BIODEGRADABILITY OF SELECT POLYCYCLIC AROMATIC HYDROCARBON (PAH) MIXTURES

A Thesis

by

ANURADHA M. DESAI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Civil Engineering

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Approved by:

Chair of Committee,	Robin L. Autenrieth
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	Timothy A. Kramer
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ABSTRACT

Biodegradability of Select Polycyclic Aromatic Hydrocarbon (PAH) Mixtures.

(December 2005)

Anuradha M. Desai, B.S., University of Pune, India

Chair of Advisory Committee: Dr. Robin L. Autenrieth

Polycyclic aromatic hydrocarbons (PAHs) are environmentally significant because of their ubiquity and the toxicity of some. Their recalcitrance and persistence makes them problematic environmental contaminants. Microbial degradation is considered to be the primary mechanism of PAH removal from the environment. Biodegradation kinetics of individual PAHs by pure and mixed cultures have been reported by several researchers. However, contaminated sites commonly have complex mixtures of PAHs whose individual biodegradability may be altered in mixtures. Biodegradation kinetics for fluorene, naphthalene, 1,5-dimethylnaphthalene and 1-methylfluorene were evaluated in sole substrate systems, binary and ternary systems using *Sphingomonas paucimobilis* EPA505. The Monod model was fitted to the data from the sole substrate experiments to yield biokinetic parameters, (q_{max} and K_s). The first order rate constants (q_{max}/K_s) for fluorene, naphthalene and 1,5-dimethylnaphthalene were comparable, although statistically different. However, affinity constants for the three compounds were not comparable. Binary and ternary experiments indicated that the presence of another PAH retards the biodegradation of the co–occurring PAH. Antagonistic interactions between substrates were evident in the form of

competitive inhibition, demonstrated mathematically by the Monod multisubstrate model. This model appropriately predicted the biodegradation kinetics in mixtures using the sole substrate parameters, validating the hypothesis of common enzyme systems. Competitive inhibition became pronounced under conditions of: $K_{s1} << K_s$, $S_1 >> K_{s1}$ and $S_1 >> S$. Experiments with equitable concentrations of substrates demonstrated the effect of concentration on competitive inhibition. Ternary experiments with naphthalene, 1,5-dimethylnapthalene and 1-methylfluorene revealed preferential degradation, where depletion of naphthalene and 1,5-dimethylnapthalene proceeded only after the complete removal of 1-methylfluorene. The substrate interactions observed in binary and ternary mixtures require a multisubstrate model to account for simultaneous degradation of substrates. However, developing models that account for sequential degradation may be useful in scenarios where PAHs may not be competitive substrates. These mixture results prove that substrate interactions must be considered in designing effective bioremediation strategies and that sole substrate performance is limited in predicting biodegradation kinetics of complex mixtures.

DEDICATION

Dedicated to my parents Mr M.V. Desai and Mrs S.M. Desai, sister Madhavi Desai, and grandmother Mrs Janaki W. Desai.

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) represent a large family of organic compounds that are considered environmental contaminants. These compounds are widespread in the environment (Harvey 1991) and can be present in quantities that pose a threat to the environment and mankind. Significant levels are detected in the air, food and water (Harvey 1991). PAHs are of principal concern due to the carcinogenicity, genotoxicity and mutagenicity of some PAHs that constitute a significant group of chemical carcinogens (Sherma 1993). PAHs appear on the USEPA's draft list of Persistent Bioaccumulative Toxins (PBTs) commonly encountered at hazardous waste sites (Kieth and Telliard 1979). The persistence, recalcitrance and toxicity of PAHs make them problematic environmental contaminants (Mueller and Cerniglia 1996). PAHs are large reduced organic molecules that are biodegradable to variable extents which has given rise to the significance of biodegradation as a suitable and natural approach for detoxification of these compounds. Bioremediation is a clean up tool in over 135 Superfund and Underground Storage Tank (UST) sites, as well as many other sites contaminated with complex mixtures of PAHs (USEPA 1989).

Bioremediation is considered environmentally friendly and technologically feasible. Degradation kinetics of individual PAH compounds by pure and mixed microbial communities have been reported by several researchers (Cerniglia 1992;

This thesis follows style and format of the *Journal of Environmental Engineering*.

Heitkamp and Cerniglia 1988; Boldrin et al. 1993; Kanaly and Harayama 2000; Wilson and Jones 1993). However, contaminated sites are commonly contaminated by complex mixtures of PAHs (Guha et al. 1999; Bauer and Capone 1988; Guha et al. 1998; Leblond et al. 2001). For bioremediation to be successfully implemented as a remediation technology, it is essential to understand the biodegradation of mixtures of PAHs. The diversity of components within a mixture, biodegradation kinetics of individual components within the mixture, the possible interactions within these components, the effects of interactions on the system and the microbial community represent some of the complicating factors in studying the biodegradation of PAH mixtures.

RESEARCH SIGNIFICANCE

Microbial degradation can be the dominant process in the fate of PAHs in the environment, but photo-oxidation and volatilization may be competitive removal mechanisms (Mueller et al. 1990). Several studies on the biodegradation of individual compounds have been reported, however at contaminated sites PAHs typically occur as mixtures of compounds (Guha et al. 1999; Bauer and Capone 1988; Guha et al. 1998; Leblond et al. 2001). PAHs are diverse both structurally and chemically. Within the family of PAHs, a wide range of solubilities exist, with the solubility generally decreasing with an increasing number of benzene rings (Mueller and Cerniglia 1996). Interactions between PAHs are possible which can alter the rate and extent of biodegradation within a mixture of PAHs (Guha et al. 1999; Knightes 2000; Beckles et al. 1998). Rarely can biodegradation patterns of single PAHs be extended to degradation patterns of their mixtures (Beckles et al. 1998). The effect of a single PAH compound on the biodegradation potential of another will be crucial in determining the efficacy and metabolic versatility of the microorganisms competent to remediate a contaminated media.

Research on the biodegradation kinetics of PAH mixtures is limited. Guha et al. (1999) reported converse effects of enhanced degradation and competitive inhibition in a ternary mixture. Sims et al. (1988) observed that high molecular weight (HMW) PAHs are more recalcitrant when present as pure compounds in soil than in the same media in complex mixtures. On the contrary, biodegradation of complex mixture creosote

revealed that the fluoranthene degradative capabilities of *Sphingomonas paucimobilis* EPA505 were inhibited in the presence of other creosote constituents (Lantz et al. 1997). They suggested that this may be due to the structural and chemical diversity of the creosote composition and lack of prexposure of *S. paucimobilis* to enhance HMW degradation. Luning Prak and Pritchard (2002) studied the degradation of a synthetic mixture containing fluoranthene, pyrene, and phenanthrene in the presence of the surfactant Tween 80 by *S. paucimobilis*. Their research demonstrated a sequential degradation where the preference from small to larger PAHs was in the order of phenanthrene > fluoranthene > pyrene. Binary, ternary and larger component systems of PAHs have been studied (Luning Prak and Pritchard 2002; Guha et al. 1999; Kelley and Cerniglia 1995; Knightes 2000). Though the simple component systems scarcely mimic the complexity inherent in contaminated environments, they do validate the effects of substrate interactions prevalent even in simple systems. It is possible that the extent of interactions observed in simple systems can become increasingly complex and pronounced in systems where a larger number of components are likely to be present. A multisubstrate model is essential to account for simultaneous utilization of substrates to estimate substrate interactions and biodegradation kinetics.

Substrate interactions between binary and ternary mixtures were demonstrated experimentally and mathematically by use of a multisubstrate model (Guha et al.1999; Knightes 2000). The multisubstrate model for competitive inhibition relies on parameters derived from sole substrate experiments which simplifies the modeling approach, yet captures the substrate interactions prevalent in a mixture. Bacteria are able

to catalyze the degradation of a wide range of PAHs (Cerniglia 1992). For example, *cis*-naphthalene dihydrodiol dehydrogenase catalyzes the degradation of other PAHs (Patel and Gibson 1976). In competitive inhibition, the substrates compete for the same enzymes. The parameters derived from the sole substrate case will also be representative for mixtures. The multisubstrate model for competitive inhibition is appropriate for those compounds that are transformed by a common enzyme system. The studies reported by Guha et al. (1999) and Knightes (2000) have successfully reviewed the use of such a multisubstrate model for simple and larger systems. Knightes (2000) studied the kinetics of a complex nine-component system and observed that inhibition became more pronounced in complex systems. The kinetics of multicomponent aqueous systems are useful in determining the rate at which different components in the system are transformed which reflects the effects the mixture is most likely to produce on the individual compounds and the microbial community. Simple systems form a basis for modeling larger component systems because the effects and interactions observed in simple component systems indicate the potential for similar or more dramatic effects in complex mixtures.

RESEARCH OBJECTIVES

Biodegradability of PAH mixtures is dependent on the biodegradation kinetics of individual compounds in the presence of multiple components and interactions between the components of the mixture. Biodegradation kinetics describe the extent and rate at which different components in a mixture undergo transformation. Substrate interactions can reveal the specificity of enzymes for substrates, between broad or narrow. This research contributes to the understanding of biodegradation kinetics of PAH mixtures. Using the kinetic data, modeling larger multicomponent systems is possible. This research isolated biodegradation as a sole process that governs the removal of PAHs from the aqueous system, where variables associated with bioavailability were eliminated by experimental design. The underlying hypothesis of this research was that all PAHs compete for the same enzyme system. The specific aims of this research are:

1. To study the biodegradation kinetics of sole substrate compounds. The Monod model will be fitted to the experimental data to generate the biokinetic parameters maximum substrate utilization rate (q_{max}) and affinity coefficient (K_s) will be estimated for sole substrate compounds. These parameters will be used for multisubstrate parameterization.

2. To evaluate the biodegradation kinetics of binary and ternary mixtures of PAHs using *Sphingomonas paucimobilis* strain EPA505. The effect of a single PAH on

the biodegradation potential of another PAH will be determined. The substrate depletion curves obtained from the sole substrate experiments will be compared to those obtained from the mixture experiments.

3. To evaluate the data obtained from the multisubstrate experiments with two different models, multisubstrate competitive inhibition model and sole substrate Monod model. The multisubstrate competitive inhibition model assumes that PAHs are utilized by a common enzyme pathway (Segel 1975). This model was used to validate the biodegradation kinetics in binary and ternary mixtures. The Monod model assumes that the presence of other substrates does not affect the behavior of a single substrate present in the mixture.

LITERATURE REVIEW

Sources, Characteristics and Fate of PAHs in the Environment

PAHs are introduced into the environment either naturally or by anthropogenic activities. These compounds are formed by the fusion of two or more benzene nuclei. Increasing industrialization and urbanization has resulted in a concomitant increase in contamination. They can be found in air, water, soils, and sediments (Harvey 1991). Once these compounds are confined to tranquil sediment bottoms, they are not of much concern to human health (Scott 1989). However, PAHs impact the aquatic environment by targeting the bottom feeders (polycaetes, bivalves and crustaceans) (Scott 1989), thus creating a disturbance in the ecological balance of the aquatic systems. PAHs may be released into the environment when the sediments are disturbed. Released PAHs become accessible to the benthic organisms and other aquatic life and are susceptible to bioconcentration in the food chain (Eadie et al. 1982). Apart from this, there are many other sources for PAHs released to the environment.

PAHs are released into the environment naturally through forest fires and volcanoes (Harvey 1991). Aside from this, microorganisms and plants during energy building reactions also contribute small amounts (Neff 1979). The naturally occurring sources of PAHs include coal and crude oil deposits (Wilson and Jones 1993). In addition, smoked food, combustion of fossil fuels and weathering of petroleum results in the formation of hydrocarbons and other byproducts (Wilson and Jones 1993). Another important source of PAHs is tobacco smoke which may induce carcinogenic effects

(Harvey 1991). The anthropogenic activities that lead to the distribution of PAHs in the environment include accidental oil spills, and oily waste sludge from petroleum refineries. Primary waste sources include creosote, coal tar, industrial discharges and gases (Mueller and Cerniglia 1996). Anthropogenic and natural activities lead to their ubiquitous environmental distribution, where their stability and persistence is governed by their chemical and physical properties.

PAHs are hydrophobic in nature. These compounds are not readily soluble in water indicated by their high octanol/water partition coefficients. PAHs are lipophilic in nature (Dabestani and Ivanov 1999); they tend to partition in the fatty tissues once organisms ingest them. The increase in the hydrophobicity and electrochemical stability is associated with an increase in the number of benzene rings and angularity of a PAH molecule (Harvey 1991). The HMW PAHs are more persistent and recalcitrant than the low molecular weight (LMW) PAHs (Wilson and Jones 1993, Cerniglia 1993). The stability and distribution of the PAHs in the natural environment is influenced by the configuration of the aromatic rings (Mueller and Cerniglia 1996), physico-chemical properties (Dabestani and Ivanov 1999) as depicted in Fig. 1. The structural and chemical configuration of the PAHs also governs their stability and distribution in the environment (Mueller and Cerniglia 1996). Once PAHs enter the environment, they are subjected to five distinct processes: volatilization, leaching, degradation, bioaccumulation and sequestration (Fig. 1). However, microbial degradation is principally responsible for removal of PAHs (Cerniglia 1993). Linear PAHs like anthracene are unstable hence these are not likely to be encountered in nature unless

confined by organic matrices (Mueller and Cerniglia 1996). The LMW PAHs are more volatile (Henry's law constant in the range 10^{-3} – 10^{-5} atm/M) than the HMW PAHs (Henry's law constant in the range 10^{-5} – 10^{-8} atm/M) (Dabestani and Ivanov 1999).

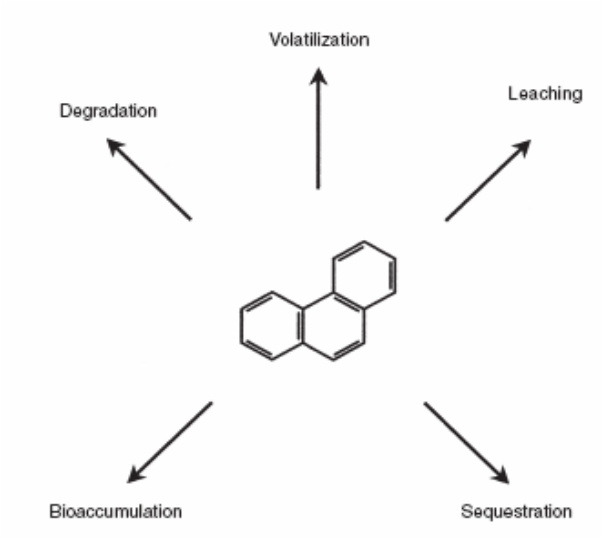


Fig. 1. Distribution of a Model PAH (phenanthrene) in Soil. (Adapted from Semple et al. 2003)

Environmental Significance of PAHs

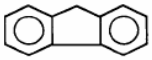
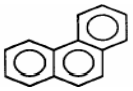
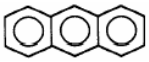
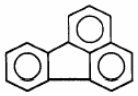
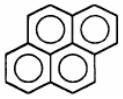
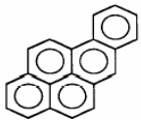
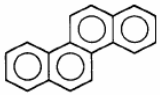
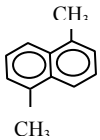
Many PAHs induce toxic effects in living organisms (Dabestani and Ivanov 1999). PAHs derive their carcinogenic and mutagenic properties from their non planarity (Dabestani and Ivanov 1999). The methyl substituted PAHs are more reactive and carcinogenic as compared to their parent compounds (Dabestani and Ivanov 1999). The genotoxicity of the PAHs increases with the number of fused benzene rings (Kanaly and Harayama 2000). Benzo[*a*]pyrene (B[a]P) is a known potent carcinogen and serves as

the benchmark for toxicity of all PAHs (Kanaly and Harayama 2000). Benz[a]anthracene, B[a]P and dibenz[a,h]anthracene show carcinogenic effects when administered orally to animals (Dabestani and Ivanov 1999). There are 17 PAHs classified by the Environmental Protection Agency EPA as priority pollutants (Kieth and Telliard 1979), the physical and chemical properties of some are listed in Table 1.

Biodegradation of PAHs

The microbial metabolism of PAHs has been studied extensively (Mueller and Cerniglia 1996) and pathways for microbial metabolism for this diverse family of compounds are available (Cerniglia 1992). Mueller and Cerniglia (1996) proposed a biodegradation pathway for the bacterial transformation of PAHs. Bacteria trigger the initial oxidation by incorporating both the atoms of molecular oxygen catalyzed by a dioxygenase, forming a *cis*-dihydrodiol, which then undergoes dehydrogenation to produce catechol (Fig. 2). Bacterial oxidation of the aromatic ring of PAHs results in the formation of metabolites (Mueller and Cerniglia 1996). The initial ring oxidation step governs the rate of the reaction and thus is a rate limiting step (Heitkamp and Cerniglia 1989); thereafter degradation proceeds faster with or without accumulation of metabolites (Herbes and Schwall 1978). For the complex fused ringed structures such as B[a]P, phenanthrene, pyrene, bacterial enzymes attack at multiple sites to form isomeric *cis*-dihydrodiols. The dioxygenases are multi-component enzyme systems comprised of three proteins with broad substrate specificities (Mueller and Cerniglia 1996). Further metabolism of *cis*-dihydrodiols by bacteria is carried out by dehydrogenation reactions

Table 1. Physical and Chemical Properties of Select PAHs*

PAH	Structure	Molecular Weight (g)	Aqueous Solubility (mg/L)	Log K_{ow}
Naphthalene		128.2	31	3.37
Acenaphthene		154.2	3.9	3.98
Acenaphthylene		152.2	16.1	4.07
Fluorene		166.2	1.89	4.18
Phenanthrene		178.2	1.11	4.46
Anthracene		178.2	0.075	4.5
Fluoranthene		202.3	0.24	4.90
Pyrene		202.1	0.132	4.88
Benzo[a]pyrene		252.3	0.0038	6.04
Chrysene		228.3	0.0019	5.63
1,5-Dimethylnaphthalene		156.2	3.19	4.38

*Complied from Mackay et al. (1992)

mediated in the presence of NAD+ to produce catechols.

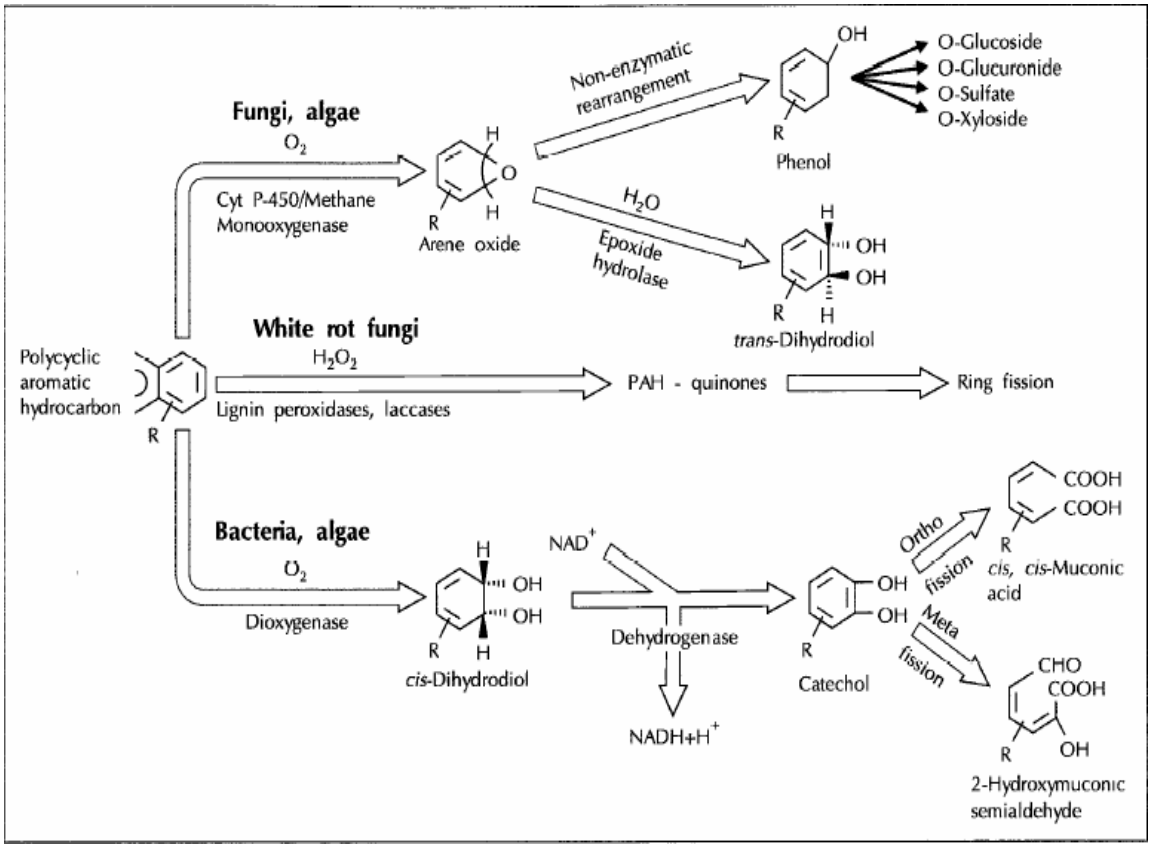


Fig. 2. Biochemical Pathway for Oxidation of PAHs (Adapted from Cerniglia 1993)

The most important step in the catabolism of PAHs is the inactivation of the aromatic ring through fission by the dioxygenase enzymes. These enzymes cause fission of the aromatic ring generating aliphatic intermediates. Fission of these *ortho*-dihydroxylated aromatic compounds takes place between the two hydroxyl groups or adjoining one of the hydroxyl groups. Each of these enzymes is specific for one type of

substrate. The intermediates produced are oxidized to generate cellular energy or for biosynthesis of cell constituents. Biodegradation is achievable only if microorganisms produce enzymes that will attack the contaminants to bring about mineralization.

Known Biodegradation Performance of Individual PAHs

In general, the use of biodegradation as a remediation technique requires an understanding of: the microorganisms that will effectively degrade a given class of organic compounds; the kinetics underlying the process; and how fast and to what extent the compounds will be degraded. Microorganisms that can degrade hydrocarbons are widespread in the soil and aquatic environments (Atlas 1995). Over the past two decades the ability of microorganisms like bacteria, fungi and algae to degrade PAHs has been proved (Cerniglia 1992). Biodegradation is a process that employs microorganisms for transforming toxic compounds, such as PAHs, to benign compounds. Biodegradation has gained attention for the clean up of contaminated sites and removal of PAHs from the environment. With bioremediation, destruction of target compounds can be achieved naturally at a relatively low cost. PAHs are the largest family of chemicals for which bioremediation was adopted at Superfund sites in United States (USEPA 1996)

Biodegradation of PAHs composed of three rings is well established and the degradation of HMW (composed of more than three rings) PAHs by bacteria was reported (Kanaly and Harayama 2000). Several bacterial genera have been identified for their ability to degrade PAHs, including the species of *Pseudomonas*, *Alcaligenes, Mycobacterium*, *Rhodococcus*, *Sphingomonas* and *Cycloclasticus* (Skerman 1967).

However, the microbial degradation of PAHs containing four or more aromatic rings is energetically less favorable as compared to LMW PAHs (Cerniglia 1992; Mueller and Cerniglia 1996). B[a]P has been the subject of comprehensive studies on biodegradation due to its hazards to human health (Kanaly and Harayama 2000). Only a few biodegradation studies demonstrate the mineralization of PAHs with more than four rings.

In 1989, Mueller et al. (1989a) documented the ability of a seven member bacterial community isolated from a creosote facility to utilize the HMW PAHs as a sole carbon source. In 1990, Mueller et al. (1990) demonstrated the ability of *Pseudomonas* (*Sphinogomonas*) *paucimobilis* EPA505 to utilize fluoranthene (HMW PAH) as the sole carbon and energy source. Ho et al. 2000 proposed a degradation pathway for fluoranthene and a cometabolism pathway for pyrene by *S. paucimobilis*. The bacterium also has the ability to metabolize the methylated forms of the PAHs (Ye et al. 1996). Ye et al. (1996) reported that *S. paucimobilis* is versatile in its specificity for PAHs, indicating that fluoranthene is able to induce enzyme(s) that can catalyze the degradation of a variety of PAHs (Mueller et al. 1990). Degradation of mixtures of pyrene, fluoranthene and phenanthrene by *S. paucimobilis* revealed competition for the same enzyme (Luning Prak and Pritchard 2002). *S. paucimobilis* mineralized the five-ring PAHs including B[a]P, benzo[b]fluoranthene, and dibenz[a,h]anthracene with the exception of dibenz[a]pyrene (Ye et al. 1996). *S. paucimobilis* used phenanthrene, naphthalene, fluoranthene, toluene, benzoic acid, 2,3- and 3,4- dihydroxybenzoic acids, 1-chloro-2,4- dinitrobenzene, anthracene and a number of other organic compounds as a

growth substrate (Story et al. 2004). These studies indicate that *S. paucimobilis* is highly competent for the degradation of many of the PAHs.

Biodegradation Kinetics

The kinetics of microbial growth and substrate utilization can be used to predict the fate and behavior of contaminants in the environment through appropriate models. Further, kinetic study results are useful in evaluating and weighing suitable treatment options for remediation and clean up of contaminated sites. Biodegradation kinetics can reveal the dependence of substrate/contaminant depletion on time. Growth and substrate depletion data may be fit with a simple Monod model (Bielefeldt and Stensel 1999). The Monod model establishes the relationship between the growth rate (μ), concentration of a single growth controlling substrate (S), and relates growth with substrate utilization (q) (Monod 1949). The parameters used in the Monod model are the maximum specific growth rate (μ_{max}) or maximum substrate utilization rate (q_{max}) and the affinity coefficient (K_s). The Monod constants q_{max} and K_s characteristically describe the biodegradability of an organic compound. The Monod equation for a single substrate is:

$$\mu = \frac{\mu_{max} S}{K_s + S} \quad (1)$$

Or

$$q = \frac{q_{max} S}{K_s + S} \quad (2)$$

Where, μ = specific growth rate (1/h); μ_{max} = maximum specific growth rate (1/h); S = substrate concentration (mg/L); K_s = affinity coefficient (mg/L); q_{max} = maximum substrate utilization rate (mg of substrate/mg of biomass/h) and q = specific substrate utilization rate (mg of substrate/mg of biomass/h). The affinity coefficient (K_s), or the half velocity constant represents the concentration at which the specific growth rate is equal to half of the maximum specific growth rate (Monod 1949). The inverse of affinity coefficient ($1/K_s$) represents the affinity of the enzyme for the substrate. Thus a lower value of K_s suggests a high affinity for the substrate. At lower values of K_s, the substrate is in the form of the enzyme substrate complex indicating that the half the maximum velocity will be achieved at a lower concentration of the substrate. At substrate concentration equal to K_s, half the maximum utilization rate is obtained.

At concentrations of $S >> K_s$, it is observed that the specific growth rate becomes equal to the maximum specific growth rate and the reaction exhibits zero order kinetics. As very low concentrations of S are approached, when $S << K_s$, the model exhibits first order kinetics. The no–growth model is used at low initial substrate to biomass concentration (S_0: X) providing a measure of the maximum utilization rate (Grady et al. 1996). The biokinetic parameters are estimated by fitting the experimental data obtained from the substrate depletion experiments to the Monod equation.

Determining degradation rates experimentally and those factors influencing them will enhance our understanding of the persistence, recalcitrance and transformation of contaminants in natural and engineered systems providing us the tools to design effective treatment systems. Biodegradation kinetics are influenced by bioavailability,

sorption and desorption processes, among other factors (Scott 1989; Cerniglia 1993). Abiotic processes, bioaccumulation, and sorption affect the rate of microbial transformation in contaminated sediments (Herbes and Schwall 1978). PAHs tend to sorb on organic matter and oily phases (Ho et al. 2000). This suggests that the processes of sorption and desorption from the organic phase, and bioavailability can be dominant factors in soil and sediment contaminated environments. In aqueous systems where the concentrations of PAHs are below their solubilities, bioavailability and desorption effects may be negligible compared to biodegradation which can emerge as the process controlling the fate of PAHs.

Variability in Estimating Kinetic Parameters

Grady et al. (1996) reviewed the possible causes in estimating biokinetic parameters. This section gives a brief overview of factors that affect parameter estimation as discussed by Grady et al. (1996). It is crucial to consider the variability in parameter estimates to ensure appropriate interpretation of the reported values. The most important factors responsible for the variability are: culture history, parameter identifiability, and the procedure used to measure the parameters.

Culture History. Environmental parameters and the period of time for which these are imposed on the culture represent the conditions of the culture prior to the experiment. These conditions are a key factor in controlling the differences in biokinetic parameters (Harder and Dijkhuizen 1986). For pure culture experiments, changes in the

environmental conditions will lead to changes in adaptation of the culture. Conditions of oxygen depletion in poorly aerated environments may cause bacteria to switch to anaerobic conditions if they are facultative. Substrate affinity is another factor that construes the concept of culture history. Harder and Dijkhuizen (1986) reported that bacteria are able to replace a low affinity system by a high affinity system when cultured under nutrient limited conditions.

Physiological Adaptation. When steady state conditions are imposed on bacteria, they can change their macromolecular composition to optimize their growth. The macromolecular composition which includes the nucleic acids, RNA and DNA, protein and the cell–envelope is known as the organism's physiological state. Physiological status is influenced by growth conditions, different growth rates result in varying physiological states. When bacteria are subjected to different growth conditions, changes occur in the metabolic activity. Thus, biodegradation kinetics are impacted by the physiological status of the organism.

Parameter Identifiability. Substrate depletion effects can be observed in batch experiments. During batch experiments, the ratio of the initial substrate concentration, S_0, to the initial biomass concentration, X_0, is one of the factors governing the identifiability of the kinetic parameters. Parameter identifiability establishes the validity and clarity of the parameters estimated based on the environmental conditions at which they were measured and thus yields a more rational and absolute estimate of a kinetic

parameter. For example, depending on the S_0/X_0 ratio, the kinetic parameters can be estimated either independently or as lumped parameters (Beilefeldt and Stensel 1999). If biomass growth and substrate utilization occur simultaneously, and follow the Monod kinetics, independent estimates of μ_{max} and K_s can be obtained when S_0/X_0 is 20/1 on a COD basis (Grady et al. 1996).

Kinetic Assay. In batch experiments, the results are impacted by the initial substrate to biomass ratio, S_0/X_0. The value of S_0/X_0 also determines the culture history. For a large ratio of S_0/X_0, changes occur in the physiological state of the culture. Conversely, if the S_0/X_0 is small the parameters will reflect the conditions existing at the time of the test.

Intrinsic and Extant kinetics. Grady et al. (1996) proposed a nomenclature to avoid confusion on the interpretation of the estimates of the biokinetic parameters. *Intrinsic kinetics* depicts the kinetic parameters measured with a high S_0/X_0 ratio; they represent the nature of the organism and substrate. At high S_0/X_0 ratio, the high substrate concentration will allow maximum growth of the biomass and cause changes in the community structure (Simkins and Alexander 1984). If the substrate is provided in small quantities relative to the amount of the biomass present, the changes in the community structure are negligible and the physiological status is maintained constant within limited time (Simkins and Alexander 1984). Extant kinetics reflect the true status of the culture as it exists at the time the culture is removed or it depicts the "currently existing" (Grady

et al. 1996) conditions. Extant kinetics eliminate experimental artifacts that can arise under other conditions such as preexposure of a microbial community to inducer or high affinity substrates. The types of kinetics, intrinsic or extant determine the type of model to be applied for estimating the kinetic parameters.

MULTISUBSTRATE BIODEGRADATION KINETICS

The mechanisms that govern the transformation of mixtures of PAHs can be complex due to the structural and chemical diversity within the PAH family. The kinetics of multicomponent aqueous systems determines the rate and extent to which different components in the system are being transformed accounting for the effects the mixture is most likely to produce on the individual compounds and the microbial community. Heterotrophic organisms support growth in a mixture of substrates rather than utilizing a single substrate (Kovárová-Kovar and Egli 1998). Sequential utilization and diauxic effects are typically observed under conditions where substrate concentrations are in excess of the growth limiting concentration (Kovárová-Kovar and Egli 1998; Lendemann et al. 1996). When organisms are fed low concentrations, simultaneous use of compounds is observed (Kovárová-Kovar and Egli 1998). This suggests that at low concentrations of substrates, growth is supported by the mixture instead of following the principle of diauxic utilization. The ability to assimilate a mixture of substrates reflects the catabolic activity of the organism in the presence of multiple substrates. A multisubstrate model is essential to desribe the simultaneous degradation of substrates. Guha et al. (1999) and Knightes (2000) reviewed the use of multisubstrate models for predicting substrate interactions between PAH mixtures.

Review of Studies on PAH Mixtures

Biodegradation studies on PAH mixtures have construed some important observations about the degradation of multiple PAHs within a system by pure cultures and mixed cultures. *Mycobacterium* strain PYR-1 degraded all six components of a synthetic PAH mixture to various extents with the exception of pyrene (Kelley and Cerniglia 1995). Bauer and Capone (1988) revealed that the rates of PAH degradation were impacted by pre-exposure to alternate PAHs and benzene. They attributed enhanced degradation of PAHs after exposure to other PAHs to enzyme induction. Beckles et al. (1998) studied the biodegradation of fluoranthene present as a sole substrate and in mixtures with naphthalene and acenaphthene in systems with and without sediments. Fluoranthene was degraded only in the presence of naphthalene and fluoranthene degradation was not induced by the presence of acenaphthene. Luning Prak and Pritchard (2002) reported the pyrene inhibition in the presence of fluoranthene and phenanthrene for *S. paucimobilis*.

Creosote is a complex mixture composed of 85 % PAHs; 10 % phenolic compounds; and 5% N-, S-, and O- heterocyclics for which biodegradation studies are not conclusive (Mueller et al. 1989b). Since creosote is a complex mixture of chemicals that exhibit diverse chemical structures (Mueller et al. 1989b), degradation of its individual components is not well understood. The fluoranthene degradative ability of *S. paucimobilis* in the presence of creosote components revealed toxic and inhibitory effects of creosote constituents (Lantz et al. 1997). Thus, bioremediation of creosote

contaminated sites is challenged by the presence of structurally diverse contaminants (Mueller et al. 1989b).

Several studies reported the ability of bacteria to utilize both pyrene and phenanthrene, indicating a metabolic similarity (Molina et al. 1999). The inhibition of phenanthrene degradation by naphthalene has been extensively studied (Stringfellow and Aitken 1995). Competitive inhibition of phenanthrene by naphthalene, methylnaphthalene and fluorene indicates that similar enzyme systems are being exploited. These results are consistent with the study reported by Luning Prak and Pritchard (2002), in which phenanthrene, fluoranthene and pyrene were found to compete for the same active site. Ye et al. (1996) demonstrated the substrate interaction phenomenon for degradation of B[a]P in the presence of other HMW PAHs by utilizing *S. paucimobilis*. The results reveal that B[a]P and the other PAHs do not share a common metabolic pathway of degradation. It is essential to take into account the substrate interactions of the antagonistic and synergistic effects to simulate or to anticipate the complexity of mixtures that is encountered in natural systems.

Interactions in PAH Mixtures

A complication in evaluating the biodegradation kinetics of PAHs is the possibility of substrate interactions (Guha et al. 1999). Within a mixture of PAHs, the substrate interactions include negative effects, which involve inhibition and/or diauxic effects (Guha et al. 1999; Beckles et al. 1998); positive effects include enhancement and cometabolism (Guha et al. 1999; Molina et al. 1999); or no effect at all (Beckles et al.

1998; Guha et al. 1999). To understand the interactions that take place in mixtures, positive and negative effects that occur within the mixtures must be evaluated. Since the underlying goal of biodegradation is to reduce the concentration of the contaminants to an acceptable level, enhanced degradation would be preferred. The interactions not only indicate the effects that occur for PAHs, but also reveal underlying mechanisms of enzymatic activity involved in the transformation of different compounds in a mixture.

The negative effects are due to competitive inhibition of multiple substrates or other means of retarding the degradation of one substrate in the presence of another. Competitive inhibition lowers the affinity of the enzyme. In competitive inhibition, multiple substrates are transformed by a common enzyme system (Stringfellow and Aitken 1995). Similar or identical enzyme systems may catalyze the degradation of compound(s) which may be structurally similar (Bauer and Capone 1988). Multisubstrate competitive inhibition captures the effect of two converse processes: enhanced degradation as result of an augmentation in the biomass population and retarding rates of degradation as a result of competition for the substrate (Knightes 2000; Guha et al. 1999). Stringfellow and Aitken (1995) demonstrated competitive metabolism between naphthalene and phenanthrene where phenanthrene degradation was inhibited by naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, and fluorene. The study further suggested that competitive metabolism may be commonly encountered among PAH-degrading organisms.

Positive effects result in the enhanced degradation of the substrate as a result of proliferation of the biomass growth on multiple substrates (Guha et al. 1999) and

enzyme induction (Bauer and Capone 1988; Luning Prak and Pritchard 2002). The presence of a suitable substrate affects the fortuitous degradation of other PAHs (Molina et al. 1999). Phenanthrene degradation by several strains decreased due to lack of pre-exposure to other PAH compounds (Molina et al. 1999). However, the responses to PAH induction are strain specific (Molina et al. 1999) and cannot be extended for all environmental media. Bauer and Capone (1988) observed enhanced degradation of PAHs as a result of pre-exposure to other aromatic hydrocarbons. In previous studies, naphthalene and phenanthrene enhanced the degradation of each of the other PAHs through cross acclimation (Bauer and Capone 1988). However, Bauer and Capone (1988) deduced that the presence of simultaneously occurring PAHs did not impact the degradation of individual PAHs. It may be reasonable to conclude that the interactions and effects encountered in a multisubstrate system are a function of the microbial community, the type of culture (mixed versus pure) and the physiological state of the community at the time of the experiment.

Multisubstrate Kinetics

Multisubstrate biodegradation kinetics describe the extent and the rate at which different components in a system are being transformed. Multicomponent systems may represent simple inhibition systems or larger multireactant systems. For a simple inhibition system, when the enzyme binds with the inhibitor and the given substrate, the Michaelis–Menten kinetics may be represented as

$$V = V_{\max} \frac{[S]}{[S] + K_m + \frac{K_m}{K_I}[I]} \tag{3}$$

where V = catalytic rate; V_{max} = maximum catalytic rate when the enzyme is saturated with S; K_I = inhibition Coefficient; I = concentration of the inhibitor and S = substrate concentration. Eq. (3) is valid for a non-reactive inhibitor (Stryer 1995). When the inhibitor is present as an alternate substrate, then the K_I is equal to K_m for the given substrate. The equation for a mixture of substrates exhibiting competitive inhibition kinetics is represented as

$$\frac{dC_i}{dT} = \frac{q_{\max i} C_i X}{K_{si} + \sum_{j=1}^{n} \frac{K_{si}}{K_{sj}} Cj} \tag{4}$$

where C_i = concentration of substrate *i* (mg/L); C_j = concentration of substrate *j* present in the mixture (mg/L); K_{si} = affinity constant for substrate *i*; K_{sj} = affinity constant for substrate *j* and X = biomass expressed as (mg/L) of protein. The model uses the parameters derived from the sole substrate case. It requires that the compounds be utilized through a common pathway (Segel 1975).

MULTISUBSTRATE BIODEGRADATION EXPERIMENTS

To evaluate biodegradation kinetics of a mixture, it is essential to measure the substrate concentration over time for each of the substrates present in the mixture. Binary and ternary mixtures of PAHs represented multisubstrate systems. Experiments were conducted in aerobic reactors under conditions of extant kinetics. The first set of compounds included fluorene, 1,5–dimethylnaphthalene and naphthalene. These compounds were selected because the rate limiting step in the biodegradation of these compounds is governed by transport kinetics (Dimitriou-Chrisitidis 2005). Since competitive inhibition requires that compounds share a common rate limiting step, the above compounds satisfied this hypothesis. In addition, these compounds exhibit medium to low q_{max} values (Dimitriou-Chrisitidis 2005) which would allow simultaneous biodegradation. The second series of experiments consisted of anthracene, naphthalene, 1-methylfluorene and 1,5– dimethylnaphthalene. The protocol for the growth of the microorganism and the experimental set up was adapted from Dimitriou-Chrisitidis (2005).

Materials and Methods

Chemicals and Chemical Analysis. Anthracene (ANT) and fluorene (FLE) were purchased from Sigma Chemical Co. (St Louis, MO). Naphthalene (NAP), 1,5–dimethylnaphthalene (15DMN) and 1–methylflourene (1MFLE) were purchased from

Alfa Aesar (Ward Hill, MA), ND Avocado Research Chemicals (Heysham, England) and Ultra Scientific (North Kingstown, RI) respectively. The purity of all chemicals was greater than 95%. Tween 80 was purchased from Sigma Chemical Co. (St Louis, MO). PAH aqueous solutions were prepared in Bushnell-Haas broth. Water used was deionized and ultra purified.

A HP 5890 Series II GC/MS coupled with a HP 5972 mass selective detector was used for quantification of the PAH compounds. The column for the GC-MS was a HP 5MS ((5%- Phenyl)-Methylpolysiloxane, 0.25mm×30m×0.25µm, (J & W Scientific). The operating conditions were: flow rate of 0.63ml/min, temperature program: 60^{o}C, 8.0 ^{o}C/min for 30 minutes to 300^{o}C. The mass spectrometer was operated in the selective ion mode (SIM).

Growth and Storage of Lyophilized Cells. The microorganism used for this research *Sphingomonas paucimobilis* DSM 7526 (strain EPA505) was purchased from DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Reconstituted cells were grown in nutrient broth for 5 days. Following the incubation period, 10% sterilized glycerol was added. After 30 minutes, the solution was transferred to cryopreservation vials and stored at -80^{o}C.

Preparation of the Mineral Salts Base (MSB). The mineral Salts Base (MSB) had the following concentrations (mg/L): $(NH_4)_2SO_4$, 1000; K_2HPO_4, 800; KH_2PO_4, 200; $MgSO_4.7H_2O$, 200; $CaCl_2.2H_2O$, 100; $FeCl_3.6H_2O$, 5; $(NH_4)_6Mo_4O_{24}.4H_2O$, 1. For the

last two salts a separate concentrated solution containing 1000 and 200 mg/L of the salts, respectively was prepared. 5 ml of this solution was added to the rest of the MSB per liter of MSB to attain the desired concentration. The final pH of the solution was adjusted to 7.0.

Preparation of Mineral Salts Base Plus Fluoranthene (MSF). To a sterilized 1 liter flask, 1 ml of fluoranthene solution in acetone (20 g/L) was added. The acetone was allowed to evaporate. A solution of Tween 80 (8000 mg/L) was prepared by adding 40 mg to 5 ml of deionized water. 200 ml of MSB was filter sterilized through 0.2 μm cellulose filter membrane and added to the flask containing fluoranthene crystals. To the same flask Tween 80 solution was filter sterilized through a syringe. The final MSF medium achieved a concentration of 100 mg/L fluoranthene and 200 mg/L Tween 80.

Growth of Sphingomonas paucimobilis DSM 7526 *on Fluoranthene*. A flask containing the sterilized MSF medium was inoculated with 5 ml of the seed culture. The solution was incubated for 72 hours at 30^{o}C in the dark with constant stirring on a horizontal shaker operating at 160 rpm (Ye et al. 1996). Following the incubation period, 10% glycerol solution was added and stored in cryopreservation vials at -80^{o}C.

Biomass for Biodegradation Experiments. The biomass for the experiments was prepared by adding 7 ml of the culture pregrown on the MSF medium to 800 ml sterilized nutrient broth containing 0.4 g glucose (Ye et al. 1996). The solution was

incubated in the dark at 30^{o}C on a horizontal shaker at 160 rpm for a period of 24 hours. Following the incubation period, the cells were centrifuged (6653×g, 10 minutes) and washed with Bushnell-Haas medium three times. After centrifugation, the supernatant was discarded and the concentrated cells were resuspended in 45 ml of Bushnell-Haas media.

Experimental Design of Biodegradation Experiments. The first set of experiments was performed using the following compounds: naphthalene (NAP), fluorene (FLE) and 1,5–dimethylnaphthalene (15DMN). Binary and tertiary mixtures were designed with combinations of these compounds and the compounds were also tested individually in the same experiment. A preliminary experiment was conducted to estimate the time required for depletion of the individual compounds and the mixtures so that sampling times could be appropriately determined. The experimental set up with initial substrate and biomass concentrations is presented in Table 2. The second set of experiments comprised the following compounds: anthracene (ANT), 1,5–dimethylnaphthalene (15DMN), naphthalene (NAP), 1–methylflourene (1MFLE). The experimental set up for the second set of experiments is shown in Table 3.

Stock solutions of individual compounds were prepared in hexane. The aqueous solutions of the PAH mixtures were prepared by adding the appropriate volume of the stock solution of the individual PAH compounds to 800 ml of Bushnell-Haas media to achieve a concentration below the aqueous solubility limit of the PAH compound, taking care that the concentration of any one of the PAH in the mixture will not exceed its

solubility. The initial concentrations of compounds were determined such that $S >> K_s$ (Dimtriou-Christidis 2005). This allows the biokinetic estimates to be determined independently (Ellis et al. 1996). The aqueous solutions were kept in the dark for three days prior to the experiment to allow complete solubilization in the aqueous solution. The experiments were conducted in 250 ml amber serum bottles (reactors) under the conditions of extant kinetics. Duplicate reactors containing 150 ml of the aqueous PAH solution were prepared. The reactors were placed on a horizontal shaker throughout the experiment. The experimental set up is depicted in Fig. 3. Required quantity of the concentrated solution of cells was added to each reactor to obtain an absorbance A595 of 0.25. The biomass concentration was quantified at the beginning of the experiment as protein by the method suggested by Bradford (1976) using BSA as a standard. Ten, 7 ml samples from each reactor were added to sampling vials at predetermined sampling times. The sampling vial was a 16 ml screw cap tube fitted with Teflon coated caps containing 3 ml of dichloromethane. Reactors containing only the aqueous PAH mixture solution without any biomass represented controls for the experiments.

The sampling vials were placed on a rotary shaker for 12 hours to allow complete partitioning of the PAHs into the dichloromethane (DCM) phase. After 12 hours, the PAHs were extracted from the DCM phase. Following this, 1 ml of the solution from the DCM phase was transferred to a GC vial and 10 µl of internal standard was added to the vial. The extracts were analyzed by using gas chromatography/mass spectrometry (GC/MS).

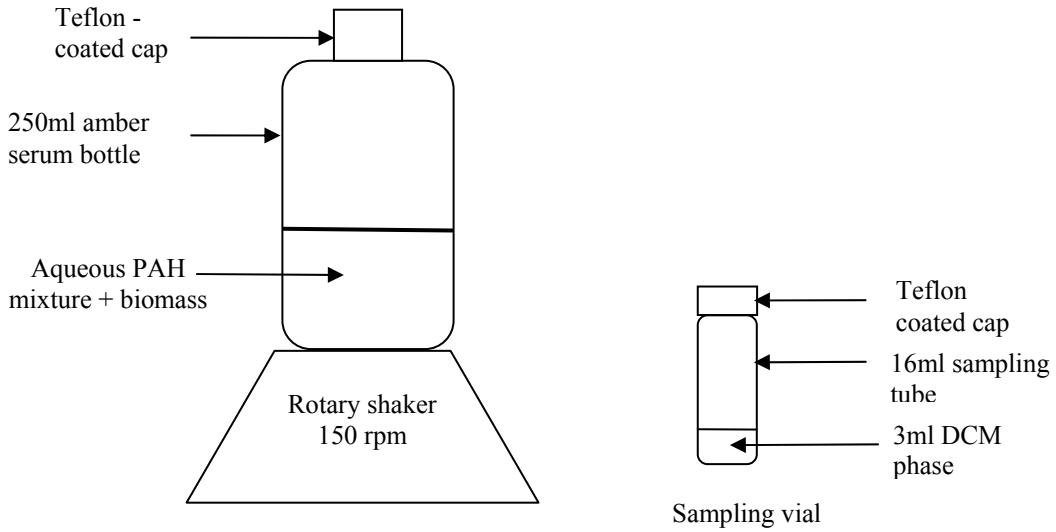


Fig. 3. Experimental Set–up for Batch Multisubstrate Biodegradation Experiments

Table 2. Set-up for First Set of Multisubstrate Biodegradation Experiments

Experiment-1	FLE[a]	15DMN[a]	NAP[a]	X[b]
Reactor A	0.35	-	-	4.06
Reactor B	-	0.183	-	4.06
Reactor C	-	-	0.74	3.61
Reactor D	0.30	0.178	-	5.21
Reactor E	0.31	-	0.75	4.80
Reactor F	-	0.18	0.65	4.75
Reactor G	0.27	0.14	0.55	4.86

[a]: Initial substrate concentrations in mg/L
[b]: Biomass concentration in mg protein/L

Table 3. Set-up for Second Set of Multisubstrate Biodegradation Experiments

Experiment-2	NAP[a]	15DMN[a]	ANT[a]	1MFLE[a]	X[b]
Reactor H	-	-	0.04	-	3.61
Reactor I	-	-	-	0.16	2.93
Reactor J	0.75	0.25	0.04	0.16	2.90

[a]: Initial substrate concentrations in mg/L
[b]: Biomass concentration in mg protein/L

MULTISUBSTRATE AND SOLE SUBSTRATE PARAMETERIZATION

Model Formulation: Multisubstrate Biodegradation Kinetics

The experimental data from the mixture experiments was evaluated using two different models. The first model is the sole substrate Monod model which can be described as a no-interaction model. The second model is the multisubstrate model with competitive inhibition. The equation describing sole substrate kinetics can be represented as

$$\frac{dC}{dT} = \frac{q_{\max} CX}{K_s + C} \tag{5}$$

This model assumes that the presence of other substrates does not affect the behavior of a single substrate. It represents the case where a compound in a mixture may behave as if it were the only compound present not accounting for the effects resulting from other existing substrates.

For a binary mixture the multisubstrate model may be represented as

$$\frac{dC}{dT} = \frac{q_{\max} CX}{K_s (1 + \frac{C_1}{K_{s1}}) + C} \tag{6}$$

For a tertiary mixture the multisubstrate model may be formulated as:

$$\frac{dC}{dT} = \frac{q_{\max} CX}{K_s (1 + \frac{C_1}{K_{s1}} + \frac{C_2}{K_{s2}}) + C} \tag{7}$$

Equations 6 and 7 assume that all PAHs are transformed by a common enzyme pathway. The model can be extended for any number of components provided the compounds exhibit competitive inhibition kinetics. The above equations are predictive since they rely on the parameters obtained from the sole substrate experiments. It captures the effect of competition among the substrates. Enhanced degradation resulting from biomass proliferation will not be accounted for due to extant conditions during the experiments. It is assumed that the biomass concentration will remain constant throughout the experiment and that the presence of additional substrates will not enhance biomass growth.

Parameter Estimation. No new parameters were estimated for the multisubstrate experiments. The parameters obtained from the sole substrate experiments formed a basis for modeling multicomponent systems. The multisubstrate competitive inhibition model was used to determine usefulness of the model for estimating multisubstrate biodegradation kinetics. The experimental data obtained from the binary and ternary mixture experiments were used to generate the model predicted curves. The experimental data consisted of substrate concentration of all the components in a given mixture monitored over a given sampling period and the initial biomass concentration (X_0). The experimental data was compared with the two models; the sole substrate model and the multisubstrate competitive inhibition model. For the binary mixture, the affinity constant (K_{s1}) for the alternate substrate C_1 represented the sole substrate parameter obtained from sole substrate modeling. The affinity constant (K_s) and the

substrate utilization rate (q_{max}) for substrate of interest C, were treated as a fitting parameters. This approach is valid since the competitive inhibition model relies on parameters obtained from sole substrate experiments. The best parameter estimates were obtained by minimizing the sum of the squared errors (SSE),

$$SSE = \sum_{i=1}^{n} (C_i^{obs} - C_i^{pred})^2 \quad (8)$$

Sole Substrate Parameter Estimation

Various methods are available for estimation of the Monod parameters (Counotte and Prins 1979; Guha and Jaffé 1996; Smith et al. 1996; Smith et al. 1998). A fairly simple approach is to linearize the Monod equations and estimate the coefficients by linear regression analysis. Linearized forms of the Monod equation are represented in the form of Lineweaver–Burk and Eadie–Hofstee plots. Simple linear regression is not appropriate for a set of non–linear equations because it transforms the error distributions (Leatherbarrow 1990). The asymptotic nature of the Monod equation makes the parameter estimation complex (Guha and Jaffé 1996). Linearizing the Monod equation makes the problem more complex if the substrate concentrations used in the experiment are low or less than K_s (Smith et al. 1996) or in the range of K_s (Counotte and Prins 1979). The use of non–linear regression techniques is widely accepted because they represent an accurate method of analysis and are practically feasible (Leatherbarrow

1990). Since the Monod equation represents a non–linear differential equation, the use of non-linear regression is a valid approach.

An alternative means of estimating the Monod parameters is by solving the integrated form of the Monod equation either in computer code or a simple spreadsheet (Smith et al. 1998). The numerical integration of the differential equations is commonly achieved by using a fourth order Runge–Kutta algorithm. Runge–Kutta methods take into account an improved slope average which predicts the values more accurately (Chapra and Canale 2002). The error between the experimental data and the model predicted data can be minimized by adapting the least–squares analysis, or by minimizing the sum of the error squares, commonly referred to as SSE.

Sole Substrate Modeling. The sole substrate parameters were estimated for naphthalene, 1,5–dimethylnaphthalene and fluorene. The constants associated with biotic and abiotic losses are assumed to be negligible. The mass balance equation representing the sole substrate system is

$$\frac{dC}{dT} = \frac{q_{\max} CX}{K_s + C} \tag{5}$$

The experimental data obtained from the sole substrate experiments comprised, C: the aqueous phase concentration of the PAH measured over time, X: initial biomass concentration and T: sampling time in hours. q_{max}, K_s and C_0 represent the system variables to be obtained by fitting Equation 5 to the experimental data. The fourth order

Runge – Kutta method was used for fitting the Monod equation to the experimental data. The extant conditions in the experiments allow a unique estimation of q_{max} and K_s (Grady et al. 1996). The fourth order Runge–Kutta method is represented as

$$C_1 = C_0 + \frac{1}{6}(K_1 + 2K_2 + 2K_3 + K_4)dT \quad (9)$$

where $K_1 = f(t, C_0)$; $K_2 = f(t + 0.5dT, C_0 + 0.5K_1dT)$; $K_3 = f(t + 0.5dT, C_0 + 0.5K_2dT)$; and $K_4 = f(t + dT, C_0 + K_3dT)$

All calculations were performed using a spreadsheet program (Microsoft Office Excel 2003, Version 11.0). A spreadsheet was prepared for obtaining the best-fit estimates of q_{max}, C_0 and K_s. An example spreadsheet for obtaining the best-fit estimates is shown in Table 4. The fitting parameters along with the constants used for curve fitting are depicted in rows 1 through 4. The biomass concentration was constant and measured at the start of the experiment. Values for the fitting parameters representing initial guesses, were transformed into the best-fit estimates by the program as the model was fitted to the experimental data. The experimental data is shown in columns A and B. Column C contains the model predicted values of concentration C. Column D contains the step size (dT), which represent an interval between two consecutive sampling measurements and are used in the Runge–Kutta algorithm. Column E contains the values obtained by using the Monod equation. Columns F through O represent the terms from the fourth Runge–Kutta algorithm used for fitting the Monod equation to the experimental data. The difference between the experimental data and model predicted

Table 4. Sample Spreadsheet for Sole Substrate Parameter Estimation

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	q_{max}	mg/mg-hr	-0.097	Fitting parameter													
2	K_s	mg/L	0.079	Fitting parameter													
3	X	mg/L	3.6100	constant													
4	C_0	mg/L	0.7997	Fitting parameter													
5	Observed data		Predicted data	Step size	dC/dT	$Ci = C_0 + \frac{1}{6}(K_1 + 2K_2 + 2K_3 + K_4)dt$										Error	Error Square
6																	
7	t	C_{obs}	Cpred	dT		K_1	$C_i + K_1/2$	K_2	$C_i+K_2/2$	K_3	C_i+K_3	K_4	$2K_2$	$2K_3$	K	C_{obs} -C_{pred}	
8	hrs	mg/L	mg/L	Hrs												mg/L	$(mg/L)^2$
9	0	0.7887	0.7997	0.57	-0.3178	-0.1811	0.7092	-0.1791	0.7102	-0.1791	0.6206	-0.1765	-0.3581	-0.3582	-0.1790	-0.0110	0.0001
10	0.57	0.6161	0.6207	0.57	-0.3097	-0.1766	0.5325	-0.1733	0.5341	-0.1734	0.4474	-0.1691	-0.3466	-0.3467	-0.1732	-0.0047	0.0000
11	1.14	0.4727	0.4476	0.57	-0.2968	-0.1692	0.3630	-0.1634	0.3658	-0.1637	0.2839	-0.1557	-0.3269	-0.3273	-0.1632	0.0251	0.0006
12	1.7	0.2947	0.2844	0.57	-0.2732	-0.1557	0.2065	-0.1439	0.2124	-0.1450	0.1394	-0.1270	-0.2878	-0.2901	-0.1434	0.0103	0.0001
13	2.27	0.1371	0.1410	0.57	-0.2237	-0.1275	0.0772	-0.0983	0.0918	-0.1069	0.0340	-0.0599	-0.1966	-0.2138	-0.0996	-0.0039	0.0000
14	2.83	0.0031	0.0413	0.57	-0.1198	-0.0683	0.0072	-0.0165	0.0330	-0.0586	-0.0173	0.0558	-0.0331	-0.1173	-0.0271	-0.0382	0.0015
15	3.4	0.0001	0.0142	0.57	-0.0531	-0.0303	-0.0009	0.0024	0.0154	-0.0324	-0.0182	0.0596	0.0048	-0.0648	-0.0051	-0.0141	0.0002
16	3.97	0.0000	0.0091	0.57	-0.0359	-0.0205	-0.0012	0.0030	0.0106	-0.0234	-0.0144	0.0442	0.0060	-0.0469	-0.0029	-0.0091	0.0001
17	4.53	0.0000	0.0062	0.57	-0.0254	-0.0145	-0.0010	0.0026	0.0075	-0.0173	-0.0111	0.0324	0.0053	-0.0346	-0.0019	-0.0062	0.0000
18	5.1	0.0000	0.0043	0.57	-0.0180	-0.0103	-0.0008	0.0021	0.0054	-0.0126	-0.0083	0.0235	0.0042	-0.0253	-0.0013	-0.0043	0.0000
19																	0.0027

values for substrate concentrations give the error (column P). The individual values from column P were squared and summed to give the sum of the error squares (SSE), cell Q19.

The SSE (cell Q19) was minimized to obtain the model predicted values by adjusting the parameter estimates. The best-fit estimates of the fitting parameters are the values for which the Solver function (Microsoft Excel 2003, version 11.0) optimizes the SSE to a minimum value. This is based on an iterative search by the Solver, where the initial guesses of the fitting parameters are replaced by the best-fit estimates in rows 1, 2 and 4. The best-fit estimates were obtained for naphthalene, 1,5-dimethylnaphthalene and fluorene. The uncertainty in the fitting parameters was determined by a method described by Smith et al. (1998).

RESULTS

Sole Substrate Experiments

Substrate depletion data was generated for the individual PAHs of fluorene (FLE), 1,5–dimethylnaphthalene (15DMN) and naphthalene (NAP), binary mixtures of these compounds and the ternary mixture. The time dependent depletion of individual PAHs is illustrated in Fig. 4. Each PAH experiment was repeated and the duplicate data are treated as independent data sets. The experimental observations represent data from duplicate sets. It can be inferred that the experiments are highly reproducible based on the minimal differences observed between sets (APPENDIX A). For example, the sole substrate depletion curves obtained from duplicate reactors for naphthalene were not significantly different since the biokinetic parameters obtained from the two data sets were reproducible (q_{max1} = 0.097 ± 6%; q_{max2} = 0.1 ± 6% mg substrate/mg protein/hr). The Monod model was fitted to the experimental data from the sole substrate experiments to yield biokinetic parameters q_{max} and K_s along with the initial concentrations for fluorene, 1,5–dimethylnaphthalene and naphthalene (Table 5). The Monod model successfully replicated the experimental data (Fig. 4). The 95% confidence intervals determined by the method described by Smith et al. (1998), are also summarized in Table 5.

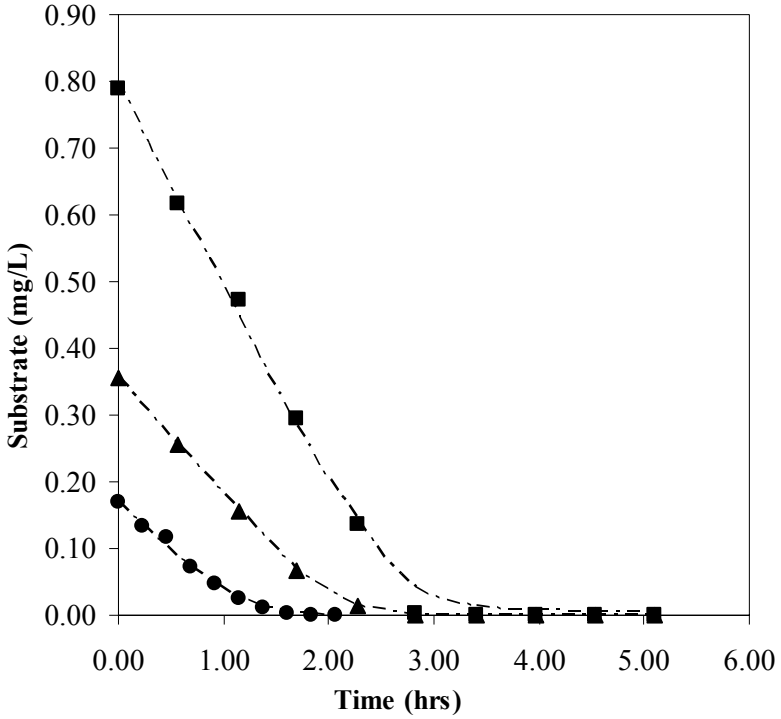


Fig. 4. Sole Substrate Degradation of Naphthalene (■), Fluorene (▲), and 1,5-Dimethylnaphthalene (●). Closed symbols represent experimental observations. Dashed lines are generated by the sole substrate Monod model.

Table 5. Estimated Parameters for Degradation of Individual PAHs

Parameters	Fluorene	1,5–Dimethylnaphthalene	Naphthalene
q_{max} (mg substrate/mg protein/hr)	0.043 ± 0.002	0.052 ± 0.003	0.100 ± 0.004
K_s (mg/L)	0.017 ± 0.001	0.043 ± 0.002	0.075 ± 0.005
q_{max}/K_s (hr^{-1} /mg protein/L)	2.60 ± 0.33	1.19 ± 0.08	1.33 ± 0.06
$C_{t=0}$ (mg/L)	0.35 ± 0.01	0.18 ± 0.01	0.78 ± 0.03

The first order rate constants which represent the ration of the maximum substrate utilization rate and half saturation constant (q_{max}/K_s) for 1,5–dimethylnaphthalene and naphthalene are comparable, but the biokinetic parameters (q_{max} and K_s) are significantly different (Table 5). The maximum substrate utilization rates for fluorene and 1,5–dimethylnaphthalene are statistically different but comparable. Fluorene has an affinity constant (K_s) less than twice that of 1,5–dimethylnaphthalene, indicating a greater binding strength for the enzyme. Naphthalene exhibits an enzyme affinity constant four times greater than fluorene suggesting a low binding strength. The K_s values for all three PAHs were below the initial concentrations used in the experiments.

The initial molar concentrations of the tested PAHs were chosen to be below the aqueous solubilities (Table 6). From the preliminary experiments, concentrations were determined such that $S >> K_s$ (Dimitriou-Christidis 2005) and degradation of all PAHs occurred without substrate toxicity. In addition, the tested PAHs exhibit low to medium q_{max} values (Dimitriou-Christidis 2005). This would allow simultaneous degradation of the compounds (Kovárová-Kovar and Egli 1998). The compounds maintained an initial molar ratio approximately 6:2:1 in the order of NAP: FLE: 15DMN, such that the concentration of any PAH in the mixture did exceed its aqueous solubility and at the same time demonstrated the effect of K_sS_1/K_{s1} on competitive inhibition. An experiment with equitable concentrations of PAHs in a mixture was performed to evaluate the effect of equitable concentrations on competitive inhibition kinetics. This data will be explained later in this section. Since the initial concentrations of PAHs and biomass

concentrations used in the experiment varied, to compare the extent of degradation, first order rate constants were calculated.

Table 6. Initial Molar Concentrations, Aqueous Solubilities and Affinity Constants of Tested PAHs

Compound	Initial Molar Concentrations (μmol/L)	Aqueous solubilities* (μmol/L)	K_s μmol/L
FLE	2.10 ± 0.06	11.3	0.100 ± 0.018
NAP	6.08 ± 0.23	241	0.58 ± 0.05
15DMN	1.15 ± 0.06	20.4	0.28 ± 0.032

* Aqueous solubilities values taken from Mackay et al. (1992) and converted into μmol/L

All three PAHs exhibit similar first order rate coefficients. Biodegradation of PAHs in soil is believed to be governed by their aqueous solubility (Wilson and Jones 1993). However, in the aqueous systems studied, there was no correlation between the aqueous solubility and extent of degradation. One possible explanation for this is that in aqueous systems where the PAH concentrations are below their solubility and all the PAHs are in the dissolved phase, bioavailability may not be a limiting factor. This observation is consistent with the study reported by Knightes and Peters (2000) which concluded that biodegradation rates in the field are attributed to physical-chemical processes. Quantitative Structure Activity Relationship (QSAR) analysis revealed that membrane transport is the rate limiting step in PAH biodegradation (Dimitriou-Christidis 2005). Knightes and Peters (2000) reported first–order rate coefficients for

naphthalene and fluorene as 1.11 and 0.026 per hour per mg protein/ L, respectively. The first–order rate coefficient for naphthalene reported by Knightes and Peters (2000) is comparable to that obtained from this study; however there is a two order magnitude difference for fluorene. The differences may be explained by the kinetic conditions of the experiment where independent estimates of q_{max} and K_s could not be obtained for fluorene and the presence of a mixed microbial community. It is possible that the microbial consortium used in their experiments had a lower population of the microorganisms that could induce enzymes for fluorene degradation or not all the organisms present in the mixed culture may have been potent PAH degraders. The pure culture used in this study was highly competent in PAH degradation.

Multisubstrate Experiments

Binary experiments were performed with combinations of fluorene, 1,5–dimethylnaphthalene and naphthalene. To evaluate the biodegradation kinetics of a mixture, measurements were conducted over time for each of the substrates present in the mixture. Substrate depletion curves for individual compounds and compounds present in binary and ternary mixtures are plotted together. The biodegradation kinetics of a single PAH were affected in the presence of multiple substrates. Enhanced degradation resulting from the presence of multiple substrates was not observed. However, enhancement would be unlikely under the extant conditions in which there is no biomass growth.

The results are indicative of substrate interactions. Substrate interactions for the binary mixtures were also predicted by the multisubstrate Monod model for naphthalene, fluorene and 1,5-dimethylnaphthalene in binary and ternary mixtures (illustrated by Figs.5,6 and 7). Evidence for the presence of common degradative enzymes for these PAHs was demonstrated by the competitive inhibition multisubstrate model developed for binary systems. In developing the model, common enzymes were assumed to be active for the tested PAHs. The multisubstrate model appropriately predicted the observed degradation behavior since estimates obtained were not significantly different from sole substrate estimates (APPENDIX B). The Monod (no-interaction) model did not adequately capture the data for the binary systems since obtained estimates were significantly different from sole substrate estimates (data not presented), indicating that the Monod model cannot be used to predict multisubstrate biodegradation kinetics. The criterion for determining the acceptability of the model was that the biokinetic parameters (q_{max} and K_s) generated by the model should not be statistically different from those generated by the Monod model.

Once competitive inhibition was determined, the fractional velocity rate equation (Segel 1975) was used to estimate the effect of multiple substrates on a single substrate. For the binary system of a substrate S and an alternate substrate S_1, the equation is

$$Vi/V_0 = \frac{K_s + S}{K_s\{1 + \frac{S_1}{K_{s1}}\} + S} \qquad (10)$$

where V_0 = catalytic rate in the absence of an alternate substrate (mg of substrate/mg of protein/hr); V_i = catalytic rate in the presence of an alternate substrate (inhibitor) (mg of substrate/mg of protein/hr); K_s = affinity coefficient for substrate S (mg/L) and K_{s1} = affinity coefficient for alternate substrate S_1(mg/L). The equation can be extended for a ternary system. Eq. (10) is valid only for substrates exhibiting competitive inhibition kinetics. The affinity constants in the above equation were those generated by the multisubstrate model since they were not statistically different from the sole substrate estimates. The fractional velocity equation was used for binary and ternary mixtures to estimate inhibition in the presence of other substrates (Table 7). The equation takes into account the substrate affinities and concentrations of co-occurring PAHs. Percent inhibition (*i)* can also be obtained from the fractional velocity data and is given by the following expression:

$$i = (1 - \frac{V_i}{V_0}) \times 100 \qquad (11)$$

Table 7. Fractional Velocities for Binary and Ternary Mixtures

Mixture	V_i/V_0		
	NAP	15DMN	FLE
FLE + NAP	0.310 ± 0.040	-	0.720 ± 0.010
FLE + 15 DMN	-	0.210 ± 0.009	0.850 ± 0.010
15DMN + NAP	0.700 ± 0.02	0.390 ± 0.010	-
Ternary mixture	0.200 ± 0.020	0.130 ± 0.009	0.710 ± 0.008

Standard deviation from the mean of duplicate measurements is indicated as ±

Initial degradation rates of naphthalene in binary mixtures were slower than the degradation of naphthalene individually, as observed from the fractional velocities (Table 7). Thus, naphthalene degradation was impacted by the presence of other PAHs in binary and ternary mixtures (Fig. 5).

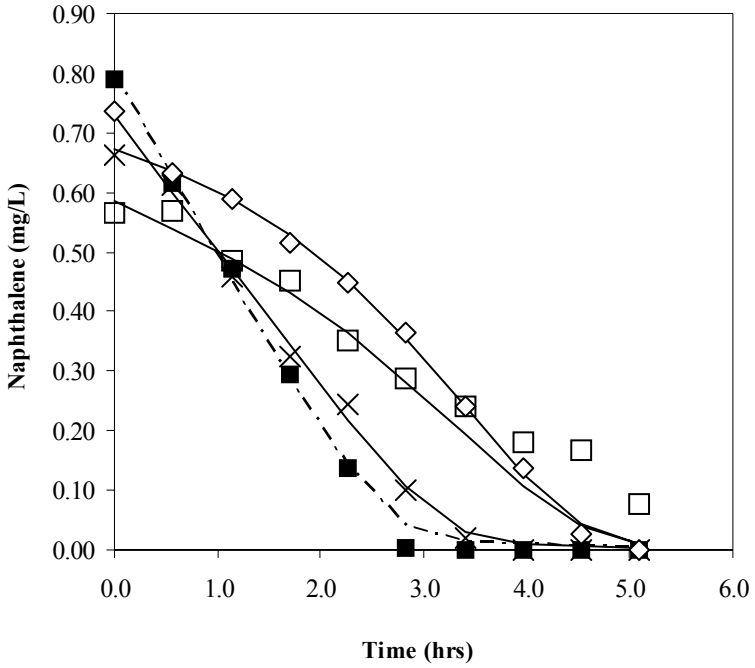


Fig. 5. Naphthalene Degradation in Sole Substrate, Binary and Ternary Systems. Closed symbols denote experimental observations for naphthalene in sole substrate system (■), open symbols denote experimental observations for naphthalene in binary mixture with fluorene (◇), 1,5-dimethylnaphthalene (×) and in a ternary mixture (□). Solid lines represent data generated by the multisubstrate competitive inhibition model. Dashed line is generated by the Monod model.

The percent inhibition for naphthalene in the presence of fluorene was 65% (Table 7), indicating a pronounced effect of fluorene on naphthalene degradation. The effect of competitive inhibition in the presence of fluorene was indicated by the high degree of curvature in the initial portion of the curve captured by the (K_sS_1/K_{s1}) term in the denominator (see equation 10). However, in the presence of 1,5–dimethylnaphthalene, the initial degradation rate of naphthalene slowed to 70% of its degradation when present individually. The substrate depletion curve, although statistically different from the sole substrate system, had a comparable slope (Fig. 5). However, if compared to degradation in the presence of fluorine, there was a significant difference between the two curves. The differences in the two curves, one in the presence of fluorene and the other with 1,5–dimethylnaphthalene, were due to the relative magnitude of substrate affinities represented by the value for K_sS_1/K_{s1}. Naphthalene degradation proceeded slowest in the ternary mixture compared to single compound behavior (V_i = 20% V_0). The initial rate of degradation of naphthalene had the following pattern: degradation individually > degradation in the presence of 1,5–dimethylnaphthalene > degradation in the presence of fluorene > degradation in the ternary mixture.

Pure competitive inhibition for naphthalene was appropriately described by the multisubstrate model for all the binary and ternary mixtures since the biokinetic parameters generated by the competitive inhibition model were not statistically different from sole substrate estimates (APPENDIX B). The validation of the substrate interactions in binary and ternary mixtures by the competitive inhibition model is indicative of common enzyme systems involved in PAH degradation.

A close look at the fractional velocities for 1,5–dimethylnaphthalene (Table 7) reveals that degradation of 1,5–dimethylnaphthalene is affected by the presence of other

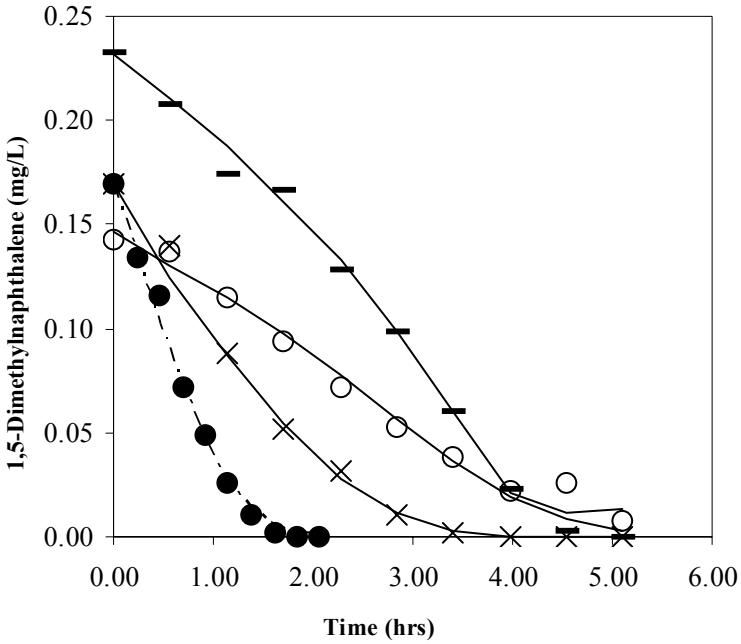


Fig. 6. 1,5-Dimethylnaphthalene Degradation in Sole Substrate, Binary and Ternary Systems. Closed symbols denote experimental observations for 1,5-dimethylnaphthalene in sole substrate system (●), open symbols denote 1,5-dimethylnaphthalene in mixture with fluorene (─), with naphthalene (×) and in a ternary mixture (○); solid lines generated by the multisubstrate competitive inhibition model. Dashed line is generated by the Monod model.

substrates. Initial rates of 1,5–dimethylnaphthalene degradation in the presence of fluorene were 21% of V_0, whereas in the presence of naphthalene, it is 39% of V_0. This suggests that competitive inhibition becomes pronounced under conditions when $K_{s1} << K_s$, $S_1 >> K_{s1}$ and $S_1 >> S$. The latter condition explains the significance of maintaining molar stoichoimetry between initial solute concentrations expressed in the form of moles/L.

The multisubstrate competitive inhibition model validated the substrate interaction for 1,5–dimethylnaphthalene in binary mixtures, suggesting that its biodegradation kinetics were governed by pure competitive inhibition (Fig. 6). The biokinetic estimates generated by the multisubstrate model for binary mixtures were not statistically different from the sole substrate estimates, further supporting the hypothesis that the sole substrate parameters are also representative of multisubstrate systems provided that their biodegradation kinetics is governed by pure competitive inhibition. The degradation of 1,5–dimethylnaphthalene in the ternary mixture was adequately simulated by the multisubstrate model, since the parameters generated by the model were not significantly different from sole substrate estimates.

Fluorene degradation in mixtures validated the observation that degradation of a single PAH is affected by the presence of a co-occurring PAH (Fig. 7). The substrate depletion curves for fluorene in binary mixtures with naphthalene and 1,5-dimethylnaphthalene almost overlapped with the sole substrate depletion curve, however they are statistically different from the individual PAH biodegradation. The sole substrate model did not validate the assumption that substrate interactions occur for

fluorene in the binary or ternary mixtures. Consequently, the biodegradation kinetics of the ternary system were not comparable to individual PAH degradation. Fluorene degradation was the slowest in the ternary system (V_i/V_0 = 0.71) as compared to its degradation in the binary and sole substrate systems. The multisubstrate competitive

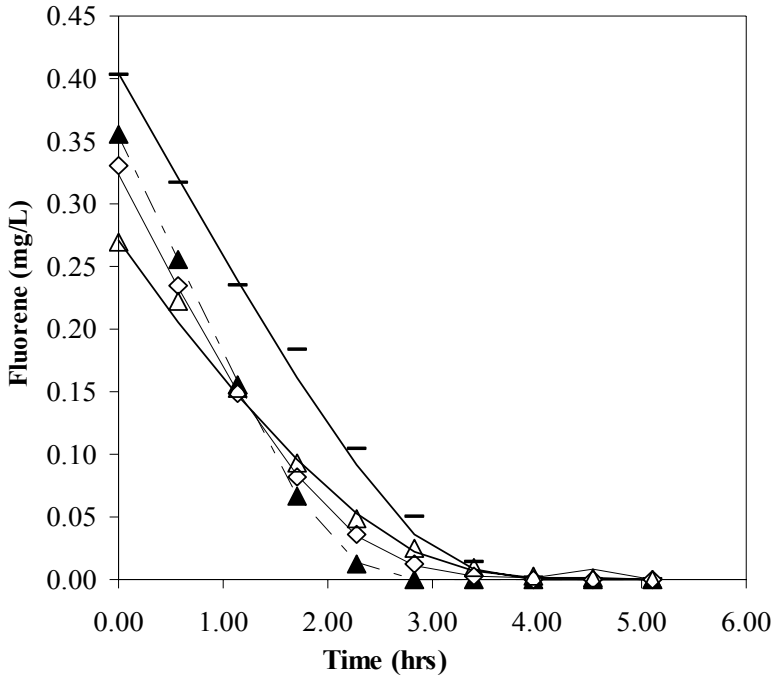


Fig. 7. Fluorene Degradation in Sole Substrate, Binary and Ternary Systems. Closed symbols denote experimental observations for fluorene in a sole substrate system (▲), open symbols denote experimental observations for fluorene in binary mixture with 1,5-dimethylnaphthalene (─), binary mixture with naphthalene (◇) and in a ternary mixture (Δ); solid lines generated by the multisubstrate competitive inhibition model. Dashed line is generated by the Monod model.

inhibition model predicted the biodegradation kinetics for fluorene in binary and ternary systems since it yielded kinetic parameters not statistically significant from individual parameter estimates.

An experiment with equitable concentrations of all three substrates was carried out to examine the effect of substrate concentrations on competitive inhibition (Fig. 8).

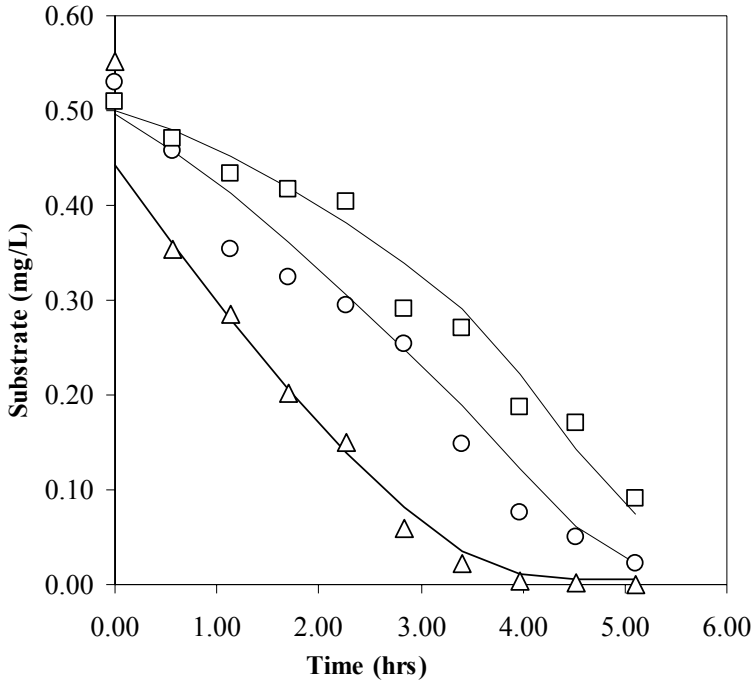


Fig. 8. Degradation of a Ternary System Consisting of Equitable Concentrations of Naphthalene (□), Fluorene (Δ), and 1,5-Dimethylnaphthalene (○). Open symbols denote experimental observations. Solid line represents degradation predicted by the multisubstrate competitive inhibition model.

As the concentrations of fluorene and 1,5-dimethylnaphthalene increased, competitive inhibition was dramatic for naphthalene ($V_i/V_0 = 0.130 \pm 0.002$) while percent inhibition for 1,5–dimethylnaphthalene was 71% ($V_i/V_0 = 0.28 \pm 0.004$). The shift in competitive inhibition was demonstrated by the term K_s ($S_1/K_{s1} + S_2/K_{s2}$), indicating that competitive inhibition is striking with the combined effects of $1/K_{s\,(15DMN)} << 1/K_{s\,(FLE)}$, $S_{(NAP)} >> K_{s\,(NAP)}$, $S_{(FLE)} >> K_{s\,(FLE)}$.

The multisubstrate model presents a simple approach towards modeling biodegradation kinetics in mixtures. The simplicity of the competitive inhibition model lies in its formulation which uses the parameters obtained from the sole substrate experiments. Thus, the parameters obtained from the sole substrate experiments are representative of the binary system as well.

The second set of multisubstrate biodegradation experiments consisted of a combination of 1–methylfluorene (1MFLE), naphthalene (NAP), 1,5–dimethylnaphthalene (15DMN) and anthracene (ANT). In addition to the previous sole substrate experiments, new experiments were performed for 1–methylfluorene and anthracene. A mixture containing 1MFLE, NAP, ANT and 15DMN was also tested in the same experiment. The sole substrate depletion curves were generated for 1–methylfluorene (Fig. 9A). The Monod model was fitted to the experimental data to yield the parameters $q_{max} = 0.105 \pm 0.022$ mg substrate/mg protein/hr and $K_s = 0.157 \pm 0.016$ mg/L. The lowest substrate affinity was found for 1-methylfluorene among the test compounds. Anthracene has a low aqueous solubility causing extensive partitioning to other surfaces, for example glassware. Consequently, it was difficult to obtain an initial

detectable concentration of anthracene in the aqueous phase since most of it was lost to the glassware. This effect was observed even for the mixture containing anthracene, in which anthracene was completely lost from the aqueous solution. The discussion and the observations are based on the premise that the resulting mixture represented a ternary system consisting of naphthalene, 1,5–dimethylnaphthalene and 1–methylfluorene. Substrate depletion curves are plotted for the ternary mixture of naphthalene, 1,5–dimethylnaphthalene and 1–methylfluorene (Fig. 9A and B).

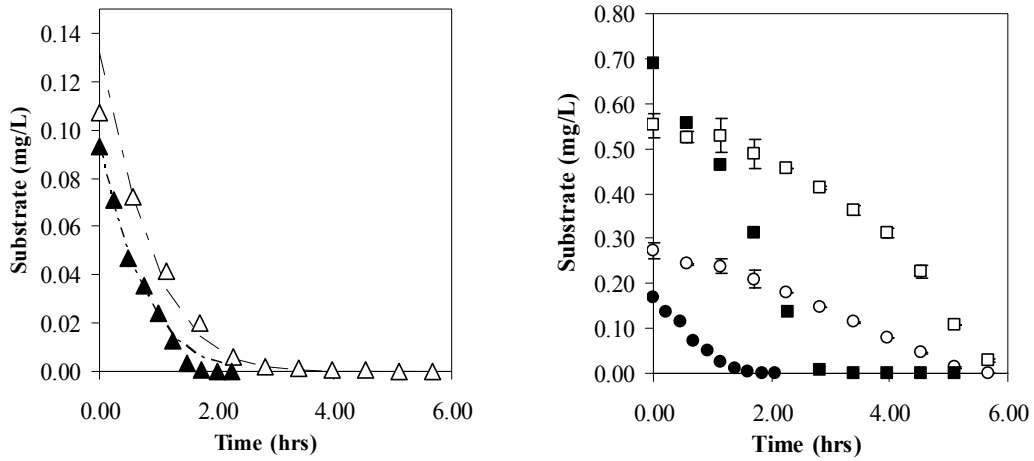


Fig. 9. Sole Substrate and Ternary System Degradation of 1-Methylfluorene (▲,Δ) (A), Naphthalene (■,□) and 1,5-Dimethylnaphthalene (●,○) (B). Open symbols denote experimental observations in the ternary system. Closed symbols denote experimental observations in the sole substrate system. Error bars represent one standard deviation from the mean. Dashed lines represent degradation predicted by the Monod model.

Standard deviation from mean of duplicate measurements was calculated only when biokinetic estimates could not be determined. Substrate interactions were evident in the ternary mixture. Reduced degradation rates of naphthalene and 1,5–dimethylnaphthalene compared to their degradation individually are indicative of antagonistic effects. The substrate depletion curves for naphthalene and 1,5-dimethylnapthalene individually and in a ternary system with 1-methylfluorene are plotted together to indicate the pronounced difference between the slopes of the two curves (Fig. 9B). Biokinetic parameters for naphthalene and 1,5- dimethylnaphthalene in the ternary system with 1-methylfluorene could not be evaluated since neither the multisubstrate model nor the Monod model predicted the biodegradation kinetics of the two compounds. However, 1– methylfluorene exhibited degradation comparable to its degradation individually (q_{max} = 0.082 mg substrate/mg protein/hr; K_s = 0.16mg/L). Consequently, 1–methylfluorene behaved as if it were the only compound present and its degradation was not affected by the presence of naphthalene and 1,5–dimethylnaphthalene. This behavior was validated by the sole substrate model which adequately captures the data of 1–methylfluorene in the ternary mixture (Fig. 9A). Preferential utilization of substrates was established in the ternary system, where degradation of naphthalene and 1,5–dimethylnaphthalene proceeded only after the complete removal of 1–methylfluorene. The multisubstrate model for competitive inhibition did not validate the substrate interactions for either naphthalene or 1,5–dimethylnaphthalene in the ternary system. The fact that competitive inhibition was not evident does not exclude the possibility that other interactions may be occurring. The

competitive inhibition model may not be valid for the following reasons: the model does not take into account sequential utilization of substrates, 1–methylfluorene may not be a competitive substrate for naphthalene or 1,5–dimethylnaphthalene indicating that 1-methylfluorene does not compete for the active site with the other substrates.

SUMMARY AND CONCLUSIONS

In all the binary mixtures, the presence of one PAH retarded the biodegradation kinetics of the co-occurring PAH. On the basis of the absence of substrate interactions in binary experiments where substrate concentrations were just below their solubility, Guha et al. (1999) concluded that substrate interactions may not be important in contaminated environments. However, this research indicated that competitive inhibition occurred in binary experiments where the concentrations of substrates are below their aqueous solubilities. Substrate interactions were not only active in the binary systems, but significantly evident in the ternary systems as well.

In the ternary mixture of fluorene, 1,5–dimethylnaphthalene, and naphthalene the presence of multiple substrates inhibited the degradation of the co-occurring PAHs (Table 7). The effect of multiple substrates on a single substrate was expressed in the form of a fractional velocity equation [Eq. (10)]. Decreased degradation rates in the presence of multiple substrates can be due to toxicity (Bouchez et al. 1995), formation of toxic metabolites and competitive inhibition (Strigfellow and Aitken 1995). Mixture biodegradation kinetics were governed by competitive inhibition demonstrated experimentally and mathematically by the multisubstrate competitive inhibition model (Figs.5, 6, 7 and 8). *S. paucimobilis* completely degraded all the components in binary and ternary mixtures; however the initial degradation rates of individual components decreased in the presence of competitive substrates (Table 7).

The extent of inhibition depended upon the number, affinities and the concentration of the co-occurring substrates. For example, naphthalene degradation in mixtures followed a pattern in the order of: degradation individually (V_0) > degradation in the presence of 1,5–dimethylnaphthalene ($0.7V_0$) > degradation in the presence of fluorene ($0.31V_0$) > degradation in ternary mixture ($0.24V_0$). Thus, competitive inhibition is a function of affinity of the alternate substrates (K_{s1}) and the relative magnitude of S_1/K_{s1} and becomes significant under conditions when $K_{s1} << K_s$, $S_1 >> K_{s1}$ and $S_1 >> S$. This is illustrated by the fact that the effect of fluorene on naphthalene degradation was greater (percent inhibition = 70%) than the effect of 1,5-dimethylnaphthalene (percent inhibition = 30%). Naphthalene and 1,5-dimethylnaphthalene did not produce dramatic effects on fluorene degradation, consequently the degradation of fluorene in binary and ternary mixtures was not comparable as indicated by the V_i/V_0 ratios. This is because fluorene had the greatest substrate affinity (K_s = 0.017mg/L) among the tested PAHs and was present in concentrations in the range of 0.27–0.36mg/L ($S >> K_s$).

Luning Prak and Pritchard (2002) found sequential degradation of equitable concentrations of substrates with *S. paucimobilis,* where the preference from small to larger molecules was in the order of phenanthrene > fluoranthene > pyrene. They attributed sequential degradation to differences in enzyme specificity (Luning Prak and Pritchard 2002). In this study, a case of preferential utilization of substrates could not be established since degradation of all the substrates proceeded concomitantly, although the initial degradation rates were significantly slower as compared to their removal

individually. A study with equitable concentrations of all the three compounds demonstrated dramatic effects of competitive inhibition for naphthalene indicating that at similar concentrations of substrates, inhibition shifted towards the substrate with the lowest affinity. The shift in competitive inhibition was demonstrated mathematically by the term $K_{s\{NAP\}} \times (S_{\{FLE\}}/K_{s\{FLE\}} + S_{\{15DMN\}}/K_{s\{15DMN\}})$. Competitive inhibition establishes that substrates compete for the same active site of an enzyme(s) revealing the presence of common enzyme systems; however, it does not disclose any information about the nature of enzymes the substrates are competing for. Another mechanism other than enzymatic transformation may be the rate determining step in biodegradation of PAHs. QSAR analysis revealed that the rate limiting step in biodegradation of PAHs is transmembrane transport which is related to binding and transport (Dimitriou–Christidis 2005). Thus, the possibility that competitive inhibition is related to membrane transport as a key step governing degradation would be consistent with the observed results.

The multisubstrate competitive inhibition model successfully estimated the biodegradation kinetics for binary and ternary mixtures of PAHs, suggesting that PAHs are competitive substrates for *S. paucimobilis*. The use of the multisubstrate model was demonstrated successfully by Guha et al. 1999 and Knightes 2000. The biokinetic parameters obtained from the sole substrate system were a representative of the binary and ternary systems as well.

Observations from Fig. 9 reveal that mechanisms other than competitive inhibition may govern the degradation of PAH mixtures. The competitive inhibition model did not validate interactions in the ternary mixture of 1–methylfluorene,

naphthalene and 1,5–dimethylnaphthalene. The results indicate that kinetics in PAH mixtures may not be necessarily governed by pure competitive inhibition and that there is a need to test and if required develop alternative models that may predict substrate interactions in mixtures where PAHs are preferentially degraded. Degradation of 1–methylfluorene in the ternary mixture was adequately predicted by the Monod model indicating that its behavior in the mixture was comparable to its degradation individually. Sequential utilization of substrates was observed where degradation of naphthalene and 1,5–dimethylnaphthalene proceeds faster after the complete removal of 1–methylfluorene. Preferential utilization of substrates was attributed to enzyme specificity and competition for the same active site (Luning Prak and Pritchard 2002). However, if 1–methylfluorene, naphthalene and 1,5–dimethylnaphthalene were to compete for the same active site, it is unlikely that 1–methylfluorene would be utilized preferentially since it exhibited the lowest substrate affinity among the tested compounds and it was present in concentrations in the range of its K_s. The results show that 1–methylfluorene is favored by the microorganism above naphthalene and 1,5–dimethylnaphthalene. As fluorene is replaced by 1–methylfluorene in the second ternary system, there is a dramatic effect on the degradation of naphthalene and 1,5–dimethylnaphthalene as compared to the effect of fluorene. It is possible that mechanisms other than competitive inhibition may be prevalent in the ternary system.

The binary and ternary experiments indicate that potential for substrate interactions exists for simple component systems where concentrations of PAHs are below aqueous solubilities. The complexity of interactions is related, to but not limited

to, the substrate affinities, concentrations of co-occurring substrates and number of components in the mixture. Biodegradation kinetics in PAH mixtures may be governed by pure competitive inhibition kinetics; however interactions other than competitive inhibition cannot be ignored in systems where the chemical and structural diversity is far more complex than these simple systems. Substrate interactions in mixtures require multisubstrate models that account for simultaneous degradation of substrates. However, simultaneous degradation may not always be the mechanism for degradation of mixtures as observed from the ternary mixture of naphthalene, 1,5-dimethylnaphthalene and 1-methylfluorene. Thus, models that account for sequential degradation of substrates may be useful in predicting biodegradation kinetics of mixtures where PAHs may not be competitive substrates. This is important because interactions between LMW PAHs and HMW PAHs can result in sequential degradation where preferential degradation of LMW PAHs can delay degradation of HMW PAHs (Molina et al. 1999). In addition, the Monod model may not be appropriate to predict multisubstrate biodegradation kinetics. Although the binary and ternary systems do not mimic the intricacy innate to complex mixtures, they indicate that interactions may become increasingly complex with the mixture composition.

NOTATIONS

The following symbols are used in this thesis:

C_i = concentration of substrate i;

C_j = concentration of substrate j;

i = percent inhibition

K_I = concentration of inhibitor;

K_s = substrate affinity constant; (mg/L)

K_{si} = affinity constant for substrate i;

K_{sj} = affinity constant for substrate j;

q = specific substrate utilization rate; (mg of substrate/ mg of biomass/h);

q_{max} = maximum substrate utilization rate; (mg of substrate/ mg of biomass/h)

S = substrate concentration; (mg/L)

V = catalytic rate;

V_0 = catalytic rate in the absence of any substrate

V_1 = catalytic rate in the presence of an alternate substrate;

V_{max} = maximum catalytic rate;

X = biomass concentration expressed as protein (mg/L);

μ = specific growth rate; (h^{-1})

μ_{max} = maximum specific growth rate; (h^{-1})

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APPENDIX A

Sole substrate depletion curves and biokinetic estimates for naphthalene (NAP), fluorene (FLE), 1,5-dimethylnapthalene (15DMN) and 1-methylfluorene in duplicate reactors.

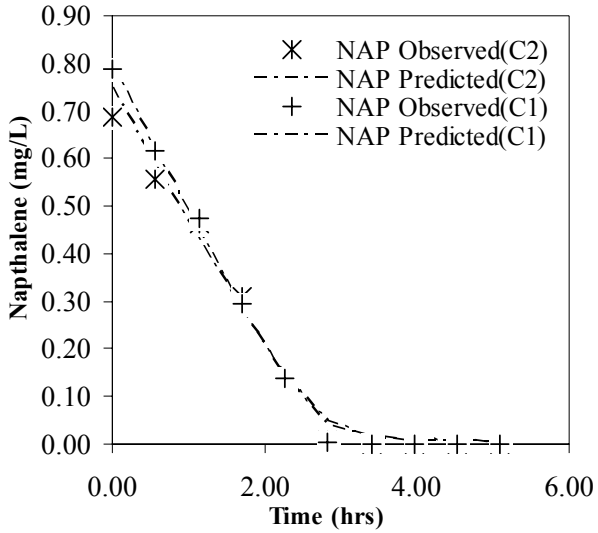

Fig.A.1. Degradation of naphthalene individually in duplicate reactors C1 and C2. Open symbols denote experimental observations and dashed lines represent the Monod model.

Table A.1. 95% Confidence intervals for sole substrate estimates of naphthalene in duplicate reactors

NAP	q_{max} (mg substrate/mg protein/hr)	K_s mg/L	$C_{t=0}$ mg/L
C1	0.097 ± 0.006	0.079 ± 0.007	0.790 ± 0.037
C2	0.100 ± 0.006	0.07 ± 0.008	0.75 ± 0.047

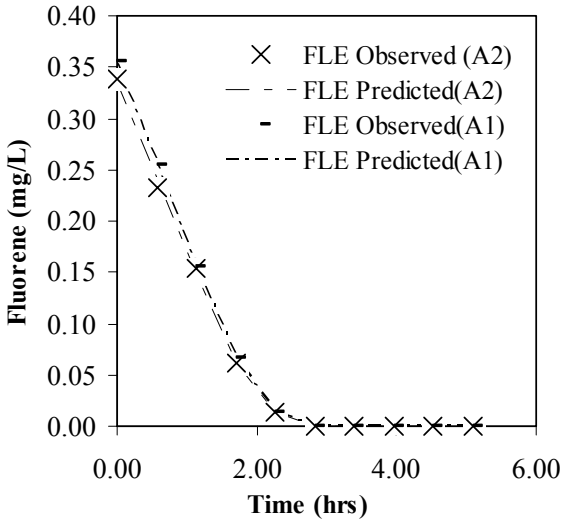

Fig.A.2. Degradation of fluorene individually in duplicate reactors A1 and A2. Open symbols denote experimental observations and dashed lines represent the Monod model.

Table A.2. 95% Confidence intervals for sole substrate estimates of naphthalene in duplicate reactors

FLE	q_{max} (mg substrate/mg protein/hr)	K_s mg/L	$C_{t=0}$ mg/L
A1	0.045 ± 0.001	0.017 ± 0.001	0.355 ± 0.001
A2	0.043 ± 0.003	0.016 ± 0.001	0.335 ± 0.020

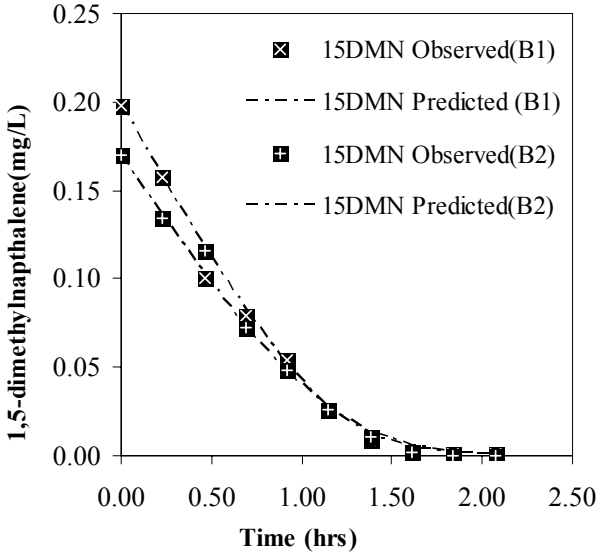

Fig.A.3. Degradation of 1,5-dimethylnaphthalene individually in duplicate reactors B1 and B2. Open symbols denote experimental observations and dashed lines represent the Monod model.

Table A.3. 95% Confidence intervals for sole substrate estimates of 1,5-dimethylnaphthalene in duplicate reactors.

15DMN	q_{max} (mg substrate/mg protein/hr)	K_s mg/L	$C_{t=0}$ mg/L
B1	0.055 ± 0.006	0.043 ± 0.004	0.198 ± 0.013
B2	0.048 ± 0.002	0.043 ± 0.001	0.17 ± 0.016

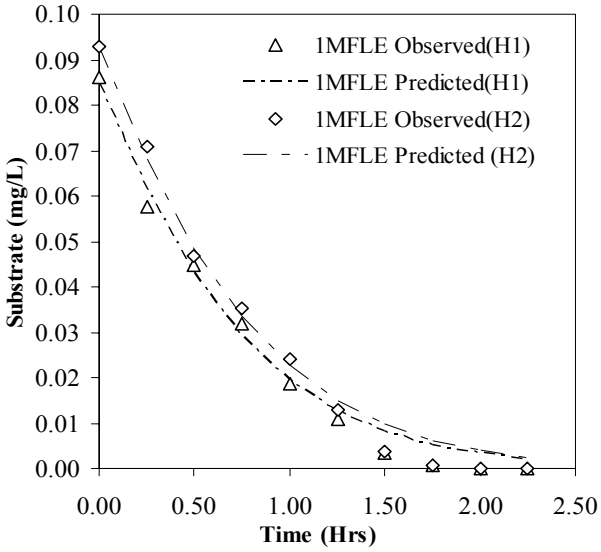

Fig.A.4. Degradation of 1-methylfluorene individually in duplicate reactors H1 and H2. Open symbols denote experimental observations and dashed lines represent the Monod model.

Table A.4. 95% Confidence intervals for sole substrate estimates of 1-methylfluorene in duplicate reactors.

1MFLE	q_{max} (mg substrate/mg protein/hr)	K_s mg/L	$C_{t=0}$ mg/L
H1	0.107 ± 0.030	0.155 ± 0.025	0.093 ± 0.002
H2	0.103 ± 0.035	0.161 ± 0.022	0.085 ± 0.003

APPENDIX B

Biokinetic estimates for naphthalene, fluorene and 1,5-dimethylnaphthalene in binary and ternary mixtures as generated by the multisubstrate competitive inhibition model.

Table B.1. Biokinetic estimates generated by the multisubstrate model for PAHs in binary and ternary mixtures

Mixture	NAP		FLE		15DMN	
Estimates	q_{max}[a] (mg substrate/mg protein/hr)	K_s[b] (mg/L)	q_{max} (mg substrate/mg protein/hr)	K_s (mg/L)	q_{max} (mg substrate/mg protein/hr)	K_s (mg/L)
FLE + NAP	0.077± 0.008	0.090 ± 0.008	0.045	0.013	-	-
FLE + 15DMN	-	-	0.035	0.014	0.045	0.046
NAP + 15DMN	0.083 ± 0.01	0.090 ± 0.007	-	-	0.055	0.039
Ternary Mixture	0.080 ± 0.015	0.1 ± 0.01	0.036	0.011	0.054	0.047

[a,b] Confidence intervals for biokinetic estimates were determined for PAHs only when estimates generated from the multisubstrate model did not lie in the confidence interval as determined for sole substrate estimates.

VITA

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