mutant was then added in

CB solution at a combined titer of approx. $1x10^8$ CFU/mL. An aliquot of 3 ml cell suspension was then carefully placed on top of 20-mL syringe barrel filled with 12 grams of sterilized Ottawa sand. The cells were allowed to interact with the column material for 1 hr, and then column was washed with 3 ml of CB solution. The first 3 mL was collected at the bottom, containing non-adherent cells, and passed it over a second column and subsequently third column to obtain adhesion deficient Tn5 insertion mutants.

Cell surface characterization

Hydrophobicity of bacterial cells was determined by measuring bacterial adhesion to hexadecane (Rosenberg et al. 1980). Four milliliter of washed cells (at initial OD_{400} =1) was mixed with 1 milliliter of hexadecane in 10 ml glass test-tube and vortexed for 5 minutes. The mixture was allowed to stand for 30 minutes to separate phases. The aqueous phase, at the bottom of the tube, was then sampled for measuring final OD_{400} with spectrophotometer. The percentage of adherence to hexadecane was then calculated by following equation-1:

$$(\text{Initial } OD_{400} - \text{final } OD_{400})/(\text{Initial } OD_{600}) \times 100 \qquad [1]$$

Adhesion experiment

The cells were grown in MSM with 200 mg/L naphthalene that also included ring-U-[^{14}C] naphthalene (sigma, > 96% pure) at 400000 dpm/mL. After 48 hrs of incubation, cells were harvested in early stationary phase, centrifuged and washed three times with CB solution. The final inoculum was re-suspended in CB solution before adhesion assay. The cell density was determined by standard plate count, and the

corresponding initial radioactivity was determined by adding inoculums (1 mL) to 10 mL of scintillation fluid and activity was counted on Packard 1500 Tri-Carb Liquid Scintillation Analyzer for 5 minutes.

The attachment assay consists of two sets of 5 ml vials; one containing sterile soil suspensions and other soil extract. The soil-water ratio and other conditions were similar to desorption and bioavailability experiments. At the end of 2 days equilibrium period, each vial was inoculated with approx 1 $x10^7$ CFU/mL. The tubes were then mixed at rotating mixer, and at predetermined time interval, the tubes were centrifuged at 500 rpm for 3 min. The radioactivity in the supernatant and soil-free control was determined by taking 1 mL sample into 10 mL scintillation liquid for activity count. The % age of cell retained in soil was calculated by the following equation-2

$$\frac{\text{(DPM / ml) in soil free control - (DPM / ml) in supernatant}}{\text{(DPM/ml) in soil free control}} \qquad [2]$$

DATA ANALYSIS: DESORPTION-BIOVAIABILITY MODEL (DBM)

Coupled biodegradation-desorption model that incorporate degradation of non-desorbable fraction was used to track total as well as liquid phase and sorbed phase naphthalene in batch slurry reactor. The overall degradation of naphthalene in a batch slurry reactor using mass balance on naphthalene can be represented by equation-3.

$$-(Vl \cdot \frac{dC}{dt} + m \cdot \frac{dS}{dt}) = (R_{bio} \cdot V + m \cdot knd \cdot Snd) \qquad [3]$$

Where m (kg) is the total mass of the soil, C (μg/L) represent naphthalene concentration in solution phase, S (μg/Kg) represent total sorbed-phase naphthalene, V(L) represents volume of the liquid, and k_{nd} is the first-order biodegradation co-efficient for non- desorbable fraction (S_{nd}) of total sorbed contaminant (S). R_{bio} is the liquid-phase biodegradation rate expression, which can be expressed by following equations:

$$R_{bio} = \frac{V_{max} \cdot C}{K_m + C} \quad \text{for Michaelis Menten kinetics,} \qquad [4]$$

or

$$R_{bio} = K_1 \cdot C \quad \text{for first-order kinetics} \qquad [5]$$

Where, V_{max} is the maximum flux that a cell can get and, K_m is the Michael-Menten constant, and K_1 first-order degradation rate co-efficient. The non-desorbable fraction is tracked by following mass balance equation

$$\frac{d S_{nd}}{dt} = -k_{nd} S_{nd} \qquad [6]$$

The total sorbed phase (S) was assumed to consist of equilibrium (S_{eq}), non-equilibrium (S_{neq}) and non-desorbable (S_{nd}) sites. The equilibrium site represents fraction that release spontaneously during desorption experiment and is described by a

linear partitioning model. The non-equilibrium site fraction allows slower release of contaminant and can be represented by first order expression. The non-desorption site is defined by Park et al. (2001) to be containing contaminant that cannot be released to aqueous solution during the experimental desorption period. Mathematically, these three-sorbed phase fractions are described as follow:

$$S_{eq} = f_{eq} K_d C \quad [7]$$

$$S_{nd} = f_{nd} K_d C_e \quad [8]$$

$$dS_{neq}/dt = \alpha (f_{neq} K_d C - S_{neq}) \quad [9]$$

Where, K_d is the sorption co-efficient, C_e is the liquid phase concentration in sorption equilibrium, t is desorption time in minutes, α is the first-order desorption rate coefficient (per minute). The three remaining terms f_{eq}, f_{neq}, and f_{nd} represent, respectively, equilibrium site fraction, non-equilibrium site fraction, and non-desorption site fraction. The three site fractions were measured from desorption experiment: f_{nd} corresponds to the plateau of the desorption rate profile; and f_{eq}, f_{neq} and α were estimated by non-linear regression analysis of desorption data with the constraint that:

$$f_{eq}+f_{neq}+ f_{nd} =1 \quad [10]$$

RESULTS

Sorption, Desorption, and Serial dilution Experiments

Sorption of naphthalene was found to be linear for the test soils (Figure 5-1). The sorption coefficients (K_d =slope of isotherms) ranged from 15 to 240 mL/g (Table 5-2). All the test soils observed non-desorbable (S_{nd}) fractions as determined by serial dilution method. The non-deosorbable fraction is calculated from the intercept of the step desorption isotherm (for total six steps) and the initial sorbed-phase concentration (Table 5-2). The calculated values were further confirmed by extracting from soils non-desorbable naphthalene at the end of last step desorption. Desorption of naphthalene from all soils were essentially complete within 15 hours (Figure 5-2). The desorbable fraction (f_d) and desorption rate coefficient (α) were determined by fitting non-linear regression equation to desorption rate data (Table 5-3). In all the experiments, the overall naphthalene mass recovery was found to be 94-97%, indicating no degradation. Biodegradation of naphthalene in soil extract for all strains was well described by Michaelis-Menten kinetics . The estimated maximum specific utilization rate co-efficient (Vmax) and the half-saturation constant (K_m) were 500 ug/L-hr and 301 ug/L, respectively. Since dissolved soil organic can potentially affect naphthalene degradation kinetics, soil extract instead of CB solution was used to determine biodegradation rate of naphthalene. Furthermore, the rate of naphthalene degradation was observed to be lower in soil extracts than in chemotaxis buffer solution (CB).

Table 5-2. Parameters for sorption of naphthalene by the A horizon of selected soils

Soil	K_d	R^2	K_{oc}	Non-desorbable fraction, fnd
CapacA	15	0.96	457	0.21 ($6x10^{-3}$)
Colwood	68	0.98	872	0.19 ($2x10^{-3}$)
Muck	240	0.997	626	0.18 ($1x10^{-2}$)

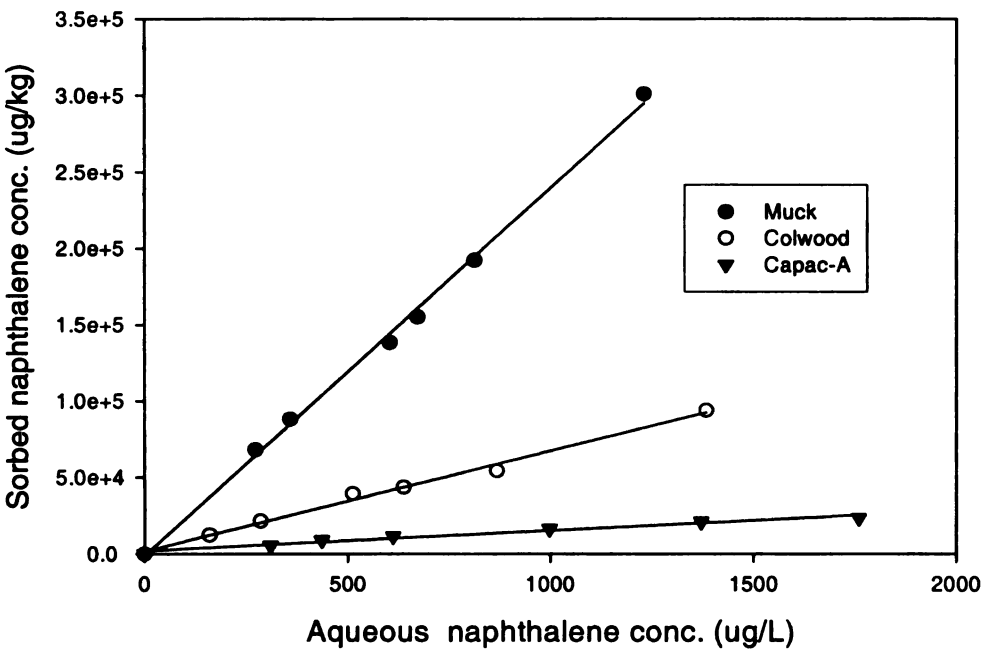

Figure 5-1. Sorption isotherm of naphthalene in natural sorbents

Table 5-3. Desorption parameters[a] evaluated by a three-site desorption model[b]

Soil	f_e	f_{ne}	f_n	α (min^{-1})	R^2
Capac	0.74(0.042)	0.1	0.16(0.009)	0.0025(0.0008)	0.94
Colwood	0.62(0.023)	0.23	0.15(0.010)	0.0016(0.0004)	0.94
Houghton Muck	0.58(0.034)	0.27	0.15(0.005)	0.005(0.0017)	0.97

[a] $f_{eq} f_{ne}, f_{nd}$= equilibrium-, non-equilibrium-, non-desorption-site fractions; α= first order desorption rate coefficient for non-equilibrium sites.

[b] Numbers in parentheses are standard deviations of the evaluated values.

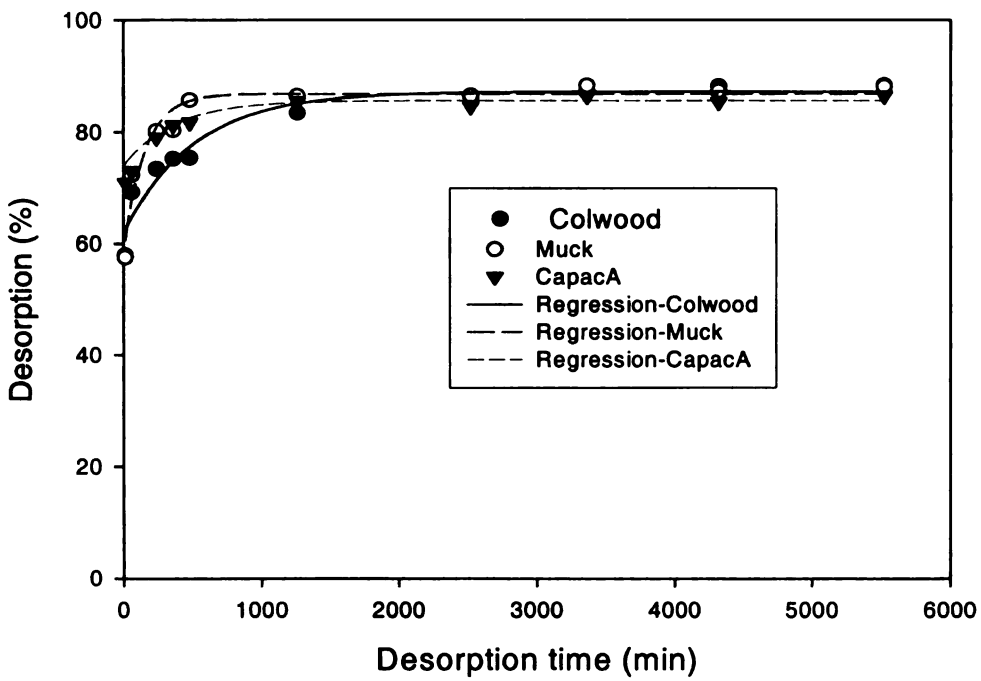

Figure 5-2. Desorption of naphthalene from three natural soils and activated carbon (GAC)

Cell Surface Properties

The bacterial surface hydro-phobicity was determined by the BATH assay (Figure5-3). The cells were grown on naphthalene then harvested, washed, and re-suspended in CB solution at the early stationary phase (40 hrs). All strains including WT

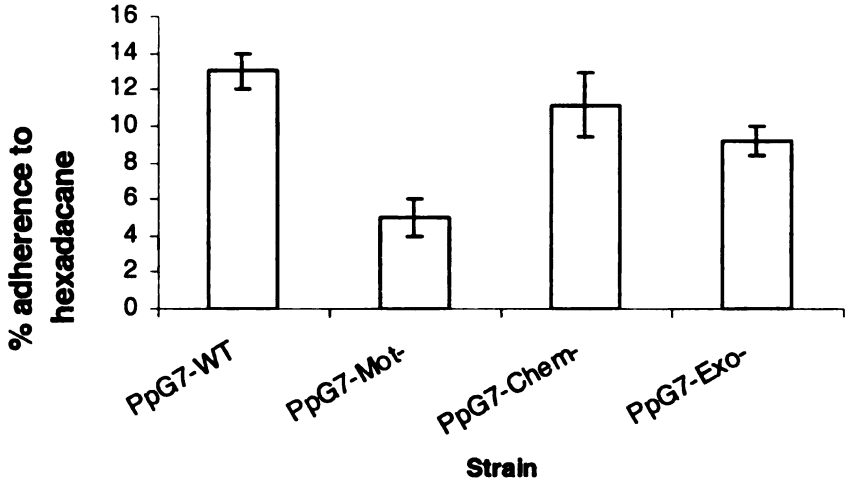

Figure 5-3. Percent of bacteria adsorbed to hexadacane in BATH test

showed less than 15% reduction in cell suspension optical density after mixing with hexadecane, indicating very low hydrophobicity (Bos et al. 1999). All strains can, therefore, be classified as hydrophilic because they remained in the aqueous phase and did no adhere to the hexadecane during BATH test. Alternatively, bacterial surface hydrophobicity and charge can be determined by measuring water content angle and cell zeta potential, respectively. The measured values of these parameters for *P. putida* G7 are given in Table 5-4. Water contact angle (θ) value for *P. putida* G7 lies between 30 and 35 indicating hydrophilic surface; bacterial surface with θ value greater than 70 is considered hydrophobic (Bastianens May 2000, AE&M). The value of zeta potential for

P. putida G-7 indicates that it surface is negatively charged at neutral pH. In summary, *P. putida* G7 is hydrophilic bacteria carry negative charge, and it is expected that its attachment properties will vary with ionic strength of chemotaxis buffer.

Table 5-4. Cell surface and hydrophobicity of *P. putida* G7 grown on different carbon sources

Strain (carbon source on which it was pre-grown)	Cell surface hydrophobicity (θw)	Cell electrophoretic mobility (u)	Cell zeta potential (ζ)
PpG7(glucose)	31± 5	-2.46± 0.39	-32.0±5.2
PpG7(naphthalene)	29±2	-2.52±0.49	-32.7±6.1

Bioavailability Experiments at Different Molar concentration of Buffer medium

Bioavailability and biodegradation rate experiments were carried out at different molar concentration (100 mM, 10 mM and 1 mM) of chemotactic buffer. Bio-availability experiments were carried out with Colwood soil and wild type *P. putida* G7, only. All the vials containing soil slurry and soil-extract were inoculated with bacterial suspension of 5 x 10^6 CFU/ml. The lowest Ionic strength (0.1 mM) of suspending solution did not affect degradation capability of bacteria. We found that the initial rate of naphthalene degradation and kinetic parameters were similar in all tested CB molar concentration.

Data from the bioavailability assays, carried out at different ionic strength in Colwood soil, are shown in Figures 5-4 (a-c). The depletion of liquid phase naphthalene was initiated first, followed by desorbable fraction of sorbed phase, and at last non-desorbale fraction of naphthalene. In all cases, WT strain could able to degrade sorbed phase naphthalene to a level below the non-desorbable level, as shown by dashed line. When bacteria have been added and start to degrade the naphthalene the concentration of dissolved naphthalene decreases. As bacteria continue to degrade naphthalene in liquid

phase, more and more naphthalene transferred from solid phase to liquid phase. Thus, mass transfer from solid to liquid phase reduces as solid phase naphthalene decreases. Within first four hours both liquid phase and desorbable fraction of sorbed naphthalene decreased to zero, indicating mass transfer limited condition. The rate of degradation of naphthalene is relatively faster during this period, as indicated by steeper and shorter part of the data. About 85% of the total naphthalene was degraded during this step and is mostly carried out by the bacteria suspend in liquid. At the end of the fist phase of degradation, a mass transfer limited degradation phase begun (represented by long and nearly horizontal part of the data). This phase is marked with non-desorbable fraction of naphthalene, which is mainly degraded by attached bacteria (micro-graph showing attached bacteria with soil)

The bio-availability data, which included both liquid phase and sorbed phase data, were fitted with the coupled desorption-biodegradation model (equation-3). The DBM model provided a reasonable description of both liquid and sorbed phase data for all three cases over the entire experimental period. The DBM model estimated degradation rate co-efficient (k_{nd}) for non-desorbable fraction (Table 5-5).

Table 5-5. Biokinetic parameters evaluated for Psudomonas putida G7-WT under varying molar concentration of chemotactic buffer solution (CB)

Soil	Molar concentration (mM)	First order degradation rate for non-desorbable fraction K_{nd} (h^{-1})
Colwood	100	0.0585 ± 0.0077
	1	0.0318 ± 0.003
	0.1	0.036 ± 0.002

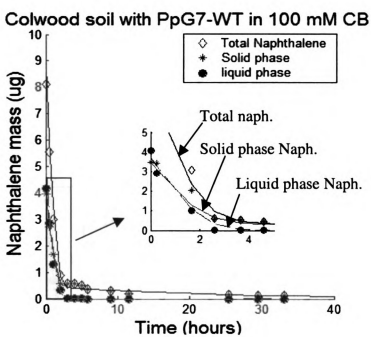

Figure 5-4a. Naphthalene degradation by Pp-G7(WT) in 100 mM CB solution

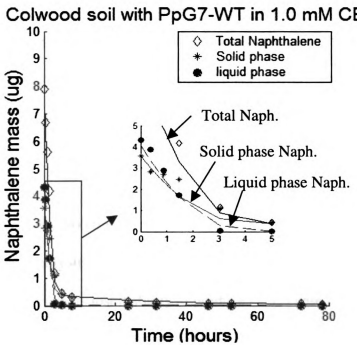

Figure 5-4b. Naphthalene degradation by Pp-G7(WT) in 1.0 mM CB solution

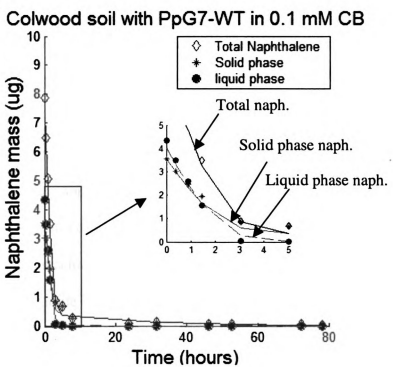

Figure 5-4c. Naphthalene degradation by Pp-G7(WT) in 0.1 mM CB solution

Isolation of Adhesion Deficient Mutants

To delineate possible role of Extra-cellular polymeric substances (EPS) on attachment, mutant was generated and isolated that had decreased attachment ability. The decrease in observed attachment ability is expected to be due to altered EPS layer.

Mutagenesis

Out of approx. 900 transposon mutants (Km^rRif^r) streaked on plate, containing 75 μg/ml Calcoflour, only 46 mutants did not florescence under long wave UV light. This showed that these mutants lost it ability to produce EPS. These selected Exo^- mutants (combined titer of ~$1x10^8$ CFU/ml) were then passed through sand columns three times. The final count of mutant collected at the bottom of third pass was determined as ~ $4x10^7$ CFU/ml. The bacterial suspension obtained at the end of third pass was taken as culture with low adhesion ability. The culture was then purified and preserved in cryo-vials at – 80 °C before use. The mutants deficient in producing EPS are hereafter denoted as Exo^-.

Batch adhesion assay

Tests for attachment to the test soil (colwood) were performed over a period of 10 hours. The initial cell concentration for each strain was ca $1x10^7$ CFU/ml. The percentage of bacteria retained in soil varied from average 20, 15, 10, and 10% for non-chemotactic strain, WT strain, non-motile strain, and Exo^- strain. For WT and non-chemotactic mutant strain, cell attachment remained almost constant over the period of time (Figure 5-5). However, non-motile and Exo^- mutants showed a steady increase (from 3 to 22%) in

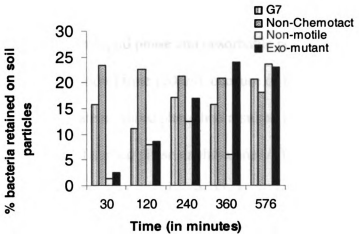

Figure 5-5. Cell attachment to Colwood soil at different time interval

attachment over a period of time. At the end of 10 hrs, the % retention for all strain remained same at around 20%.

Bioavailability Experiments with Wild type and Mutant Strains

The bio-availability assays were repeated for WT *P.putida* G7, and three mutant strains. The three mutant strains were non-motile, non-chemotatic, and mutant deficient in producing EPS layer (Exo^-). The cell density was kept low at $5x10^5$ CFU/ml in all experiments to avoid rapid degradation in liquid phase as was observed in experiment with relatively higher cell density ($1x\ 10^7$ CFU/ml). Previous research has also recommended use of low density cells for isolating role of bacterial phenotypes (chemotaxis, attachment etc) of interest (Marx and Aitken 1999). Data from the bioavailablity assays for four strains in colwood soil are shown in Figure 5-6. All strains showed almost similar degradation profile in both liquid phase as well as solid phase. All strains, including Exo^- mutant, could able to degraded sorbed phase naphthalene to levels

below he non-desorbable level, as shown by dashed line. In contrast to earlier study with high cell density, both liquid phase and desorbable portion were degraded very slowly over an extended period of time (20hrs), because of low cell density. It appeared that slower degradation rate in liquid phase had resulted in a prolong availability of the desorbable fraction of sorbed phase naphthalene. After all desorbable naphthalene degraded, the degradation of non-desorbable fraction started at same time for all the strains (later part of the data). The non-desorbable fraction was then degraded very slowly by all strains over a period of 120 hrs.

The bio-availability data were then fitted with the coupled desorption-biodegradation model (DBM). The model provided a reasonable description of sorbed phase data for all strains over the entire experimental period. The DBM model estimated degradation rate coefficient of non-desorbable fraction (knd) and is shown in Table 5-6. For all strains, the estimated k_{nd} values were determined to be statistically similar. Thus, non-desorbable fraction was apparently degraded at same rate and to same extent, indicating that all strains possess similar capabilities to degraded non-desorbable naphthalene.

DISCUSSION

Recently, several researchers observed enhanced bioavailability of sorbed HOCs in which rate of degradation by bacteria was faster than the rate of abiotic desorption. The enhance bioavailability, however, requires existence of a mechanism that facilitates degradation of sorbed chemical. Previous studies have suggested that the phenomenon of

Table 5-6. Biokinetic parameters evaluated for non-adhesion mutant along with three other strains of PpG7.

Soil	Strain	K_{nd} (hr^{-1})	Michaelis-Menten parameters	
			K_m (mg/L-hr)	V_s (mg/L)
Colwood	WT	0.025+0.001	90	50
	Non-chemotactic	0.023+0.004	30	10
	Non-motile	0.027+0.007	90	50
	Non-adhesion (Exo^-)	0.0196+0.001	90	50

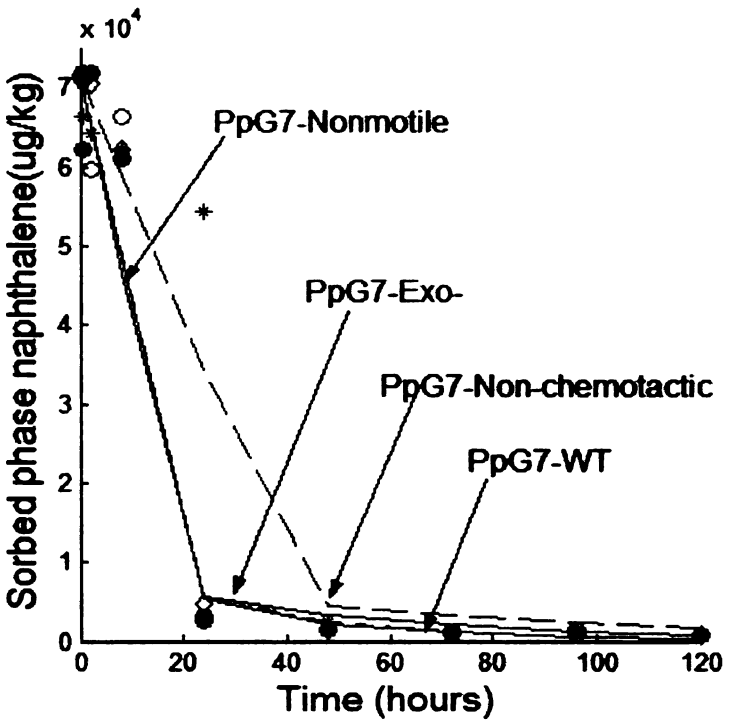

Figure 5-6. Naphthalene degradation in soil-slurry-solution by PpG7- WT, and its three mutants: non-chemotatic. non-motile. and non-adhesion

enhances bioavailability is likely dependent on the specific bacterial trait e.g., production of bio-surfactant or bacterial attachment. It has recently been shown that neither synthetic nor microbially produced surfactant aided in utilization of sorbed HOCs by bacteria (Calvillo and Alexander 1996). However, numerous studies cited attachment as a plausible explanation for enhanced bioavailability of soil-sorbed HOCs.

The previous studies attributed observed difference in rate of sorbed phase degradation and corresponding abiotic desorption rate to bacterial attachment. However, the role of bacterial attachment in direct uptake of soil-sorbed HOCs is still not clear. The effect of attachment on degradation of HOCs has been widely studied yielding controversial results. For example, Mukherji and Weber (1998) suggested that microbial attachment could even hinder both mass transfer and biodegradation.

In this study an attempt has been made to examine the factors affecting bacterial attachment and consequently enhance bio-degradation. The factors studied included affect of molar concentration of buffer medium and EPS layer of *Pseudomonas putida* G7. By varying these factors, we intended to isolate affect of attachment on bioavailability of sorbed phase naphthalene. Initial studies on *Pseudomonas putida* G7 were conducted to determine characteristics of its surface properties. Evaluation of BATH test and other cell surface properties (zeta potential and contact angle) indicated that wild-type *Pseudomonas putida* G7 has very low hydro-phobicity with corresponding low zeta potential. It was, therefore, expected that initial cell attachment would be controlled by electrostatic interaction, the magnitude of which could be manipulated by varying molar concentration of buffer medium at constant pH.

Bio-availability experiments carried out at varying molar concentration of CB solution and constant pH values, however, did not yield any distinguishable difference. Bacteria could equally access and degraded all fractions (i.e., liquid, desorbable, and non-desorbable) of naphthalene under varying molar concentration of buffer medium. This finding seems to rule out affect of electrostatic interaction on initial attachment of bacteria. Both BATH test and soil attachment assay showed 15-20 % of cell attachment, and this fraction of cells attachment could either due to hydrophobic site on cell surfaces or electrostatic attraction between negative site on bacterial surface and localized positive sites on soil clay. This fraction of attached cells were concluded to be unaffected by electrostatic interaction as was observed from bioavailability experiments at different ionic strength. The fraction of 15-20% attached bacteria were able to degrade non-desorbable fraction of sorbed phase naphthalene.

The probable reason for this finding can be explored in term of complex nature of soil. Previous research with bacterial attachment mostly dealt with clean sand or artificial surfaces e.g., Teflon and glass. The interaction of bacteria with these uniform surfaces cannot be reproduced with complex system like soil. Lahlou et al. (2000) demonstrated that attachment results from glass and Teflon couldn't be reproduced with soil materials. For example, the author concluded that one strain showed significant attachment to teflon and glass but did not show any affinity toward soil components. The soil used in this experiment (i.e., Colwood) consists of a complex mixture of quartz (75%), clay (15%), and organic (8%) matter carrying either negative or positive surface charge depending on solution pH. Since the pH of buffer medium was kept near neutral (pH =6.5), all soil components mostly carried negative charge. However, clay minerals may also exhibit

fixed positive charge resulting from breakage at the edges to expose trivalent or tetravalent positive ions or from the exchange of OH groups (Stotzky 1985). Marshall and coworker showed adsorption of bacterial cells to clay particles resulting from positive charges on platelets clay edges and breakage at the edges(Marshall 1976).

Another plausible explanation is that decreasing ionic strength might have reduced electrostatic repulsion between bacterial and negative site, but did not affect neutral site or even positive site (of clay surfaces due to broken edges). In presence of positive charges on clay surfaces, lowering of ionic strength even caused opposite effect i.e., reduction in electrostatic repulsion between bacterial surfaces and clay positive surface and hence greater attachment. Thus, it is highly probable that due to presence of both negative and positive charge surfaces in soil, lowering of ionic strength might not have effected overall attachment of bacteria. Furthermore, at neutral pH, *P. putida* G7 is relatively more negatively charged than soil surfaces. Due to large difference in negative charges between bacterial and particle surface charge, the change in Gibbs free energy of adsorption due to electrostatic interaction is negligible, regardless of ionic strength.

After verifying that charge at bacterial surface did not affect bioavailability of sorbed phase naphthalene, we turned toward the possibility of role of specific structure on the outer membrane (e.g., EPS and LPS layer). *Pseudomonas putida* G7 is known to possess both extra-cellular polymeric substances (EPS) as well as smooth and rough lipo-polysaccaride (LPS)(Kachlany et al. 2001). EPS may be secreted as a loose layer of carbohydrates called slime layer or a more rigid layer of protein and is known as capsule. Several researchers have demonstrated significance of EPS and LPS on adhesion of bacteria to solid surfaces (Allison and Sutherland 1987; Pringle et al. 1983). The EPS and

LPS promote adhesion by adsorption to surfaces; polymers bridge the distance between cells and the surfaces and may cause adhesion even when the cells do not experience attraction forces (Figure5- 7).

By selecting for mutants with altered surface characteristics, we can obtain strains that have significantly different adhesion properties than parent strain. Transposon mutagensis was used to generate cells with altered surface characteristics. Screening on Calcoflour plate is a convenient method for isolating non-EPS producing mutant, especially when wild type strongly fluoresces under UV light. In case of *Pseudomonas putida*, we observed a weak fluoresce signals that made it difficult to screen for non-EPS producing mutants. In absence of alternative screening system, we picked colonies that were observed being dull and opaque under UV light. Passing sequentially non-EPS mutants through a sand column, however, allowed us to collect non-adhesive mutants.

Both, Bath test and batch adhesion test revealed that non-adhesive mutant had lower retention with hexadance and live soil, respectively. The batch test however indicated that non-adhesive mutant recovered it attachment capability with time. The plausible explanation for this trend is that non-adhesive mutant re-developed its attachment phenotype under starvation condition. This trend can be explained base on earlier observations that bacteria employ different modes by which it can acquire surface characteristics that influence its attachment and detachment during exogenous energy and nutrient deprivation (Neu and Marshall 1990). In the beginning of the batch adhesion test with non-adhesive mutant, substrate was readily available in dissolved as well as desorbable form. However, as naphthalene depleted with time, bacteria somehow

acquired adhesion trait, by unknown mechanism, to get access to otherwise non-desorbable , difficult to get substrate in the system.

The observation, as described in previous paragraph, can equally be applicable to our bioavailability experiment, where non-adhesive strains could equally degrade non-desorbable naphthalene. After depletion of readily available fraction, naphthalene was present under mass transfer limited condition. This condition created an apparent starvation condition that forced bacteria to make-up for its inability to attachment by some other mechanism (e.g. increase in surface hydrophobicity etc). Our results also substantiated previous research that observed that degradative ability of the adhesion-deficient and wild type were similar under substrate limiting conditions (Deflaun and Fletcher 1999).

Even though mutagensis is an invaluable tool in isolating bacterial surface component, yet it success demands several confirmatory experiments. The confirmation of successful mutagensis requires large alterations in adhesiveness, multiple replicate adhesion assay, and statistical comparisons (Williams and Fletcher 1996) . Thus, screening for correct mutants is very difficult and time-consuming. This exercise of selecting non-adheisve mutant is even more difficult because attachment can employ variety of surface polymer (including protein ligand, carbohydrates and lipid on the bacterial surface) under different substratum chemistry and environmental conditions (Williams and Fletcher 1996).

In conclusion, the results of this study suggest that factors promoting bacterial attachment (i.e. removal of exo-polysaccharides, presence of flagella and reduction of buffer medium concentration) do not contribute to biodegradation of non-desorbable

fraction of soil-sorbed naphthalene in soil-slurry system. Further research is clearly needed in establishing this finding under a well defined system, since bacterial attachment to soil matrix is complex and unlikely to be explained by one or other property of the cell surface (Bos et al. 1999). Different cells employ different polymers in mediating attachment depending on different substratum chemistries and medium composition. For example, some polymer (eg pilli and LPS) may mediate in attachment to hydrophobic surface; whereas, other polymer (EPS) help bacteria to attach with hydrophilic surfaces. Certain combinations of bacterial, solid surface and liquid medium properties could result in large deviations from the null hypotheses.

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CHAPTER 6. SUMMARY AND CONCLUSIONS

SUMMARY

The phenomenon of the enhanced bioavailability of soil-sorbed organic molecules is rapidly gaining attention. It is defined as the availability of organic compounds for bacterial uptake at a rate greater than a-biotic de-sorption rate from the sorbent. The aim of this study was to investigate the role of specific physiological traits of bacteria in the enhanced bioavailability of soil-sorbed organic contaminants. The specific bacterial traits considered included: production of biomaterial, variation in the attachment to solid surfaces, and chemotaxis/motility. To accomplish the aim, three main objectives were pursued in sequence. All of the identified traits were tested in a systematic way in stirred and unstirred soil slurry reactors using a representative PAH, naphthalene. Wild type naphthalene degrading bacteria along with its mutant cultures (non-chemotectic, non-flagellated, and non-adhesive) were used for studying the role of these traits. The pure wild type, *Pseudomonas putida* G7, has been reported previously to uptake sorbed phase naphthalene directly in the soil slurry. Furthermore, it has been characterized as being a motile and chemotectic strain.

The research work involved generating, isolating, and characterizing mutants with one of the missing phenotypes (motility, chemotaxi, adhesion). Sorption isotherms and single and series dilution desorption experiments were conducted to evaluate the distribution coefficient, de-sorption rate, and amount of non-desorbable naphthalene. The initial degradation rate was measured for wild type as well as for all mutants in the soil extract solution. All the studies were carried out assuming that initial degradation kinetics

of all mutants remained the same as that of wild type strain. Bioavailability experiments involved first establishing sorption equilibrium, inoculating the systems with bacteria, and measuring naphthalene concentrations in both sorbed and dissolved phases over the period of time. The rate of naphthalene degradation by each culture in stirred batch systems was fitted with a non-linear regression model (DBM model) to determine the sorbed phase degradation rate coefficients. Bioavailability data were compared for each type of organism for the extent and rate of sorbed phases degradation to evaluate the role of each phenotype.

The first objective was to observe phenomenon of enhanced bio-availability by comparing the removal of naphthalene in a soil-slurry system using bacterial strain *P. putida G7*, Tenax beads extraction, and serial extraction with the 0.1 M buffer solution alone. Also explored under this objective was the affect of excreted biomaterial on enhanced bioavailability. Comparison of the results of the Tenax beads extraction and serial dilution with PpG7 biodegradation showed that the rate and extent of naphthalene removal by bio-degradation was at all times significantly greater than the abiotic removal rate and extent in the sorbed phase. It seemed that naphthalene did not have to be desorbed in the aqueous phase for bio-degradation by PpG7. Apparently, PpG7 were able to overcome the mass-transfer limitation by an unknown mechanism and could able to directly uptake sorbed phase naphthalene. The activity of the extra-cellular enzyme was ruled out as no significant naphthalene loss was observed with bacterial the filtrate during 3 days of the incubation period. Also, the possible effect of extra-cellular polymer on sorption/deosprion parameters was minimal. Since all bioavailability experiments were

carried out at 2 mg/L of naphthalene, the resulting biomaterial production was expected to be low enough to have caused any affect on partition co-efficient (and consequently mass transfer) of sorbed naphthalene.

For the second objective, we evaluated the role of chemotaxis/motility on naphthalene degradation in sorbent-slurry system under stirred and quiescent conditions. In the stirred system, containing natural soils, the mixing had completely masked the role of chemotaxis and motility due to the presence of an isotropic concentration and mixing-mediated adhesion of cells to sorbent particles. However, in the stirred system with GAC the role of chemotaxis/motility became apparent at a longer incubation time. The presence of a large pool of non-labile naphthalene, which is released over an extended period of time, attracted more cells at the activated carbon surface than what could be accumulated due to mixing alone. The increasing number of cells accumulation due to chemotaxis/motility caused a greater rate of non-labile naphthalene degradation as compared with the system inoculated with a mutant. Experiments carried out with soils under a no-mix condition, however, revealed that none of the strains were able to degrade the non-desorbable fraction of naphthalene. The present study suggested that in a heterogeneous matrix (i.e., soil) , containing medium to high content of clay and organic, the role of mixing is very important in successful bioremediation. The lack of mixing reduced the effective diffusion of molecules in soils due to solid phases, dead-end pores and high tortousity of the system. Also, the lack of mixing reduced the number of sites available for bacterial attachment for direct uptake due to pore sizes exclusion and retardation affect (due to sorption).

The third objective was to examine the factors affecting bacterial attachment and consequently the rate and extent of biodegradation of naphthalene in a soil-slurry reactor. The factors studied included effect of ionic strength and EPS layer of pseudomonas Putida G7. Bio-availability experiments were carried out with: 1) PpG7-WT strain under variable ionic strength of suspending solution, and 2) different strains of PpG7 possessing variable attachment capabilities under constant ionic strength of suspending solution. Three orders of decrease in molar concentration of buffer medium did not affect rate and extent of sorbed phase naphthalene degradation by *P.putida* G7. Likewise, important phenotypes (e.g., EPS, flagella, attachment) of PpG7-WT apparently did not contributing toward enhanced bioavailability of sorbed phase naphthalene.

CONCLUSIONS

1. The effect of extra-cellular enzyme and polymers was not observed in enhanced bioavailablity of soil-sorbed naphthalene.
2. In natural soils, chemotaxis/motility traits do not play any role on enhanced bioavailability. Chemotaxis/motility contributed in enhance degradation in a system with granualr activated carbon, which contain greater fraction of non-desorbable naphthalene.
3. Factors promoting bacterial attachment (i.e. removal of exo-polysaccharides, presence of flagella and reduction of buffer medium concentration) do not contribute to biodegradation of non-desorbable fraction of soil-sorbed naphthalene in soil-slurry system.
4. Mixing plays an important role in bioavailablity of sorbed contaminant in natural soils.