Multisubstrate Biodegradation Kinetics of Naphthalene, Phenanthrene, and Pyrene Mixtures

Saumyen Guha,* Catherine A. Peters, Peter R. Jaffé

Department of Civil and Environmental Engineering, Princeton University, Princeton, New Jersey 08544; telephone: (609) 258-5645; fax: (609) 258-2799; e-mail: cap@princeton.edu

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Abstract: Biodegradation kinetics of naphthalene, phenanthrene and pyrene were studied in sole-substrate systems, and in binary and ternary mixtures to examine substrate interactions. The experiments were conducted in aerobic batch aqueous systems inoculated with a mixed culture that had been isolated from soils contaminated with polycyclic aromatic hydrocarbons (PAHs). Monod kinetic parameters and yield coefficients for the individual compounds were estimated from substrate depletion and CO2 evolution rate data in sole-substrate experiments. In all three binary mixture experiments, biodegradation kinetics were comparable to the sole-substrate kinetics. In the ternary mixture, biodegradation of naphthalene was inhibited and the biodegradation rates of phenanthrene and pyrene were enhanced. A multisubstrate form of the Monod kinetic model was found to adequately predict substrate interactions in the binary and ternary mixtures using only the parameters derived from sole-substrate experiments. Numerical simulations of biomass growth kinetics explain the observed range of behaviors in PAH mixtures. In general, the biodegradation rates of the more degradable and abundant compounds are reduced due to competitive inhibition, but enhanced biodegradation of the more recalcitrant PAHs occurs due to simultaneous biomass growth on multiple substrates. In PAH-contaminated environments, substrate interactions may be very large due to additive effects from the large number of compounds present. © 1999 John Wiley & Sons, Inc. Biotechnol Bioeng 65: 491–499, 1999.

Keywords: PAH; polycyclic aromatic hydrocarbon; biodegradation kinetics; multisubstrate; Monod kinetics; naphthalene; phenanthrene; pyrene; competitive inhibition

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are important environmental contaminants because of their known or suspected carcinogenicity, and because of their extensive occurrence as environmental pollutants. Significant interest in the potential for bioremediation of PAH contaminated sites has resulted in considerable progress toward understanding biodegradation of PAHs and degradation pathways (Evans et al., 1965; Gibson and Subramanium, 1984; Bossert and Bartha, 1986; Mueller et al., 1989; Weissenfels et al., 1990; Bouwer et al., 1992; Cerniglia, 1992). Much of this research has focused on individual compounds, whereas at contaminated sites PAHs usually occur as mixtures of compounds (Luthy et al., 1994).

Biodegradation kinetics in PAH-contaminated environments are complicated by the possibility of substrate interactions. Few studies have investigated the kinetics of growth and substrate depletion in systems containing mixtures of PAHs. An early study on the impacts of PAHs on the rates of disappearance of other PAHs in sediments revealed that interactions may result from enzyme inductions that occur due to pre-exposure of a culture to a PAH compound (Bauer and Capone, 1988). The study by Keck et al. (1989) on PAH biodegradation in prepared mixtures and in complex wastes showed both positive and negative substrate interactions. The half-lives of PAHs in sole-substrate systems were shorter than those in PAH mixtures, indicating competitive inhibition. However, when the PAH mixtures were present in matrices containing other degradable hydrocarbons (refinery waste, creosote), cometabolic interactions overrode the competitive inhibition effects. A study that combined PAHs with polar creosote-related compounds (Millette et al., 1995), showed that phenanthrene biodegradation may be inhibited by the presence of more soluble and degradable compounds. In a study using six different bacterial strains and mixtures of six PAHs, Bouchez et al. (1995) observed a variety of substrate interaction effects depending on the choice of bacterial strain and the PAH mixture. Kelly and Cerniglia (1995) observed that the mineralization rates of individual PAHs were reduced by the presence of other PAH compounds. Furthermore, they inferred that the initial oxidation step of PAH biodegradation is not compound-specific, as degradation was observed for a range of different PAHs. Another recent study (Stringfellow and Aitken, 1995), reported competitive inhibition of...
phenanthrene uptake by naphthalene, methylnaphthalene, and fluorene, providing compelling evidence of common enzyme systems for biodegradation of a number of PAHs. Shuttleworth and Cerniglia (1996) also observed inhibition of phenanthrene degradation in the presence of naphthalene for three different microbial strains.

This brief overview of the literature reveals that substrate interactions for PAH mixtures have been observed and there is no simple rule for predicting these interactions. Hence, simple models that do not account for the combined effects of inhibition due to the presence of preferred substrates and enhanced degradation due to the presence of multiple growth substrates may not be suitable for predicting biodegradation rates of PAHs in contaminated environments. The objective of this study was to examine substrate interactions in simple PAH mixtures, and to evaluate the potential of a multisubstrate Monod kinetic model to describe the biodegradation kinetics. This paper presents results from biodegradation experiments with naphthalene, phenanthrene and pyrene as sole substrates, and as binary and ternary mixtures. The experiments were conducted using aerobic aqueous systems and a mixed culture under conditions approaching intrinsic kinetics for the compounds studied. Monod kinetic parameters and yield coefficients for individual compounds were estimated from experimental observations of substrate depletion and carbon dioxide production rates in sole-substrate systems. These parameters were used in the multisubstrate Monod kinetic model to predict the biodegradation rates of the PAHs in binary and ternary mixtures. The experimental observations from the multisubstrate experiments are presented and compared with the model predictions to examine the validity of this modeling approach. Finally, the substrate interaction findings are explained using simulations of biomass growth kinetics in multisubstrate systems.

THEORY OF MULTISUBSTRATE MONOD KINETIC MODELING

Substrate interactions may result from the dual effects of (i) competitive metabolism in which one substrate inhibits the utilization of another because of competition for the active binding site of an enzyme and (ii) the fortuitous growth of biomass due to the presence of multiple substrates. The first will negatively impact a substrate’s biodegradation rate and the second will enhance it. The multisubstrate Monod kinetic model has the capability to capture both these interaction effects.

In a multisubstrate system in which all the substrates can serve as primary substrates for growth, biomass growth is due to the utilization of all the compounds:

\[ \mu_T = \sum_{i=1}^{n} \mu_i, \]  

(1)

where \( \mu_T \) is the total specific growth rate and \( \mu_i \) is the specific growth rate on substrate \( i \), and the summation is taken over the \( n \) substrates. The specific growth rate, \( \mu_i \), is related to the concentrations of the substrates through the multisubstrate Monod growth relationship:

\[ \mu_i = \frac{\mu_{\text{max},i} C_i}{K_{Si} + \sum_{j=1}^{n} K_{Sj} C_j}, \]  

(2)

where \( \mu_{\text{max},i} \) is the maximum specific growth rate from substrate \( i \), \( C_i \) is the concentration of substrate \( i \), \( K_{Si} \) is the half-saturation constant for substrate \( i \), and \( K_{Sj} \) is the half-saturation constant for each substrate \( j \). Eq. (2) assumes that all the components in the mixture share a common rate-limiting enzyme reaction pathway. This relationship is analogous to the theoretical multisubstrate enzyme kinetic expressions derived by Segal (1975) and presented by Costa and Malcata (1994), Yoon et al. (1977), Machado and Grady (1989), Chang et al. (1993), and Stringfellow and Aitken (1995). Eq. (2) is different from the substrate interaction models discussed by others, in which the summation in the denominator is replaced with \( C_i + K_i \), where \( K_i \) is an empirical interaction parameter (Klecka and Maier, 1988; Oh et al., 1994). The relationship in Eq. (2) is fully predictive in the sense that the parameters can be determined from independent measurement in sole-substrate systems.

The use of this model as a predictive tool implies an assumption that the microbial community in the multisubstrate system is comparable to that in the sole-substrate systems with respect to physiological state. This assumption is valid, even in a mixed culture system, provided that all the substrates are utilized by a common enzyme system. For PAHs, this is a fairly good assumption in light of the findings of Kelly and Cerniglia (1995) and Stringfellow and Aitken (1995) discussed above. Hence, despite the complexities present in a mixed culture system, this relatively simple predictive model may be useful to describe biodegradation kinetics of PAH mixtures in the field or in engineered systems where mixed cultures exist.

The mathematical implication of the summation term in the denominator of Eq. (2) is that \( \mu_i \) in the multisubstrate case is less than the specific growth rate that would be predicted by sole-substrate Monod kinetics. However, since the total biomass growth rate in the multisubstrate case is larger than what would occur if there was only one substrate (Eq. (1)), the actual substrate depletion rate can be enhanced. These two competing effects are captured in the substrate depletion rate equation:

\[ \frac{dC_i}{dt} = -\frac{\mu_i X}{Y_i}, \]  

(3)

where \( X \) is the biomass concentration and \( Y_i \) is the biomass yield coefficient from growth on substrate \( i \). The \( n \) different substrate depletion relations (Eq. (3)) combined with an expression for biomass growth rate

\[ \frac{dX}{dt} = \mu_T X, \]  

(4)
represent a system of differential equations coupled through the dependence of each μi on the concentrations of all the substrates. Depending on which substrates are present, their initial concentrations, the initial biomass concentration, and their respective affinity constants and maximum specific growth rates, the biodegradation rates for the individual compounds in a mixture may be enhanced or reduced relative to the comparable sole-substrate case. These effects are discussed in detail in the section “Biomass Growth Simulations.”

MATERIALS AND METHODS

Chemicals and Chemical Analysis

The PAHs used in this study were naphthalene, phenanthrene, and pyrene. The sole-substrate experiments were performed with 14C-labeled compounds, purchased from Sigma Chemical Company. Radiolabeled naphthalene was uniformly labeled. Radiolabeled phenanthrene was labeled at the 9th carbon. In the radiolabeled pyrene, the 4th, 5th, 9th, and 10th carbons were labeled. The multisubstrate experiments were conducted with nonradiolabeled compounds, purchased from Aldrich Chemical Company. The purity of the nonlabeled compounds were 99+, 98%, and 99% for naphthalene, phenanthrene, and pyrene, respectively. The aqueous solubility values at 20°C, reported in the literature are, 31, 1.4, and 0.14 mg/L for naphthalene, phenanthrene, and pyrene, respectively (Mackay et al., 1992).

Radiolabeled compounds were analyzed using a Packard Tri-carb 1900TR liquid scintillation counter. Disintegrations per minute (DPM) data were converted to concentrations of solute, expressed as equivalent carbon, from a standard curve prepared from the stock solution of known solute concentration and radioactivity.

A Hewlett Packard high-pressure liquid chromatograph (HPLC) with UV and fluorescence detection was used to quantify the nonradiolabeled PAH compounds. The column was HP Spherisorb ODS 2, 5 mm, 125 × 4 mm. The operating conditions were flow rate of 1.5 mL/min, temperature of 30°C, injection volume of 10 mL, mobile phase of acetonitrile and water, a uniform gradient from 40:60 (acetonitrile/water) to 80:20 during the run time of 10 min. Naphthalene was detected with the diode array UV detector at 220 nm for the concentration range of 0.75–31 mg/L. Fluorescence detection (ex. 218 nm and em. 357 nm) was used for concentrations below 0.75 mg/L. The detection limit for naphthalene was 0.01 mg/L. Phenanthrene was detected using the fluorescence detector at ex. 248 nm and em. 395 nm, with a detection limit of 0.002 mg/L. Pyrene was detected using the fluorescence detector at ex. 230 nm and em. 387 nm, with a detection limit of 0.001 mg/L.

Biomass and Biomass Analysis

An enrichment culture was isolated from a contaminated soil sample collected from the Fire Training Area 02 (FTA-02) of the Wurtsmith Air Force Base, 13–15 ft below land surface. An analysis of the soil (Clayton Environmental, 1995) showed that it was contaminated with 24 mg/kg of naphthalene, 1.3 mg/kg of phenanthrene, as well as traces of other PAHs. The enrichment culture was maintained as a stock culture by periodically replenishing with fresh nutrient solution (BOD dilution water made from the buffer pillows obtained from HACH Company) and a mixture of naphthalene, phenanthrene, and pyrene dissolved in a small amount of methanol. Prior to each biodegradation experiment, a sample from the stock culture was washed 5 times with ~1000 mg/L of Brij 35 surfactant followed by 3 washes with BOD dilution water until the concentration of Brij 35 was reduced below detection. The sample was then maintained in the absence of a growth substrate for 24 h prior to inoculation of the experiment vessel. A sample of the inoculum was extracted with octanol and the extract analyzed to quantify any carryover solute. Often a very small amount (0.001–0.005 mg/L) of pyrene was detected and the initial concentration estimate of the solute in the experiment system was corrected accordingly.

Quantification of biomass was carried out by two independent procedures. Bio-Rad protein assay was used to measure biomass as total protein (Bio-Rad Laboratories, Richmond, CA). The cell protein in the sample was solubilized by putting the sample vial into an ultrasound bath for 12 h followed by steam bath at 120°C for one half hour. The samples were then analyzed using the Bio-Rad assay procedure which is based on the Bradford method, with bovine γ globulin as the protein standard. All the samples were analyzed in duplicate and at two different dilutions. Biomass was also quantified as Total Organic Carbon (TOC), in a Coulometrics Inc., TOC-TC analyzer. Liquid samples were oxidized at 900°C in a pure oxygen environment in the presence of barium chromate and reduced silver catalysts. The carbon dioxide produced was measured in a CO2 Coulometer (ASTM D4129-82). The TOC of the biomass was computed from the difference between the bulk sample and the filtered (through 0.2-μm inorganic filters) sample. Five replicates of each sample were analyzed.

Biodegradation Experiments

Sole-substrate biodegradation experiments were conducted in 25-mL reaction vessels that contained in their headspace a brass cup containing KOH solution to trap evolved CO2. The headspace was sufficient to provide oxygen to the aqueous culture which was stirred to facilitate mass transfer to the gas phase. The oxygen concentration was measured before and after the experiment to verify aerobic conditions existed. Biomass concentrations were measured only at the beginning of the experiment for a determination of initial conditions. The solutions from the reaction vessel and the KOH cup were analyzed at the beginning and at certain time intervals to monitor substrate concentration and carbon dioxide production. A total of approximately 120 experimental observations covering two different initial biomass con-
centrations and a full range of solute concentrations (near zero to near aqueous solubility) were obtained for each compound. Independent abiotic batch experiments were conducted to estimate the partitioning onto the apparatus, partitioning onto the biomass, volatilization kinetics and gas/liquid phase equilibrium. The endogenous decay coefficient, $b$, was measured in an experiment in which biomass was aerated in the absence of a growth substrate and biomass concentration was measured with time. The details of sole-substrate experimental procedures have been previously reported (Guha and Jaffe, 1996).

The biodegradation experiments with binary and ternary mixtures of PAHs were performed in 30 mL crimp-sealed serum vials with Teflon-lined caps. Because of the use of nonlabeled substrates, no carbon dioxide data were obtained. The vials were sacrificed periodically and duplicate samples were withdrawn using a syringe and expressed through a presaturated 0.2-µm inorganic syringe filter, Anotop 10 from Whatman. The samples were analyzed using HPLC. Abiotic control experiments were performed in parallel to estimate the volatilization kinetics and equilibrium partitioning. The initial concentrations of solutes and biomass used for the multicomponent experiments were similar to those used for the sole-substrate experiments and are listed in Table I. The aim was to use high initial substrate concentrations so as to approach intrinsic kinetic conditions (Grady et al., 1996), while being below the aqueous solubilities of the compounds.

**PARAMETER ESTIMATION AND MODELING**

**Estimation of Monod Kinetic Parameters and Yield Coefficients**

The mass-balance differential equations for substrate and biomass concentrations for the sole-substrate experimental system have been previously presented (Guha and Jaffe, 1996). Partitioning of the compounds onto the apparatus and biomass were modeled as equilibrium partition isotherms. Liquid to gas phase partitioning was modeled as a dynamic process. Biodegradation kinetics were modeled by the sole-substrate Monod kinetic formulation,

$$
\mu_i = \frac{\mu_{max}C_i}{K_S + C_i},
$$

(5)

All the parameters describing physical/chemical processes were determined through independent experiments, leaving only the Monod biokinetic parameters for empirical fitting to the sole-substrate experimental data.

Parameter estimation was accomplished by combining the differential mass balance equations with a nonlinear parameter estimation algorithm. For each PAH, the data set consists of experimental observations of substrate concentration ($C$) and carbon dioxide concentration ($CO_2$) with time. For each data point, the residuals are defined as

$$
e_c = \hat{C} - C \quad \text{and} \quad e_{CO_2} = \hat{CO}_2 - CO_2
$$

(6)

where the $\hat{}$ denotes the predicted values. A maximum likelihood estimation method was used to estimate the Monod kinetic parameters $\mu_{max}$, $K_S$, and yield coefficient $Y$. The objective function subject to minimization was (Bard, 1974)

$$
f(\theta) = \frac{n}{2} \log[\det \mathbf{M}(\theta)],
$$

(7)

where $n$ in Eq. (7) is the number of observations in the data set, $\theta$ is the parameter matrix, and $\mathbf{M}(\theta)$ is the moment matrix of the residuals given by

$$
\mathbf{M}(\theta) = \begin{bmatrix}
\sum e_c^2 & \sum e_c e_{CO_2} \\
\sum e_c e_{CO_2} & \sum e_{CO_2}^2
\end{bmatrix}
$$

(8)

Minimization was accomplished using Broyden–Fletcher–Goldfrab–Shanno algorithm. The optimum parameter values were found to be robust as determined from repeated minimization runs using different initial guesses for the parameters.

**Multisubstrate Biodegradation Modeling**

By analogy with the mathematical modeling of the sole-substrate biodegradation experiments, the mass-balance equations describing the behavior of substrates and of biomass in the multisubstrate biodegradation experiments are developed. The first equation is the differential equation for the concentration of substrate $i$ in the liquid phase, $C_i$,

$$
\frac{dC_i}{dt} = \frac{1}{Y_i} \mu_i X - k_{wg,i} \left( C_i - \frac{C_{g,i}}{K_{H,i}} \right)
$$

(9)

where the symbols that have not been previously defined are

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Initial substrate concentration [mg/L]</th>
<th>Initial biomass concentration as protein [mg/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene (1) and Phenanthrene (2)</td>
<td>16.05 0.81</td>
<td>— 1.2</td>
</tr>
<tr>
<td>Naphthalene (1) and Pyrene (3)</td>
<td>18.73</td>
<td>0.83 0.075</td>
</tr>
<tr>
<td>Phenanthrene (2) and Pyrene (3)</td>
<td>21.0</td>
<td>0.11 0.05</td>
</tr>
</tbody>
</table>
as follows: $K_{dg,i}$ is the equilibrium partition coefficient for sorption onto the apparatus [dimensionless], $K_{bms,i}$ is the equilibrium partition coefficient for sorption onto biomass [L/mg], $K_{H,i}*$ is the equilibrium partition coefficient for partitioning to the headspace [dimensionless], $k_{wg,i}$ is the mass transfer rate coefficient between the water phase and head space [h$^{-1}$], and $C_{g,i}$ is the concentration of $i$ in the gas phase [mg/L]. Because volatilization to the headspace is modeled kinetically, a second mass balance equation is needed for the concentration of substrate in the gas phase:

$$\frac{dC_{g,i}}{dt} = k_{wg,i} f_w \left( C_i - \frac{C_{g,i}}{K_{H,i}^*} \right),$$

(10)

where $f_w$ and $f_g$ are the fractional volumes of water and head space, respectively [dimensionless]. Finally, a mass balance equation is written for biomass in the liquid phase:

$$\frac{dX}{dt} = \sum_{i=1}^{n} \mu_i X - bX,$$

(11)

where $b$ is the first-order endogenous decay coefficient [h$^{-1}$]. The multisubstrate Monod kinetic growth relationship in Eq. (2) is used for $\mu_i$ in Eq. (9) and (11) using the Monod parameters and yield coefficients estimated from the sole-substrate experimental data.

The abiotic parameters were estimated from independent batch experiments, for which the methods have been previously described (Guha and Jaffé, 1996). The parameters $K_{dg,v}$, $k_{wg,i}$ and $K_{H}*$ were estimated in multisubstrate systems. The partition parameter for the biomass ($K_{bms,i}$) was estimated for each compound in the sole-substrate systems, and it was assumed to remain the same for the multisubstrate mixture. The endogenous decay coefficient, $b$, was assumed to be the same for all the biodegradation experiments. The measured values of this parameter and the abiotic parameters are listed in Table II. The set of coupled nonlinear ordinary differential equations (Eqs. (9–11)) was solved numerically using a Picard iteration scheme with Euler backward time stepping. The initial concentrations of substrates and biomass were set to experimentally measured values (Table I).

**RESULTS AND DISCUSSION**

**Monod and Abiotic Parameters for Individual Substrates**

Table III summarizes the Monod biokinetic parameter values that were obtained from the sole-substrate experiments. Because the experiments were necessarily conducted at concentrations that are quite low, there is the concern that $\mu_{max,i}$ cannot be independently determined (Grady et al., 1996). Examination of the error surface over $\mu_{max}$ and $K_s$ revealed that the estimated parameters are somewhat correlated. However, the objective function (Eq. (7)) was indeed found to have a true minimum at the parameter values reported in Table III. We conclude that the experimental data did contain sufficient information about both parameters but that their error structures are correlated. Because of this correlation, meaningful confidence intervals for the parameters cannot be estimated. However, one can consider their precision to be within a factor of 2.

The values of $K_{S,i}$ are roughly equivalent to the aqueous solubilities of the individual PAHs. The values of $\mu_{max,i}$ decrease by roughly an order of magnitude for each additional aromatic ring. This implies that at high concentrations in sole-substrate systems naphthalene would degrade more quickly than phenanthrene, which would degrade more quickly than pyrene. At solute concentrations well below $K_{S,i}$, first-order kinetics will dominate. The values of $\mu_{max,i}$/$K_{S,i}$ are quite comparable indicating that at low concentrations the biodegradation rates of these compounds are not very different from each other. Note that these inferences regarding biodegradation rates represent inherent biodegradability in liquid cultures. The experiments were carefully designed to account for physical/chemical processes governing bioavailability, and as such the biokinetic parameters are independent of these processes.

**Multisubstrate Biodegradation: Binary Systems**

The symbols in Figs. 1–3 show the experimental observations of substrate concentrations over time from the multisubstrate experiments involving binary mixtures. The error bars are the pooled standard deviations using the replicate measurements for each vial sacrificed at a particular time point. For some of the observations the error bars are smaller than the symbols. Also shown in these figures are the predicted substrate depletion curves according to the multisubstrate model (solid curves). For comparison, the

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**Table II.** Abiotic and endogenous decay parameter values for the multisubstrate biodegradation experiments.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Naphthalene</th>
<th>Phenanthrene</th>
<th>Pyrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{dg}$</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$K_{s,av}$ [L/mg]</td>
<td>–</td>
<td>0.0914</td>
<td>0.191</td>
</tr>
<tr>
<td>$k_{wg}$ [h$^{-1}$]</td>
<td>0.002</td>
<td>0.01</td>
<td>0.015</td>
</tr>
<tr>
<td>$K_{H}*$</td>
<td>0.5</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>$f_w$</td>
<td>0.57</td>
<td>0.57</td>
<td>0.57</td>
</tr>
<tr>
<td>$f_g$</td>
<td>0.43</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>$b$ [h$^{-1}$]</td>
<td>0.0039</td>
<td>0.0039</td>
<td>0.0039</td>
</tr>
</tbody>
</table>

– experiments did not show any significant positive values.

**Table III.** Monod kinetic parameters and the yield coefficient values obtained from the sole-substrate biodegradation experiments.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Naphthalene</th>
<th>Phenanthrene</th>
<th>Pyrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_{max}$ [h$^{-1}$]</td>
<td>0.23</td>
<td>0.037</td>
<td>0.8 x 10$^{-3}$</td>
</tr>
<tr>
<td>$K_{s}$ [mg/L]</td>
<td>23.75</td>
<td>0.8</td>
<td>0.11</td>
</tr>
<tr>
<td>$Y$</td>
<td>0.485</td>
<td>0.497</td>
<td>0.502</td>
</tr>
</tbody>
</table>
dashed curves show the solutions of the same equations with the initial concentrations of the other substrates set to zero. That is, the dashed curves indicate how this substrate would behave if it was the only substrate present, according to the biokinetic parameters determined from the sole-substrate experiments.

In each of the binary systems in which naphthalene was present, the presence of the other solute had no significant effect on the biodegradation rate of naphthalene (see Figs. 1a and 2a). Similarly, phenanthrene behaves essentially the same whether it is present with either naphthalene or pyrene or whether it is the sole substrate (Figs. 1b and 3a), and pyrene behaves essentially the same whether it is present with either naphthalene or phenanthrene or whether it is the sole substrate (Figs. 2b and 3b). The lack of substrate interaction for binary mixtures is not only experimentally observed, but it is also what is predicted by the multisubstrate Monod kinetic model as is evidenced by the similarity between the solid and dashed curves. Given that the substrate concentrations in these experiments are the highest possible (just below aqueous solubility), if substrate interactions were operative they probably would have been evident. These findings suggest that substrate interactions in binary PAH mixtures are not likely to be significant in contamination scenarios in the environment under aerobic conditions.

**Multisubstrate Biodegradation: Ternary Systems**

The experimental results from the multisubstrate experiment involving the mixture of all three PAHs are shown as symbols in Fig. 4. Again, the solid curves are the predicted substrate depletion curves according to the multisubstrate Monod kinetic model, and the dashed curves show the predicted substrate depletion if the solute was present as a sole substrate. Unlike in the binary systems, in the ternary system there is a significant difference between the observed substrate depletion rates (symbols) and the sole-substrate depletion rates (dashed curves) for each substrate. Naphthalene biodegradation is slower in the ternary mixture than in a sole-substrate system. Phenanthrene biodegradation, al-
though a little slower at the beginning of the experiment, has increased significantly in the later period. Pyrene biodegradation is faster in the ternary mixture over the entire time period.

We conclude that the potential for substrate interactions in PAH mixtures does exist, and at least three compounds must be present for these interactions to be evident. Furthermore, these interactions may be negative as in the inhibition effect for naphthalene, positive as in the enhancement effect for pyrene, or a mixture of inhibition and enhancement as for the intermediate compound, phenanthrene. Note that in Fig. 4c (pyrene) there is a slight biphasic shape to the curve predicted by the multisubstrate model. At approximately 40 h there is a transition from one first-order decay regime to another with a stronger decay rate. This is due to the depletion of the other substrates, and is mathematically captured by the $K_{si}C/K_{sj}$ terms in the denominator of Eq. (2). Explanations of these types of effects are discussed in the next section.

For all three compounds, the multisubstrate Monod kinetic model predictions (solid lines) match the experimental observations fairly well. This is especially true for naphthalene. For phenanthrene, the substrate depletion curve has changed from an exponential decay type of curve in the sole-substrate system to an S-shaped curve in the ternary system. The multisubstrate model predictions capture this well. For pyrene, there is considerable uncertainty in the concentration measurements as is indicated by the size of the error bars, as well as greater variability across experimental vessels as is indicated by the lack of smoothness in the observation points. Neither model adequately captures the data in the first 30 h of the experiment. However, the faster depletion of pyrene in the ternary system as compared to the sole-substrate system is evident, and this general trend is well predicted by the multisubstrate model especially at...
longer time periods. This result is remarkably promising given that the model is predictive, i.e., based on independently determined parameters from abiotic experiments and sole-substrate biodegradation parameters.

**Biomass Growth Simulations**

The biodegradation rate of a compound is proportional to both the specific growth rate on that compound and the biomass concentration in the system (Eq. (3)). The presence of other substrates reduces the value of the specific growth rate for a given compound, \( i \) (Eq. (2)), and the extent of this reduction depends on the relative magnitudes of the concentrations of the other substrates and the ratios of \( K_{S_i} \) to the \( K_{S_j} \) values of the other substrates. The effect of retardation of the specific growth rate can be compensated and sometimes reversed if the biomass present is substantially greater in the multisubstrate system. Through numerical simulation we examine this process and how it is directly coupled with the substrate behavior shown in Figs. 1–4.

Simulations of biomass concentration as a function of time, \( X(t) \), were generated using the estimated model parameters and the initial conditions in the experimental systems. Simulation results rather than experimental results are presented because the changes in biomass with time are too small to have been precisely detected in the experimental systems. Figures 5a–d illustrate the predicted biomass growth kinetics in the four multisubstrate systems studied (solid lines). For comparison, the predicted biomass growth kinetics when only one substrate is present with the same initial concentration as in the mixture are also shown (dashed lines). Whenever naphthalene is present, considerably more biomass is in the system than what would be present with phenanthrene or pyrene alone. So, while multisubstrate Monod kinetics dictates that there is a retardation effect for every compound in the mixture, this may be offset by significant growth on the more degradable and abundant substrate (in this case, naphthalene) resulting in enhanced degradation of the less degradable compounds. In the binary experiments, these effects were not significant enough to be observed.

In the ternary system (Fig. 5d), the biomass growth rate in the mixture is somewhat slower than that predicted by naphthalene degradation alone, but the system ultimately produces more biomass than could be generated by any one substrate. The effect on naphthalene was a net inhibition of the biodegradation rate (Fig. 4a), explained by the retardation of the specific growth rate and the resulting delay in biomass growth. Initially, phenanthrene also experienced an inhibition effect (Fig. 4b), but after substantial biomass growth both phenanthrene and pyrene degraded faster than they would have if present as sole substrates. In general, because of the additive nature of the retardation term in the denominator of Eq. (2) and of the total specific growth rate

![Figure 5](image_url)

**Figure 5.** Simulated biomass growth in the four multisubstrate experiments: (a,b,c) Binary systems and (d) Ternary system. Solid curves represent multisubstrate systems; dashed curves represent sole-substrate systems. The initial substrate and biomass concentrations are the same as the four experimental conditions.
(Eq. (1)), the more compounds that are present the greater will be the substrate interaction effects, whether positive or negative.

**CONCLUSIONS**

This study demonstrates the potential for significant substrate interactions in multisubstrate systems involving PAHs. While competitive inhibition may cause retardation of the biodegradation rate of one compound, enhanced biomass growth may increase the biodegradation rate of another compound. In general, in a mixture of PAHs the rates of the more degradable compounds will be inhibited and the rates of the more recalcitrant compounds will be enhanced. The extent of these effects is directly related to the number of substrates that are present. So, while this study was limited in scope to very simple mixtures, substrate interactions were observed and were found to be larger in the ternary system compared to the binary systems. Substrate interactions are likely to be greater in environmental contamination scenarios where PAHs are often encountered in very complex mixtures. This suggests that caution should be employed in universal application of simple first order rate parameters for individual PAH compounds in the field.

The observed substrate interaction effects for PAHs are well described by the multisubstrate Monod kinetic model, indicating the importance of coupled substrate and biomass mass balance equations. So, despite the seeming complexity of PAH biodegradation kinetics in mixed substrate systems, the predictive capability of this model has the potential to greatly simplify this problem because of the reliance on sole-substrate parameters. The validity of this model relies on the contention that naphthalene, phenanthrene, and pyrene are utilized simultaneously by a common enzyme system in the mixed culture. This assumption must be tested before inferences are drawn about underlying mechanisms. While the predictive capability of this model will need to be verified for more complex mixtures, it holds great promise for simplifying the mathematical representation of complex biodegradation kinetics in the field.

**References**


