

MULTISUBSTRATE BIODEGRADATION KINETICS FOR BINARY AND COMPLEX MIXTURES OF POLYCYCLIC AROMATIC HYDROCARBONS

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Abstract—Biodegradation kinetics were studied for binary and complex mixtures of nine polycyclic aromatic hydrocarbons (PAHs): Naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, 2-ethylnaphthalene, phenanthrene, anthracene, pyrene, fluorene, and fluoranthene. Discrepancies between the observed biodegradation rates and those predicted by a sole-substrate model indicate that significant substrate interactions occurred in both the binary and complex-mixture experiments. For all compounds except naphthalene, biodegradation was enhanced. The observations were compared to predictions from two multisubstrate biodegradation kinetic models: One that accounts for competitive inhibition, and one that does not. Both models are fully predictive in that parameters had been determined from an independent set of sole-substrate experiments. In the binary experiments, the major multisubstrate effect was biomass enhancement as a result of growth on naphthalene. Substrate interactions were orders of magnitude larger for most compounds in the complex mixtures, but significant competitive inhibition effects counteracted some of the biomass enhancement effect. The present study has demonstrated that the sole-substrate model is inadequate to describe multisubstrate biodegradation kinetics for a broad range of PAH mixtures. Whereas the multisubstrate model without inhibition did an adequate job of predicting the observed effects in some cases, we advocate the use of the multisubstrate model with inhibition for similar modeling efforts in light of the evidence that the model was correct more often than not. Theory supports its use because of the common enzyme pathways for biodegradation of PAHs.

Keywords—Polycyclic aromatic hydrocarbons Biodegradation Kinetics Mixtures Competitive inhibition

INTRODUCTION

Many contaminants exist in the environment as chemical mixtures. For example, polycyclic aromatic hydrocarbons (PAHs) typically coexist in very complex mixtures, such as coal tar, creosote, and diesel fuel [1]. Such mixtures can occur as distinct organic phases, often called nonaqueous-phase liquids, or as dissolved or colloid-associated solutes in groundwater and sorbed species associated with soils and sediments. Many PAHs are suspected to be carcinogenic, and 16 PAHs are listed by the U.S. Environmental Protection Agency as priority pollutants [2]. Significant interest exists regarding bioremediation of PAH-contaminated sites, because biodegradation is a significant process for PAH removal, especially under aerobic conditions. Although PAHs most commonly are found as complex mixtures, much of the research on PAH biodegradation kinetics has focused on systems having only one PAH substrate. Significantly less research has been done regarding PAH mixtures and the effects of multiple substrates on biodegradation kinetics.

A multisubstrate system contains the possibility of both positive and negative interactions between the substrates. First, the presence of multiple carbon sources may result in faster biomass growth compared to that in a sole-substrate system. Because the rate of substrate depletion is a function of the biomass concentration, the biodegradation rate for each compound may be increased if common populations are responsible for their degradation. Second, if common enzymes are involved, the presence of multiple substrates may result in competition, thereby inhibiting degradation. The net result of these two effects could be an increase in the rate of biodeg-

radation as a result of enhanced biomass growth, a decrease in biodegradation rate from competitive inhibition, or no noticeable effect because the two effects balance and cancel each other out.

Previous studies have found evidence of enhanced biodegradation rates as well as decreased biodegradation rates for PAHs present in mixtures [3–15]. These studies provided valuable insights regarding the significance for potential substrate interactions in the biodegradation of mixtures of PAHs, but when examined collectively, no clear pattern to the substrate interactions was found. Because of the range of substrate and biomass conditions represented, it certainly is possible that biomass-enhanced biodegradation was dominant in some cases whereas multisubstrate inhibitions were dominant in other cases. The absence of routine measurement of biomass growth and the differences in initial substrate concentrations and experimental designs make it difficult to infer underlying mechanisms.

Only a few studies have investigated kinetics of PAH multisubstrate biodegradation in aqueous systems, which eliminate confounding effects deriving from kinetically limiting sorption to and from soils and sediments. Even fewer studies have used enzyme kinetic modeling, rather than empirically inferring inhibitory effects, to investigate these kinetics. Seminal work in this area was performed by Stringfellow and Aitken [11], who studied biodegradation kinetics of phenanthrene (PHN) in binary mixtures with naphthalene (NPH), 1-methylnaphthalene (1MN), 2-methylnaphthalene (2-MN), and fluorene (FLR). Those authors observed competitive inhibition of PHN degradation, suggesting that these PAHs probably share a common enzyme system within each of the two species tested. Despite the inhibition effect, enhanced oxygen uptake was observed

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in some of their binary experiments, implying enhanced biomass growth as a result of the presence of the second substrate. Guha et al. [16] studied substrate interactions in binary and ternary mixtures of NPH, PHN, and pyrene (PYR) using a PAH-degrading consortium. That work demonstrated the importance of substrate interactions in simple mixtures and demonstrated the feasibility of using predictive multisubstrate modeling, laying the groundwork for its use in more complex systems [17]. Lotfabad and Gray [12] measured the biodegradation kinetics of PAH mixtures with a mixed culture and examined the data using a multisubstrate model. Competitive inhibition explained biodegradation kinetics in creosote-contaminated soils but not for the PAHs added to pristine soils.

The goal of the research presented in this paper was to investigate substrate interactions in simple and complex PAH mixtures and to examine the extent to which multisubstrate modeling can predict biodegradation kinetics for these systems. Three different mixtures were studied: Two binary mixtures of NPH with 1MN and with PHN, and a complex mixture of nine PAHs (NPH, 1MN, 2MN, 2-ethylnaphthalene [2EN], PHN, anthracene [ANTH], PYR, FLR, and fluoranthene [FLN]). The experiments were conducted in aqueous systems to eliminate soil and sediment sorption phenomena, thereby isolating the biodegradation processes of interest. The experiments were further designed to account for confounding physicochemical processes introduced in the laboratory that could not otherwise be eliminated. The binary experiments were performed to exhibit the importance of multisubstrate effects in simple systems and to permit mechanistic inference by minimizing confounding factors related to the presence of numerous substrates. The nine-PAH mixture was used to investigate a system that is more representative of the types of mixtures that typically occur in environmental contamination scenarios. To our knowledge, this is the first report of the systematic examination of the multisubstrate biodegradation kinetics of a complex, nine-component mixture.

Biodegradation reaction pathways for a wide range of PAHs are similar, and a dioxygenase enzyme is responsible for the initial step in the aerobic biodegradation of many PAHs [18]. In the present work, we examine PAH-mixture biodegradation in the theoretical context of multisubstrate competitive inhibition for enzyme-mediated reactions [19]. In addition to their application to PAH mixtures, multisubstrate models that describe competitive inhibition in microbiological systems have been used to model utilization of mixtures of sugars [20–22], BTEX (benzene, toluene, ethylbenzene, and xylene) [23–26], and trichloroethylene [11,16,27]. Theoretically, when multiple substrates are utilized within a common enzyme-mediated pathway, the mathematical description of competitive inhibition is accomplished solely with single-substrate parameters. If this theory holds, then no need exists for empirically determined binary- or higher-order parameters to describe substrate interactions. One of the objectives of the present study was to examine the extent to which theoretical multisubstrate models could predict biodegradation kinetics for binary and complex mixtures of PAHs. The kinetic parameters were taken from a related study in which PAH biodegradation kinetics were measured in sole-substrate systems [28]. Predictive modeling is used in the present work to provide a basis for comparison of experimental results with sole-substrate systems, to determine which multisubstrate model best describes experimental observations, and to provide a theoretical basis for explaining multisubstrate effects.

MATERIALS AND METHODS

Biodegradation modeling

Here, we present the theoretical basis for modeling biodegradation kinetics in sole-substrate and multisubstrate systems when the biodegradation is mediated by a common enzyme pathway. For a given PAH substrate, the rate of change in concentration as a result of biodegradation is modeled as

$$\frac{dC_i}{dt} = -q_i X \quad (1)$$

where C_i is the concentration of the PAH substrate i (mg/L), q_i is the biomass-normalized substrate utilization rate (mg substrate/mg protein/h), X is the biomass concentration (mg protein/L), and t is time (h). The rate term, q_i , may take different functional forms depending on whether inhibition effects are modeled. The change in X with time may be modeled differently depending on what is assumed about whether growth is supported by a single or multiple substrates.

Sole-substrate model. The simplest means of describing the biodegradation rate is the sole-substrate biodegradation model, based on traditional Monod kinetics:

$$q_i = q_{\max,i} \frac{C_i}{K_{S,i} + C_i} \quad (2)$$

where $q_{\max,i}$ is the maximum substrate utilization rate per unit biomass for substrate i (mg substrate/mg protein/h) and $K_{S,i}$ is the half-saturation coefficient for substrate i (mg/L). In the present work, the predictions from this model serve as the scenario to which the effects of multiple substrates are compared. It describes substrate behavior as independent of the concentrations of other substrates, thus representing the case of the absence of any substrate interactions. The change in biomass concentration is modeled as if substrate i were the only growth substrate present:

$$\frac{dX}{dt} = q_i Y_i X \quad (3)$$

where Y_i is the yield coefficient for substrate i (mg protein/mg substrate).

Multisubstrate model with no inhibition. The multisubstrate model with no inhibition represents the case in which multiple growth substrates are available to the microorganisms:

$$\frac{dX}{dt} = \sum_{i=1}^n q_i Y_i X \quad (4)$$

where n is the number of growth substrates present. However, the additional substrates do not affect the individual substrate utilization rates, which are represented as being the same as in the sole-substrate model (Eqn. 2). According to this model, the additional carbon sources increase the growth of biomass, thereby increasing the biodegradation rates of compounds relative to their sole-substrate biodegradation rates. If this model holds, the only possibility is an enhancement of biodegradation rates when substrates are present in mixtures. This model serves to constrain the upper limit of biodegradation rate enhancement.

Multisubstrate model with competitive inhibition. The multisubstrate model with competitive inhibition represents the case in which multiple substrates are available to the microorganisms but the substrates compete for the limited number of enzymes present. Biomass growth is described in the same

manner as for the multisubstrate model without inhibition (Eqn. 5), but the substrate utilization rate is modeled as

$$q_i = q_{\max,i} \frac{C_i}{K_{S,i} + \sum_{j=1}^n \frac{K_{S,i} C_j}{K_{S,j}}} \quad (5)$$

where $K_{S,j}$ is the half-saturation coefficient for substrate j (mg/L) and C_j is the concentration of substrate j (mg/L). This model captures the competing effects of biodegradation enhancement and competitive inhibition. These opposing processes result in an initially deterred biodegradation rate (compared to the non-inhibition multisubstrate and sole-substrate models) that subsequently increases as the additional biomass grows faster than the sole-substrate scenario (see Guha et al. [16] and references therein).

Chemicals

The PAHs were purchased from Aldrich Chemical (Milwaukee, WI, USA), and all had purities of 98% or greater except for ANTH, which had a purity of 97%. Methanol and acetonitrile, the organic solvents used for high-pressure liquid chromatography, were of Optima quality and purchased from Fisher Scientific (Fairlawn, NJ, USA). The water used was deionized and ultrapurified using HYDRO Picosystem Plus (Hydro Service and Supplies, Durham, NC, USA). The PAH stock solutions were prepared using BOD nutrient buffer solution (Hach, Loveland, CO, USA) as reported elsewhere [28].

Biomass

The enrichment consortium used for these experiments is the same culture that was used in the sole-substrate biodegradation experiments [28]. The consortium has been determined via 16S rDNA analysis to have one dominant species, *Sphingomonas yanoikuyae* (21 of 24 samples). For months before these experiments, the consortium had been maintained on the nine PAHs involved in the present study in a BOD nutrient medium made of KH_2PO_4 , K_2HPO_4 , CaCl_2 , MgSO_4 , Na_2HPO_4 , NH_4Cl , and FeCl_3 . Previous work had shown that all nine PAHs were biodegraded by this consortium. Acenaphthene, which had been included in the sole-substrate biodegradation study, was found to be not degradable and was not included in the multisubstrate experiments.

Before each experiment, the consortium was washed clean of all PAHs using a series of Tween 80 (Imperial Chemical Industries Americas, Wilmington, DE, USA; obtained from Aldrich, Milwaukee, WI, USA) washing cycles and BOD nutrient buffer rinsing cycles. The details and effectiveness of this procedure are presented elsewhere [29]. Biomass concentrations were measured using the Bio-Rad protein assay, based on the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) and compared to a bovine gamma-globulin protein standard. The detection limit for biomass samples was 0.14 mg protein/L. The procedure is described in detail elsewhere [29].

Chemical analysis

Aqueous PAH samples were analyzed using a Hewlett-Packard Series 1050 high-pressure liquid chromatograph (Agilent Technologies, Palo Alto, CA) followed by ultraviolet and fluorescence detection (FLD). For each sample, a 20- μl injection volume was used with a mobile phase of acetonitrile and water, with flow rates ranging from 0.5 to 0.7 ml/min. Four replicate samples were taken from each vessel. For the vessels in the experiment involving nine PAHs, only two FLD mea-

surements could be made because of the inability to adequately separate all elution peaks to permit switching of FLD wavelengths. Wavelengths for ultraviolet and FLD detection are the same as those presented elsewhere [29], except that FLR was detected using a FLD of $\lambda_{\text{ex}} = 225$ nm and $\lambda_{\text{em}} = 335$ nm. The optimal detection methods allowed detection limits for NPH, 1MN, PHN, and PYR of 0.005 mg/L, and FLR, 2MN, 2EN, and FLN had detection limits of 0.001 mg/L. The detection limit of ANTH was 0.0005 mg/L. These methods allowed detection limits well below the aqueous solubilities of each PAH.

Experimental design

For each PAH combination, two experiments were performed in an attempt to separately examine the effects of biomass growth and competitive inhibition. This was accomplished by varying the concentration of NPH, because it has the greatest potential to promote growth. In one experiment for each mixture, NPH was present at a concentration comparable to those of the other substrates, and in the second experiment, NPH was present at an appreciably larger concentration. The experiments with the smaller BPH concentrations maximize our ability to infer the effects of competitive inhibition without the confounding effects of a large increase in biomass from growth on one substrate. The experiments with large NPH concentrations were designed so that a relatively large increase in biomass would occur over the time period of the experiment. This allows one to infer the impact of biomass growth enhancement on biodegradation rates of all PAHs present.

Each experiment consisted of parallel series of abiotic and biotic reactor vessels. The biotic series consisted of 15 individual reactor vessels, and the abiotic series consisted of seven reactor vessels. The observation of substrate and biomass concentrations at a given point in time was done by sacrificing one reactor vessel. Two to four replicate aqueous samples and five replicate biomass samples were taken from each vessel. Kinetically limiting abiotic substrate losses, substrate partitioning to apparatus and biorelated material, and endogenous decay of the biomass had all been quantified using independent, identical experiments [28]. The initial concentrations and the mixture components for each experiment are listed in Table 1. For each PAH, the initial substrate concentration was determined from the measured concentration of the stock solution using mass-balance principles to account for substrate that sorbed both to the apparatus and to biomass [28]. Initial biomass concentrations were below the detection limit of the assay. The values given in Table 1 were calculated from the measured concentration of the stock biomass solution multiplied by the appropriate dilution factor [28].

Experiments (EXPTs) 1A and 1B studied NPH and 1MN. These compounds were chosen because in a previous study of biodegradation kinetics in sole-substrate systems, the Monod kinetic parameters and yield coefficients for both PAHs had been determined with a high degree of confidence, which was not the case for the remaining PAHs [28,30]. In EXPT 1A, NPH and 1MN were present at similar initial concentrations so that neither substrate would dominate biomass growth. In EXPT 1B, the NPH concentration was more than an order of magnitude higher than that of 1MN, permitting investigation of the impact of a large increase in biomass concentration on the biodegradation rates of both substrates.

Experiments 2A and 2B studied NPH and PHN. This binary

Table 1. Multisubstrate experiments (EXPT) and initial conditions for nine polycyclic aromatic hydrocarbons: Naphthalene (NPH), 1-methylnaphthalene (1MN), 2-methylnaphthalene (2MN), 2-ethylnaphthalene (2EN), phenanthrene (PHN), anthracene (ANTH), pyrene (PYR), fluorene (FLR), and fluoranthene (FLN)

EXPT	Initial substrate concentration (mg/L)									Initial biomass concentration (mg protein/L)
	NPH	1MN	2MN	2EN	PHN	ANTH	PYR	FLR	FLN	
1A	3.1	1.5								0.035
1B	7.3	0.36								0.035
2A	2.2				0.91					0.040
2B	10				0.86					0.040
3A	0.77	0.31	0.23	0.084	0.47	0.030	0.072	1.2	0.086	0.033
3B	7.1	0.32	0.27	0.13	0.45	0.024	0.070	1.2	0.080	0.033

pair was chosen because these two compounds have similar molecular structures, consisting entirely of aromatic carbons. Under aerobic conditions, bacteria degrade these compounds only via dioxygenation of the aromatic ring. These experiments therefore avoid the complication of studying substrates with potentially different biodegradation pathways. Alkyl-substituted PAHs, such as 1MN, can be degraded by dioxygenation of the ring or by mono-oxidation of the alkyl group [31]. In EXPT 2A, PHN and NPH had similar initial concentrations, whereas in EXPT 2B, NPH was at an initial concentration an order of magnitude larger than that of PHN.

Experiments 3A and 3B studied a mixture of nine PAHs. All PAHs were present at similar initial concentrations for EXPT 3A, whereas EXPT 3B had an initial concentration of NPH approximately an order of magnitude larger than the initial concentrations of the other PAHs. In EXPTs 3A and 3B, the initial concentrations of all the compounds were low as a result of the low aqueous solubilities of some of the PAHs (ANTH, PYR, and FLN).

The initial concentrations of PAHs in all experiments were sufficiently small that the dissolved oxygen concentrations were effectively constant throughout the experiments, thereby maintaining aerobic conditions. Initial biomass concentrations were designed to be sufficiently low to allow an orders-of-magnitude increase in biomass concentration, resulting in dramatically enhanced the effects of biomass concentration on biodegradation rates. Preliminary modeling calculations showed that the optimal sampling time was every 2 to 4 h.

Experimental system modeling

For the experimental system, a mathematical model was developed to describe the biodegradation process as well as other physicochemical processes. The mathematical modeling was undertaken to enable comparison of experimental observations with model predictions of sole-substrate and multi-substrate biodegradation kinetics. Furthermore, model simulations accounted for the unique initial conditions in each experiment, thereby allowing comparisons to be drawn across different experiments.

Despite all efforts to eliminate and minimize processes other than biodegradation in the experimental systems, abiotic processes were significant. This is not surprising given the volatility and hydrophobicity of PAHs. To account for these confounding processes, the mathematical model representing substrate concentration dynamics incorporated kinetically limiting abiotic losses as well as fast partitioning to both the apparatus and biomass. Based on mass-balance principles, the rate of change of concentration of substrate i is modeled as

$$(1 + K_{a,i} + K_{b,i}X) \frac{dC_i}{dt} = -q_iX - k_{a,i}C_i - K_{b,i}C_i \frac{dX}{dt} \quad (6)$$

where $K_{a,i}$ is the abiotic equilibrium partitioning coefficient (dimensionless; accounts for fast sorption to the apparatus), $K_{b,i}$ is the biotic equilibrium partitioning coefficient (L/mg protein; accounts for fast sorption to the biomass), $k_{a,i}$ is the kinetic abiotic loss term (/h; accounts for slow abiotic losses), and q_i is defined according to the biodegradation model (see Eqns. 2 and 5). Endogenous biomass decay was incorporated in the mass-balance equation for X by subtracting b , the endogenous decay rate (/h), from the total change in biomass concentration (Eqns. 3 or 4). The parameters $K_{a,i}$, $K_{b,i}$, $k_{a,i}$, and b were all determined previously through independent experiments [28], and these parameters were not adjusted or calibrated for the multisubstrate experiments.

The present study had $n + 1$ coupled differential equations, where n is the number of PAHs present. These equations were solved using a fourth-order Runge-Kutta numerical method with programming code written using Matlab (Ver 5.2.0; The Mathworks, Natick, MA, USA). Model simulations were conducted using the initial conditions for substrate and biomass concentrations in Table 1.

Biodegradation parameters

The biodegradation rate parameters and yield coefficients are based on parameters determined from previous sole-substrate experiments [28] and are listed in Table 2. For seven of the PAHs, q_{\max} and K_S could not be uniquely determined because of the low solubility limits of these compounds [30]. For each of these compounds, the first-order biodegradation rate coefficient, q_{\max}/K_S , was estimated instead. To model mul-

Table 2. Sole-substrate biodegradation parameters based with minor modification on determinations from a previous study^a

Compound	q_{\max} (mg/mg/h)	K_S (mg/L)	Y (mg/mg)	q_{\max}/K_S (L/mg/h)
Naphthalene	0.64	0.57	0.4	1.1
1-Methylnaphthalene	0.62	5.3	0.5	0.12
2-Methylnaphthalene	0.6 ^b	3.1 ^b	0.4	0.19
2-Ethylnaphthalene	0.6 ^b	2.7 ^b	0.4 ^c	0.21
Phenanthrene	0.6 ^b	2.2 ^b	0.4 ^c	0.27
Anthracene	0.6 ^b	2.5 ^b	0.4 ^c	0.24
Pyrene	0.6 ^b	0.69 ^b	0.4 ^c	0.75
Fluorene	0.6 ^b	24 ^b	0.4 ^c	0.026
Fluoranthene	0.6 ^b	1.7 ^b	0.4 ^c	0.32

^a Knights and Peters [28].

^b Specified value of q_{\max} , and K_S estimated as $0.6/(q_{\max}/K_S)$.

^c Estimated as $Y = 0.4$.

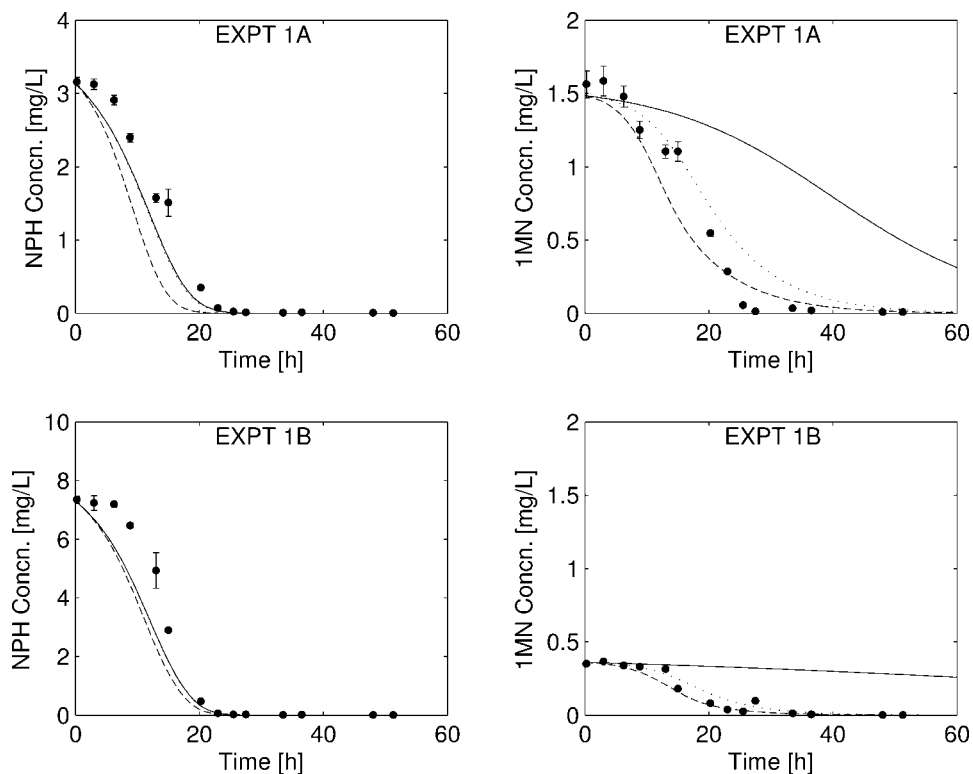


Fig. 1. Experiments (EXPTs) 1A and 1B: Substrate concentrations: Comparison of multisubstrate experimental observations (●) for naphthalene (NPH) and 1-methylnaphthalene (1MN) to sole-substrate (solid line), multisubstrate with competitive inhibition (short dashes), and multisubstrate with no inhibition (long dashes) models. In EXPT 1A, similar initial concentrations for NPH and 1MN were used; in EXPT 1B, the initial concentration of NPH was one order of magnitude greater than the initial concentration of 1MN. Error bars represent the standard deviation.

tisubstrate competitive inhibition (Eqn 5), however, both Monod parameters are needed. To overcome this apparent obstacle, an arbitrary value of q_{\max} for PAHs other than NPH and 1MN was assumed as 0.6 mg/mg/h. This value was chosen because it was similar to the value estimated for NPH and 1MN (the two PAHs for which q_{\max} was uniquely estimated). The corresponding value of K_S was calculated by dividing by the first-order rate coefficient (q_{\max}/K_S). Using this method, the resulting K_S is large enough so that $K_S \gg C$ for the duration of the experiments. One goal of EXPT 2 was to verify that this assumption is effective in multisubstrate modeling. A sensitivity analysis was done to ensure that the modeling results, under the concentration conditions relevant to these experiments, were not sensitive to this choice of q_{\max} . The analysis showed that the model was relatively insensitive to the choice of q_{\max} as q_{\max} increased, but sensitivity increased as q_{\max} approached zero.

Because of the lack of confidence in the stoichiometric yield coefficients for the PAHs with low initial concentrations (FLR, 2EN, PHN, ANTH, FLN, and PYR), Y_i was estimated as 0.4 mg protein/mg PAH. The uncertainty in the yield coefficient for the high-molecular-weight PAHs does not generate large uncertainty in predicted biomass yield because of the low concentrations of these compounds and their minimal contribution to biomass growth.

RESULTS

The experimental observations and modeling predictions for the multisubstrate experiments are presented in Figures 1 through 5. The substrate concentration data are presented in linear space for EXPTs 1A, 1B, 2A, and 2B, whereas the

concentrations for EXPTs 3A and 3B are presented in logarithmic space because of the wide range of substrate concentrations. The symbols represent the average values of replicate samples, with their error bars representing the standard deviation. Many error bars are smaller than the size of the symbol; this is particularly true in log-space.

Binary experiments: Substrate observations

The substrate concentration plots are presented in Figure 1 for EXPTs 1A and 1B. In both experiments, the experimental data for both substrates follow the general Monod trend, in which initially slow substrate utilization as a result of logistic growth is followed by first-order decay. Naphthalene and 1MN are very similar in their maximum utilization rates, but the half-saturation coefficient of NPH is an order of magnitude smaller than that for 1MN (Table 2). One would expect that when these substrates are present individually, NPH would be degraded significantly faster than 1MN. This is what is predicted by the sole-substrate model, which is shown as solid curves in the Figure 1. In both binary experiments, it was observed that the two compounds degraded at roughly the same rate.

Substrate concentration plots are presented in Figure 2 for EXPTs 2A and 2B. The first-order biodegradation rate coefficient (q_{\max}/K_S) for PHN is roughly one order of magnitude less than that for NPH (Table 2), and the sole-substrate model predicts that if each of these compounds were present individually, then PHN would degrade much more slowly than NPH. In these binary experiments, PHN was observed to degrade at a rate that is only slightly slower than NPH.

The dramatic differences between the experimental data and

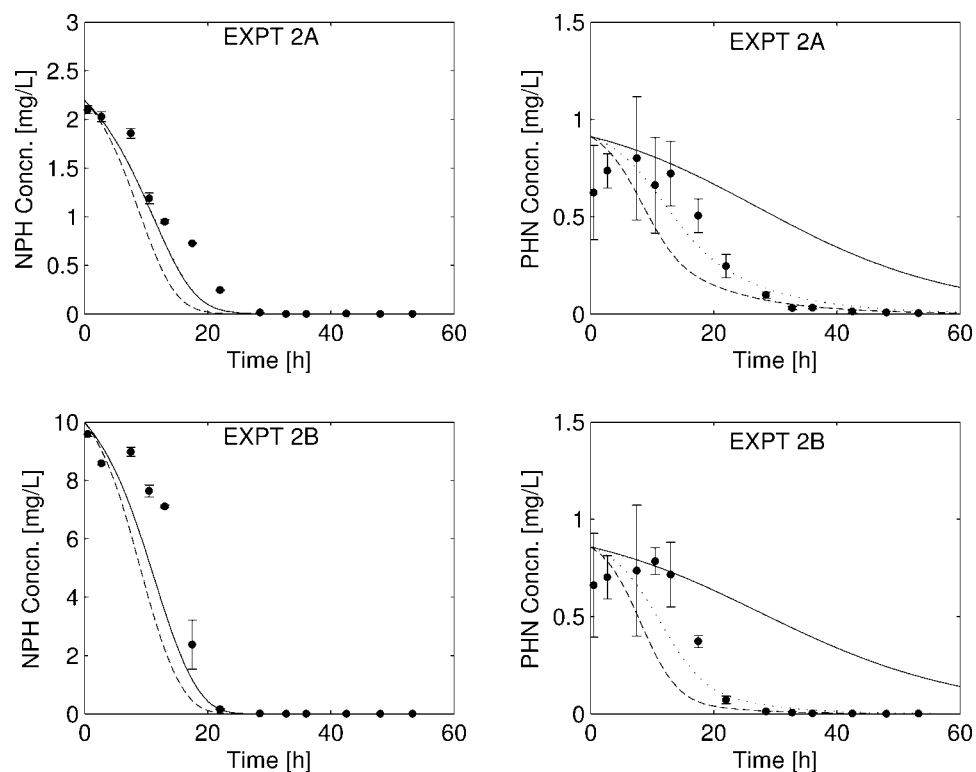


Fig. 2. Experiments (EXPTs) 2A and 2B: Comparison of multisubstrate experimental observations (●) for naphthalene (NPH) and phenanthrene (PHN) to sole-substrate (solid line), multisubstrate with competitive inhibition (short dashes), and multisubstrate with no inhibition (long dashes) models. In EXPT 2A, initial concentrations for NPH and PHN were similar; in EXPT 2B, the initial concentration of NPH was one order of magnitude greater than the initial concentration of PHN. Error bars represent the standard deviation.

the sole-substrate model predictions demonstrate that multisubstrate effects are pronounced for 1MN and PHN in binary experiments with NPH. In the two binary experiments involving PHN, the time for the concentration to reach half its initial value is overestimated by a factor of two using the sole-substrate model. The time to reach half the initial concentration of 1MN in EXPT 1A is similarly overestimated. In EXPT 1B, the sole-substrate model overestimates the time to reach half the initial concentration by a factor of more than six (beyond the scale of the plot). For both substrates, significant errors would be incurred if one were to predict biodegradation rates for mixtures involving NPH based on data and parameters inferred from sole-substrate experiments.

Also shown in Figures 1 and 2 are the multisubstrate model predictions. In all cases, substrate depletion is predicted to be the fastest in cases when increased biomass growth is the result of multiple substrates and inhibition effects are absent. Substrate depletion predicted by the multisubstrate model that includes competitive inhibition is slower, but it is faster or similar to that predicted by the sole-substrate model. Under other conditions (different from those studied here), it is possible that the extent of inhibition is so great that the sole-substrate model would predict faster substrate depletion than the multisubstrate with inhibition model. This phenomenon has been observed in previous work [16].

In all four binary experiments, the NPH data are reasonably well represented by the sole-substrate model. The biodegradation rate of NPH is not dramatically affected by the presence of the additional substrates of either 1MN (EXPTs 1A and 1B) or PHN (EXPTs 2A and 2B). The apparent lack of substantial multisubstrate effects for NPH biodegradation is explained by

examining the multisubstrate model predictions. The multisubstrate model without inhibition predicts only slightly enhanced biodegradation relative to the sole-substrate model predictions. The increased rate of NPH degradation predicted by this model would be caused by the additional growth on the other substrate present. This effect is negated in the multisubstrate model that accounts for inhibition such that the sole-substrate and multisubstrate inhibition model predictions coincide. The minimal impact of 1MN or PHN is consistent with the multisubstrate inhibition theory, because neither of these substrates can significantly affect growth. Also, their K_S values are relatively large compared to the K_S value for NPH, so the inhibition term in the denominator of Equation 5 is small. The model predictions and experimental results for NPH suggest that the sole-substrate biodegradation formulation would adequately model this substrate. However, if multisubstrate modeling were used, the competitive inhibition model would be the best representation.

The discrepancies between the observations for 1MN and PHN and the sole-substrate model predictions are well explained by the multisubstrate models. The presence of the more degradable NPH, which sometimes is present at significantly higher concentrations, increases biomass concentration, which leads to enhanced biodegradation of the other substrates. The difference between the predictions of the two multisubstrate models is not very dramatic for 1MN and PHN in any of the binary systems. The potential inhibitory effect of NPH is minimal compared to the more significant biomass enhancement effect. The small difference in the model predictions relative to experimental error makes it difficult to judge which model is more accurate, but clearly, either of the multisubstrate mod-

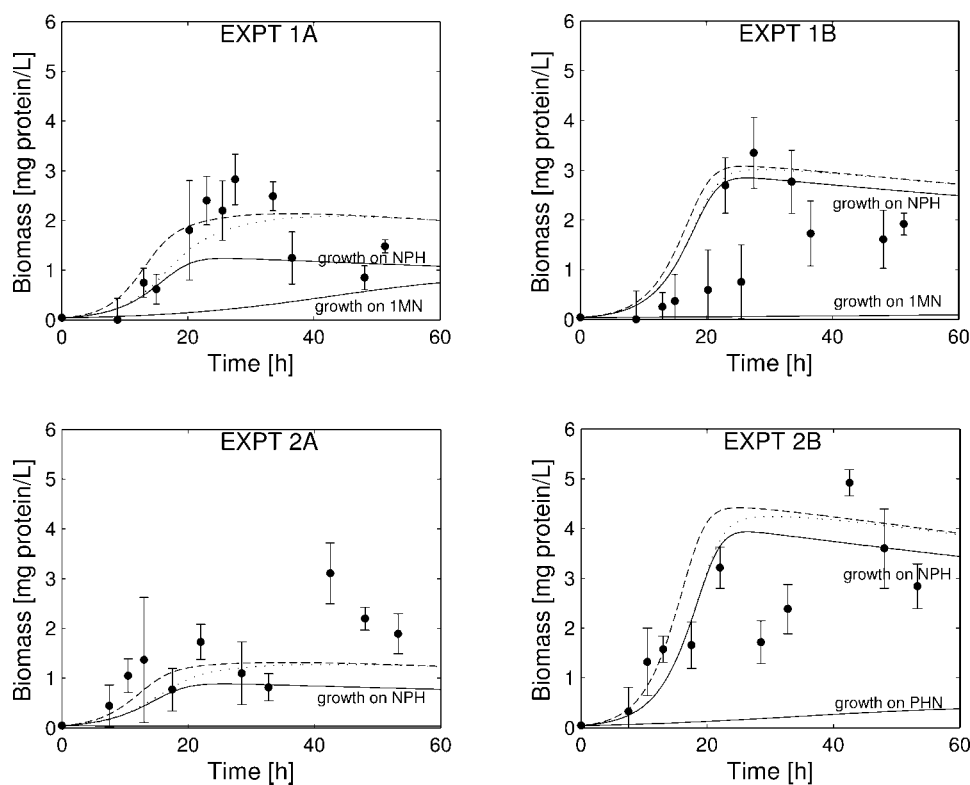


Fig. 3. Experiments (EXPTs) 1A, 1B, 2A, and 2B; Biomass concentrations: Measured observations (●) compared to multisubstrate with no inhibition (long dashes) and multisubstrate with no inhibition (short dashes) biodegradation models. The sole-substrate growth on naphthalene (NPH), 1-methylnaphthalene (1MN), or phenanthrene (PHN; solid line) denoted with text. Error bars represent the standard deviation.

els does a better job of predicting biodegradation rates of 1MN and PHN compared with the sole-substrate model.

Binary experiments: Biomass observations

The observed biomass concentrations and biomass modeling predictions are presented in Figure 3 for EXPTs 1A, 1B, 2A, and 2B. For the first 9 h, biomass measurements were below the detection limit. After 9 h, growth was sufficient that biomass concentrations were measurable. Biomass growth was greater in EXPTs 1B and 2B compared to that in EXPTs 1A and 2A, as expected because of the larger amounts of available NPH substrate.

For EXPT 1A, the biomass concentration increased 10-fold within 15 h, then increased another 10-fold over the next 15 h. The difficulty in accurately measuring the biomass concentration is apparent but does not dismiss its importance in interpreting the observations from these experiments. The biomass concentrations clearly increase in the first half of the experiment, which is associated with the corresponding decrease in substrate concentrations, and then level off or decrease when substrate is depleted.

Four different models predicting biomass growth are plotted along with the biomass observations. The predictions of the multisubstrate model without inhibition place an upper bound on the predicted biomass growth rates. The multisubstrate model with competitive inhibition predicts that the biomass growth is slightly slower than that without inhibition. Two solid curves are plotted, representing the two sole-substrate growth models, which represent the hypothetical scenarios of biomass growth resulting from only one substrate. In the plot for EXPT 2A, growth on PHN alone would be insignificant. For EXPTs 1B and 2B, little difference is observed between

the two multisubstrate models and the sole-substrate NPH model. Naphthalene is both more degradable and more abundant than the other substrates, so the growth kinetics, even in the multisubstrate models, are dominated by growth on NPH. In EXPT 1A, where NPH and 1MN have initial concentrations of the same order of magnitude, the sole-substrate model shows that growth on NPH would be more rapid but that growth solely on 1MN would be slower but appreciable.

The scatter in the data and the associated error bars make it difficult to identify the most successful model and mechanistically infer underlying multisubstrate growth kinetics. The differences between the multisubstrate biodegradation kinetics models are not large enough for distinction. However, sole-substrate modeling of growth on 1MN in EXPTs 1A and 1B and growth on PHN in EXPTs 2A and 2B clearly would have resulted in significant underprediction of the biomass concentration.

Complex-mixture experiments

The experimental data and model predictions for the complex-mixture experiments are presented in Figures 4 and 5 for EXPT 3A and EXPT 3B, respectively. In EXPT 3B, all PAH concentrations decreased over time with respect to their abiotic controls (abiotic concentrations are not shown). This confirms that the consortium was able to degrade all the PAHs that were present.

For most of the PAHs in the present study, the observations of substrate concentration fall below the sole-substrate model predictions, implying significant biodegradation enhancement. In EXPT 3A, this was the case for all compounds except NPH, and in EXPT 3B, this was the case for all compounds except NPH, PYR, and FLN. This suggests that a sole-substrate bio-

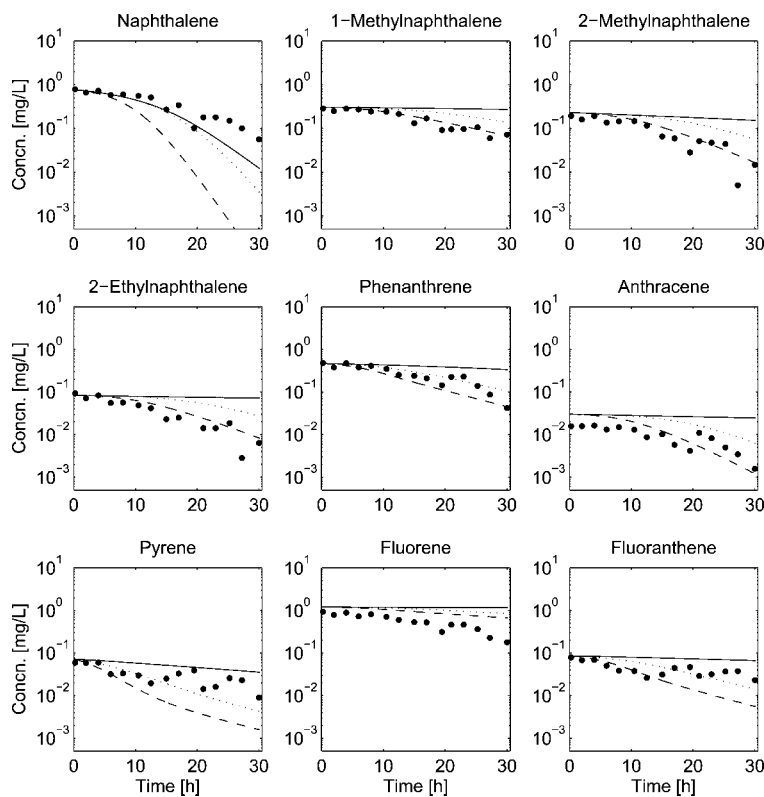


Fig. 4. Experiment 3A: Comparison of multisubstrate experimental observations (●) for nine polycyclic aromatic hydrocarbon (PAH) mixture to sole-substrate (solid line), multisubstrate with competitive inhibition (short dashes), and multisubstrate with no inhibition (long dashes) models. The initial concentration of naphthalene was the same as those for other PAHs. Error bars represent the standard deviation.

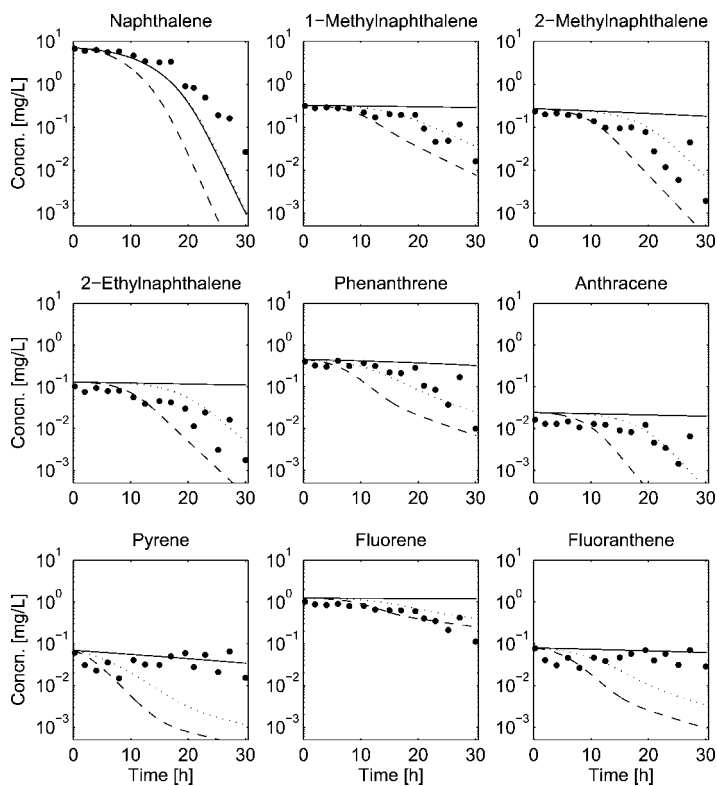


Fig. 5. Experiment 3B: Comparison of multisubstrate experimental observations (●) for nine polycyclic aromatic hydrocarbon (PAH) mixture to sole-substrate (solid line), multisubstrate with competitive inhibition (short dashes), and multisubstrate with no inhibition (long dashes) models. The initial concentration of naphthalene was an order of magnitude higher than those of the other PAHs. Error bars represent the standard deviation.

degradation model is ineffective in representing the biodegradation of these substrates when present in a complex mixture. In both experiments, NPH depletion was slower than the depletion rate predicted by the sole-substrate model; this phenomenon had been observed to a smaller extent in the binary experiments.

Also shown in Figures 4 and 5 are the predictions from the two multisubstrate models. The non-Monod shapes of these curves are evident by comparison with the Monod shape of the sole-substrate model predictions, which are linear after an initial downward convexity. (Good non-Monod examples are PHN, PYR, and FLN in EXPT 3B.) As with the binary systems, the multisubstrate model without inhibition predicts the fastest biodegradation, and the predictions of the multisubstrate model with inhibition are slower than this but faster than the sole-substrate model predictions. Unlike the binary systems, the multisubstrate model with inhibition curves are significantly different from those of the multisubstrate model without inhibition (note the log scale), implying that inhibition effects are predicted to be substantial as a result of the presence of a large number of other substrates. Despite the large differences between the two multisubstrate model predictions, it is still difficult to determine which model best describes the data for those compounds for which enhanced biodegradation was observed. In EXPT 3A, the multisubstrate model without inhibition clearly is the better model for 1MN, 2MN, 2EN, and ANTH, and in EXPT 3B, the multisubstrate model with inhibition clearly is the better model for 1MN, PHN, ANTH, and FLR. For the other compounds with enhanced biodegradation, it is not clear which model is best.

For NPH in EXPT 3B, the predictions from the sole-substrate and multisubstrate with competitive inhibition models were indistinguishable, as was the case in all four binary experiments. The multisubstrate model with inhibition is insufficient to explain the substantial inhibition of NPH biodegradation that was observed in both complex-mixture experiments. One conclusion that can be drawn is that a multisubstrate model with no inhibition certainly does not adequately represent the biodegradation of NPH in this complex mixture. Rather, either the sole-substrate or multisubstrate with competitive inhibition model more adequately represents the system.

Discrepancies in model predictions and experimental observations may be attributable to errors in the determination of initial conditions for the model simulations. This appears to be the case for ANTH in EXPTs 3A and 3B. The initial ANTH concentrations appear to be approximately 20% too large, causing the model simulation curves to be vertically offset. This demonstrates the importance of accurate determination of initial simulation conditions, both for interpretation of experimental observations and for prediction of biodegradation kinetics in the field. A mathematical model that is exactly correct with regard to underlying theory and kinetic parameters will make erroneous predictions if the initial conditions are in error.

Biomass concentrations measured for EXPTs 3A and 3B demonstrated that biomass growth was substantial during the length of these experiments (data not shown). Experiment 3B appeared to have more biomass growth than EXPT 3A, as would be expected because of the larger amount of substrate available in EXPT 3B.

DISCUSSION

Significant substrate interactions were observed in both the binary experiments and in the complex-mixture experiments. This was inferred by the discrepancy between the observed substrate depletion rates and those predicted by the sole-substrate model. In the binary experiments, biodegradation of both 1MN and PHN was significantly enhanced by the presence of NPH. In the complex-mixture experiments, significant biodegradation rate enhancement was observed for almost all compounds except NPH. These effects were much larger (order-of-magnitude scale) than those observed in the binary experiments. The presence of a larger number of substrates resulted in a greater potential for multisubstrate interactions. Naphthalene was the only compound for which biodegradation was inhibited, slightly in the binary experiments and substantially in the complex-mixture experiments.

We can definitively conclude that the sole-substrate model is inadequate to describe multisubstrate biodegradation kinetics for the PAH mixtures in these experiments. It is less clear which of the two multisubstrate models best describes the experimental observations. In the binary experiments, both multisubstrate models did an adequate job of predicting this biodegradation rate enhancement. The two multisubstrate model predictions were not much different from each other, indicating that the major multisubstrate effect was biomass enhancement as a result of growth on NPH. The inhibition component of the overall effect was not very large.

In the complex-mixture experiments, large differences were found between the predictions of the two multisubstrate models, implying that inhibition effects could significantly counteract some of the biomass enhancement effect. Some PAH depletion rates were best matched to the multisubstrate model without inhibition, whereas others were best matched to the multisubstrate model with inhibition. It is difficult to explain the observations with regard to the extent to which the substrate interaction effects are the result of biomass enhancement, competitive inhibition, or both. Clearly, however, some amount of inhibition has occurred, because the multisubstrate model without inhibition overestimates the biodegradation rate for many of the compounds. This is especially evident in the complex-mixture experiment with the high NPH concentration (Fig. 5).

Neither of the two multisubstrate models was clearly optimal in the present study, but we advocate the use of the multisubstrate model with competitive inhibition for similar modeling efforts. The empirical argument to support this position is that its predictions were right more often than they were wrong, which was not the case for the multisubstrate model without inhibition. The theoretical argument to support its use is that substantial evidence indicates common enzyme pathways exist for PAHs, presenting opportunities for competitive substrate binding. Other mechanisms of biodegradation inhibition in PAH mixtures have been reported, most notably those that arise from buildup of metabolites, which could be dead-end products or intermediates that degrade much more slowly than their parent compounds [31–34]. Biodegradation of PAHs produces organic acids, which can result in unfavorable acidification [35]. Metabolites from PAH biodegradation also have been shown to have cytotoxic effects [36,37], which would result in a decrease in biodegradation rate of mixture components. Mathematical models could be derived for these more complex mechanisms, but they would neces-

sarily depend on the concentrations of important metabolites and byproducts, which are difficult to measure and only rarely determined in the field.

The multisubstrate model with competitive inhibition has great utility because of its fully predictive nature. No additional parameters are needed over those estimated via sole-substrate experiments. As such, its use is no more cumbersome than the multisubstrate model without inhibition. As is the case with any predictive model, accurate estimation of initial conditions is essential for accurate prediction. When predicting biodegradation of PAHs in complex mixtures, one needs the initial concentrations of all substrates—not just the initial concentration of the compound(s) of interest.

In these experiments, it was important to measure biomass growth and to include that measure in model predictions. In the natural environment and in experiments using bacterial consortia, the biomass concentration can be represented using a lumped measure, X , as was done in the present study. Such lumped measures may be inadequate when modeling biomass growth on mixed substrates because of the preferential response of some species. The importance of this complexity was not determined in the present study and warrants further examination when considering systems for which biomass growth is a dominant process. The consortium used in these experiments was composed almost entirely of *S. yanoikuyae*, so shifts in community populations are unlikely to have occurred.

With an environmental system in which growth is negligible or a pseudo-steady state is already established with respect to biomass, one would typically use first-order kinetics to describe biodegradation rates. For a mixture of PAHs, however, such a model would not capture the effects of competitive inhibition. In this case, the appropriate model is the combination of Equations 1 and 5, in which X is a constant. This model would predict that biodegradation rates are always slower than predicted by the sole-substrate model, and the magnitude of this inhibition effect is directly related to the concentrations of the other substrates. Biodegradation enhancement as a result of biomass growth was significant in the present study, but the observations also revealed that inhibition was occurring and was, potentially, a very large effect in complex mixtures. By deduction, biodegradation rates in the field would be significantly slower than those that would be predicted if one failed to account for the concentrations of competitive substrates and their multisubstrate inhibition effects.

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