Biodegradation Kinetics of Aromatic Hydrocarbon Mixtures by Pure and Mixed Bacterial Cultures

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Microbial growth on pollutant mixtures is an important aspect of bioremediation and wastewater treatment. However, efforts to develop mathematical models for mixed substrate kinetics have been limited. Nearly all models group either the microbial population (as "biomass") or the chemical species (e.g., as biological oxygen demand). When individual chemical species are considered, most models assume either no interaction or that the nature of the interaction is competition for the same rate-limiting enzyme. And when individual microbial species are considered, simple competition for the growth substrate is the only interaction included. Here, we present results using *Pseudomonas putida* F1 and *Burkholderia* sp. strain JS150 growing individually and together on benzene, toluene, phenol, and their mixtures and compare mathematical models to describe these results. We demonstrate that the simple models do not accurately predict the outcome of these biodegradation experiments, and we describe the development of a new model for substrate mixtures, the sum kinetics with interaction parameters (SKIP) model. In mixed-culture experiments, the interactions between species were substrate dependent and could not be predicted by simple competition models. Together, this set of experimental and modeling results presents our current state of work in this area and identifies challenges for future modeling efforts. *Key words:* benzene, biodegradation kinetics, mixed growth substrates, phenol, *Pseudomonas putida* F1, toluene. *Environ Health Perspect* 110(suppl 6):1005–1011 (2002).
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Organic chemical mixtures are prevalent in wastewater from industrial and municipal sources as well as in contaminated groundwater. Common examples of chemical mixtures that often become pollutants include gasoline and other petroleum fuels, pesticides, and wood-treating substances. Landfill leachates are complex mixtures that contaminate groundwater supplies around the world. Pollutant mixtures may contain only organic chemicals or may also include inorganics, heavy metals, or radionuclides. The occurrence of contaminants in mixtures is an important problem because the removal or degradation of one component can be inhibited by other compounds in the mixture and because different conditions may be required to treat different compounds within the mixture. The work reported here was motivated by the first of these issues as it applies to pollutant biodegradation.

Researchers have noted that microbial degradation (metabolism) of a compound in a mixture can be strongly affected by other substituents of the mixture (*1–4*). This has been observed not only for mixtures of toxic chemicals (bioremediation) but also for mixtures of pollutants and readily degraded compounds (wastewater treatment) and mixtures of sugars (fermentation). To understand mixture effects, one must consider the metabolic role each compound plays for the microorganisms. The terms "homologous" and "heterologous" have been proposed by Harder and Dijkhuizen (*5*) for compounds that serve the same or different roles, respectively.

The effects of other compounds in a mixture of homologous carbon and energy substrates on the biodegradation of a chemical can be positive, as in the case of increased growth at low substrate concentrations (*6,7*) or induction of required degradative enzymes (*8*). More commonly, negative interactions are reported. Reasons for decreased biodegradation rates include competitive inhibition (*9–11*), toxicity (*12*), and the formation of toxic intermediates by nonspecific enzymes (*13,14*).

Although mathematical models of mixed homologous substrate consumption and microbial growth have been proposed [e.g., (*2,11,15–19*)], this body of literature is much smaller than that for the modeling of single-substrate growth kinetics. Most models have been tested with only two substrates, and their applicability to larger mixtures has been assumed without validation. More recently, models have been proposed and tested for larger mixtures. Examples include the growth of *Escherichia coli* on six sugars (*16*), the growth of a mixed culture on benzene, toluene, ethylbenzene, and *o*- and *p*-xylene (BTEX compounds) (*11*), and the biodegradation of three polycyclic aromatic hydrocarbons (*20*).

In addition to the interactions among chemical components of a mixture undergoing biodegradation, the interactions among microbial species in a mixed culture may be important. For example, Lewandowski and co-workers (*21*) studied the biodegradation of phenol by several two-species mixed cultures. Excellent agreement between pure-and-simple competition theory and experimental data occurred when the two species in an experiment were both isolated from the same environment. However, when a mixture was composed of two organisms from different environments, there was no agreement with the pure-and-simple competition model. Conversely, in research by Murakami and Alexander (*22*), interspecies interactions beyond pure-and-simple competition, including interactions harmful to one species while the other was unaffected, occurred between members of a binary culture isolated from the same sewage treatment plant.

At Colorado State University, our group has been studying the biodegradation kinetics of chemical mixtures for several years. The long-term goal of this research is to understand (and model mathematically) the biodegradation of complex chemical mixtures by microbial communities. Our strategy is to first learn from simpler (but representative) systems: pure cultures degrading mixtures and mixed cultures degrading single chemicals (Figure 1). In this report, we review our results at this intermediate level and then describe the results from a simple chemical mixture–microbial mixture experiment. Finally, we discuss some preliminary results that may provide insights on the observations made previously.

This work has focused on the biodegradation of benzene, phenol, and toluene. These monoaromatic compounds are ideal representatives of chemicals found in pollutant mixtures. They are produced in very large quantities for use as fuels, solvents, and starting

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materials for chemical syntheses (*23*). As an outcome of this prevalent use, monoaromatics are widespread environmental contaminants, usually in mixtures. Thirty monoaromatics are listed in the U.S. Environmental Protection Agency's *Priority Pollutants* (*24*), and 11 of these compounds are in the top 100 chemicals on the Agency for Toxic Substances and Disease Registry's *Priority List of Hazardous Substances* (*25*). Two bacterial strains were used in the work presented here: *Pseudomonas putida* F1 and *Burkholderia* sp. JS150. *P. putida* F1 uses toluene dioxygenase (TDO) to initiate the metabolism of toluene, benzene, phenol, and other aromatics (*26*). In contrast, *Burkholderia* sp. JS150 can express at least three initial dioxygenases (*12*). These strains are thus interesting and distinct model systems for the study of mixture biodegradation kinetics.

Materials and Methods

Microorganisms. *P. putida* F1 is a well-characterized aromatic hydrocarbon–degrading bacterium that can use toluene, benzene, ethylbenzene, phenol, and other aromatics as sole carbon and energy sources (*26*). The biodegradation of toluene by *P. putida* F1 begins with the oxidation of the aromatic ring by TDO to form *cis*-toluene dihydrodiol (*26–28*), which is then dehydrogenated to form 3-methylcatechol. This molecule is then cleaved at the *meta* position and then converted in three steps to acetaldehyde and pyruvate (*29–31*). TDO also catalyzes the oxidation of benzene (*28,32,33*) and phenol (*34,35*). In both cases, catechol is formed after dehydrogenation and is then further degraded by *meta* ring cleavage and other reactions to tricarboxylic acid cycle intermediates. Thus, *P. putida* F1 uses the same metabolic pathway to metabolize toluene, benzene, and phenol.

Burkholderia sp. JS150 is a nonencapsulated mutant of *Burkholderia* sp. JS1 obtained after ethyl methane sulfonate mutagenesis of strain JS1 (*12*). In addition to toluene, benzene, and phenol, this species is able to degrade a wide range of substituted aromatic compounds. Strain JS150 has a much greater metabolic capability than *P. putida* F1, with the ability to synthesize at least four ring-fission pathways and use three separate initial dioxygenases (including a nonspecific TDO) when grown on various substrates (*12*).

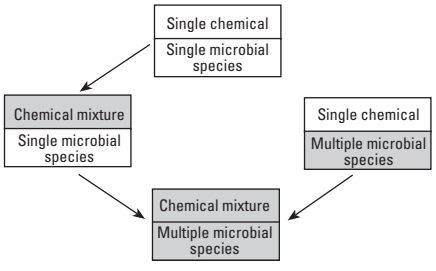

Figure 1. Levels of complexity in biodegradation kinetics research.

Media. For all experiments, a modified Hutner's mineral base was used as the carbon-free medium (*36*), and toluene, benzene, and/or phenol was added. Phenol was added before autoclaving, but toluene and benzene were added after autoclaving to minimize losses from volatilization (*37*). For strain maintenance, cultures of both bacteria were grown on toluene vapors and stored at –70°C in 10% glycerol.

Chemicals. Benzene (Sigma, St. Louis, MO, USA; HPLC grade), toluene (Baker, Phillipsburg, NJ, USA; HPLC grade), and phenol (Sigma, >99.5% pure) were used as the carbon sources. Chloroform and *p*-xylene (both from Baker; GC grade) were used to prepare samples for gas chromatography (GC). All chemicals used for media preparation were reagent grade.

Analytical methods. Cell concentrations were measured as optical density at 600 nm (OD_{600}) with a Bausch & Lomb Spectronic 21 spectrophotometer (Bausch & Lomb, Rochester, NY, USA) and correlated to biomass concentration (*37,38*). To quantify the two cell populations in the mixed culture experiments, a fluorescence *in situ* hybridization (FISH) method was developed (*39*). In this procedure, 30 µL of a culture sample was applied to slides, which were then dried, fixed, and dehydrated. The samples were then exposed to species-specific oligonucleotide probes that were 5′-end labeled with fluorescein isothiocyanate (*40*), rinsed, and prepared for counting under a Leitz epifluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with a BioQuant image analysis system (R&M Biometrics Inc., Nashville, TN, USA)(*39*). For species *Burkholderia* sp. JS150, correlations were also developed between cell concentration (cells/mL or cfu/mL) and biomass concentration (mg/L): 3.5×10^6 cells/mL equivalent to 1.0 mg/L biomass for cells grown on toluene, and 2.4×10^6 cells/mL equivalent to 1.0 mg/L biomass for cells grown on phenol.

Benzene, toluene, and phenol concentrations were measured by GC. Aqueous samples were extracted with chloroform, and *p*-xylene was used as an internal standard (*37*). Samples were stored at 4°C in 2-mL screw-cap vials with Teflon-lined rubber septa, until analysis. Benzene, toluene, and phenol standards were prepared as aqueous solutions and extracted with chloroform/*p*-xylene. The detection limit of this method for each of the three compounds was 5 µM.

Protocol for batch biodegradation experiments. All data for biodegradation kinetics modeling were obtained from batch bioreactor cultivations inoculated from shake flask cultures grown on the same carbon source(s) used in the bioreactor. Two 3-L Applikon batch bioreactors (Applikon, Foster City, CA, USA) were used for the biodegradation kinetic experiments. The total initial substrate concentration was approximately 0.5 mM in the liquid phase, regardless of the number of substrates involved. In mixture experiments, the substrates were added in approximately equimolar amounts. Henry's law was used to calculate the amount of toluene or benzene to be added. All experiments were run at the operating and initial conditions found to provide intrinsic biodegradation kinetics, including a low inoculum size (expressed as the ratio of substrate to cell mass; a value of 300 was used) (*37*). The bioreactor was run as a closed system with no air sparging to eliminate the substrate loss due to volatility. The system operated aerobically (dissolved oxygen levels remained above 5 mg/L), 30°C, and without pH control (although the pH remained in the range of 6.7–6.9). Less than 1% of toluene and phenol was lost in sterile control experiments. Biodegradation experiments were performed in duplicate, and replicates were not performed simultaneously. Additional experimental details can be found in Reardon et al. (*37*).

Determination of biodegradation kinetics model parameters. Several mathematical models were compared for their ability to fit or predict the experimental biodegradation kinetics data. The values of all required model parameters were determined by performing nonlinear curve fitting to the experimental data using SimuSolv, a modeling and simulation package (Dow Chemical Company, Midland, MI, USA). Simusolv employed a Gear method to solve the differential equations and maximized the log of the likelihood function (LLF) to optimize the unknown parameters and discriminate between models (*37*). The model with the maximum LLF value and most homogeneous errors residual plots was chosen. For each final model, the percent variation explained (PVE; similar to r^2 value for linear regression) was calculated using the LLF. The average value for each of the parameters was found by separately determining the values for each of the duplicate experiments and then averaging these two values.

The tested models were those for cell growth kinetics (as a function of growth substrate consumption). An equation was also needed to model substrate depletion. For the relatively nonvolatile substrate phenol, the rate of consumption was described as

$$\frac{dS}{dt} = -\frac{\mu X}{Y_{X/S}}, \qquad [1]$$

where S is the substrate concentration, t is time, μ is specific growth rate, $Y_{X/S}$ is the biomass yield, and X is biomass concentration. To determine the yield, $Y_{X/S}$, the concentration of cells produced (cells/mL) was divided by the concentration of substrate consumed (mM).

Because toluene and benzene are volatile, Equation 1 required modification to account for the presence of toluene in both the gas and liquid phases in the bioreactor. Microbial growth rates depend on the liquid-phase substrate concentration only, whereas the biomass yield is a function of the change in total mass of substrate. Because the cultivation conditions were chosen to ensure that mass transfer rates (from gas to liquid phase) were always faster than biodegradation rates (*37*), the masses of toluene in the liquid and gas phases could be related using Henry's law, yielding

$$m_{TOT} = m_L + m_G = m_L\left[1 + \left(\frac{H}{RT}\right)\left(\frac{V_G}{V_L}\right)\right] = \alpha m_L. \quad [2]$$

Here, m refers to the mass of toluene in the gas phase (subscript G), liquid phase (L), or the entire system (TOT). H is the Henry's law constant, R is the gas constant, T is the temperature, and V_G and V_L are the gas and liquid phase volumes. Henry's law constants of 8.08×10^{-3} atm·m^3/mol for toluene and 7.31×10^{-3} atm·m^3/mol for benzene at 30°C were used (*41*). The temperature and volume of liquid remained essentially constant during an experiment, and therefore the rate of substrate consumption can be written as

$$\alpha\frac{dS_L}{dt} = -\frac{\mu(S_L)X}{Y_{X/S}}. \quad [3]$$

In most experiments, a certain amount of lag time was observed before any measurable depletion of substrate or growth of organisms occurred. Because the models do not account for this lag time, time zero for modeling was defined as the time when 2% of the substrate had been consumed.

Results

Biodegradation of chemical mixtures by pure cultures of* P. putida *F1. The first set of experiments involved the use of *P. putida* F1 to biodegrade benzene, toluene, phenol, and their binary and tertiary mixtures. In the single-substrate experiments, the growth kinetics were well fit by the Monod model,

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{\mu_{max}S_L}{K_S + S_L}, \quad [4]$$

in which μ_{max} is the maximum specific growth rate and K_S is the Monod half-saturation constant. Equation (3) was used to model the consumption (biodegradation) of toluene and benzene. The Monod model parameter values for each of the three substrates are listed in Table 1. The Monod model provided the best fit for the biodegradation of toluene and benzene by *P. putida* F1, although substrate inhibition has been reported for growth on toluene by other microorganisms (*10*). However, in the case of growth on phenol, well known as an inhibitory substrate, the fit to the experimental data was slightly improved by use of the Andrews model (*37*):

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{\mu_{max}S_L}{K_S + S_L + S_L^2/K_i}, \quad [5]$$

where K_i is an inhibition parameter. In addition, the growth pattern with phenol as a substrate was different than that for toluene in that biomass production continued for approximately 10 hr after phenol was depleted. This is indicative of the transient production of an intermediate that was than consumed for growth, and we therefore tested various models that included such an intermediate. However, none of these yielded an improved fit to the data (*37*). We chose to use the Monod model rather than the Andrews model because the differences between the model fits were small and because use of the Andrews model with its additional parameter did not improve the prediction of mixture experiments.

The results of a biodegradation experiment with toluene and phenol are shown in Figure 2. Toluene was consumed before phenol, and phenol biodegradation did not begin until toluene was nearly depleted. Although this sequential substrate consumption is reminiscent of diauxic growth, the classic definition of that phenomenon (induction or derepression of catabolic enzymes) does not apply here because *P. putida* F1 uses the same enzymes to metabolize both substrates (*35*). Similarly, when this species was grown on a 50:50 mixture of benzene and phenol, benzene was degraded first, and phenol consumption did not begin until benzene concentrations were near zero (Figure 3). In the case of the toluene–benzene mixture, *P. putida* F1 consumed both of these substrates simultaneously during most of the cultivation, but toluene biodegradation began before that of benzene, and toluene was depleted first (Figure 4).

A common model for cell growth on homologous substrate mixtures is a no-interaction sum kinetics model, in which the specific growth rate is the sum of the specific growth rates on each substrate i (μ_i). The rate of consumption for substrate i can be

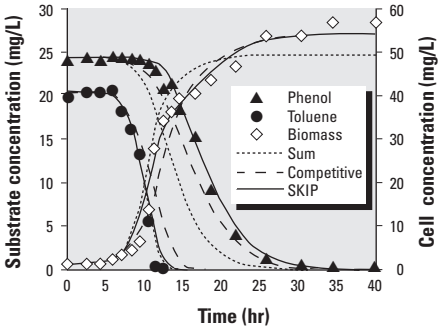

Figure 2. Experimental data and model output for batch biodegradation of a toluene–phenol mixture by *P. putida* F1. Symbols indicate measurements of liquid-phase toluene (●), phenol (▲), and biomass concentrations (◇). Lines are predictions from the sum kinetics, no-interaction model (dotted lines), competitive inhibition model (dashed lines), and SKIP model (solid lines). Adapted from Reardon et al. (*37*).

Table 1. Parameters for Monod and SKIP models of biodegradation of mixtures.[a]

Microorganism	Growth substrate	μ_m (per hour)	K_S (mg/L)	$Y_{X/S}$ (g/g)	$I_{1,2}$ (-)	$I_{2,1}$ (-)	PVE
P. putida F1	Toluene	0.86 ± 0.01	13.8 ± 0.9	1.28 ± 0.13	N/A	N/A	98.4
	Benzene	0.73 ± 0.03	0.12 ± 0.02	1.20 ± 0.05	N/A	N/A	86.6
	Phenol	0.11 ± 0.01	32.0 ± 2.4	0.80 ± 0.07	N/A	N/A	93.9
	Toluene–phenol	*	*	*	55 ± 5	0.01 ± 0.002	98.1
	Toluene–benzene	*	*	*	5 ± 0.3	0.01 ± 0.003	95.7
	Benzene–phenol	*	*	*	18.5 ± 1.5	0.01 ± 0.002	94.2
	Toluene–benzene–phenol	*	*	*	*	*	96.7
Burkholderia sp. JS150	Toluene	0.39 ± 0.01	1.01 ± 0.28	1.03 ± 0.09	N/A	N/A	96.3
	Phenol	0.31 ± 0.03	0.51 ± 0.38	0.88 ± 0.005	N/A	N/A	99.1
	Toluene–phenol	*	*	*	80.6 ± 6	0.6 ± 0.03	97.3

[a]For the parameters $I_{1,2}$ and $I_{2,1}$, subscript 1 refers to the first chemical in the pair. The notation "N/A" is shown when a parameter was not used to model growth on the substrate indicated; *indicates that previously determined values of that parameter (from single-substrate experiments) were used.

modeled using Equation 1 or Equation 3, as appropriate. Because the Monod model was found to be suitable for biodegradation of each of the three monoaromatics individually, the no-interaction sum kinetics model is

$$\mu = \frac{\mu_{max,1} S_1}{K_{S,1} + S_1} + \frac{\mu_{max,2} S_2}{K_{S,2} + S_2}, \qquad [6]$$

where the subscripts 1 and 2 refer to each of the two substrates. The predictions of this model for the toluene–phenol mixture are shown in Figure 2. Comparison of these predictions with the toluene–phenol data clearly reveals mixture effects because phenol biodegradation occurred later and at a lower specific (per cell) rate than predicted by the model. Thus, the presence of toluene inhibited phenol biodegradation. However, phenol had little effect on toluene consumption. Benzene also inhibited phenol biodegradation, although phenol did not have a significant impact on the rate of benzene metabolism (*37*). Finally, when the model was applied to toluene–benzene mixtures, the biodegradation of benzene was predicted to be earlier and faster than was actually measured, suggesting that the presence of toluene inhibited the degradation of benzene. In contrast, the presence of benzene had little effect on toluene consumption (*37*). Thus, mixture effects (i.e., nonadditivity) were found with all three pairwise combinations of these three monoaromatics.

Because *P. putida* F1 uses TDO to initiate catabolism of all three chemicals, one might expect that these mixture effects are due to competitive inhibition of this enzyme. A sum kinetics model incorporating purely competitive substrate kinetics (*18*) is

$$\mu = \frac{\mu_{max,1} S_1}{K_{S,1} + S_1 + \left(\frac{K_{S,1}}{K_{S,2}}\right) S_2} + \frac{\mu_{max,2} S_2}{K_{S,2} + S_2 + \left(\frac{K_{S,2}}{K_{S,1}}\right) S_1}. \qquad [7]$$

Predictions from this model are shown in Figures 2–4 for each of the binary mixtures. In the case of the toluene–phenol mixture, the model prediction for phenol degradation represented the data better than did the no-interaction model, but the agreement with the toluene data was worse. Thus, the one-sidedness of the mixture effect was not well predicted. Similar phenomena occurred when Equation 7 was used to predict the biodegradation of benzene–phenol and toluene–benzene mixtures. Models incorporating noncompetitive and uncompetitive interactions have also been tested, but none gave satisfactory results (*37*).

To account for these mixture effects, an alternative model was formulated by incorporating an interaction parameter $I_{i,j}$ into the sum kinetics framework (*37*):

$$\mu = \frac{\mu_{max,1} S_1}{K_{S,1} + S_1 + I_{2,1} S_2} + \frac{\mu_{max,2} S_2}{K_{S,2} + S_2 + I_{1,2} S_1} \qquad [8]$$

Here, $I_{i,j}$ indicates the degree to which substrate *i* affects the biodegradation of substrate *j*, with larger values corresponding to stronger inhibition. Yoon et al. (*18*) were the first to propose a model of this type, which we call sum kinetics with interaction parameters (SKIP). To obtain the values of the interaction parameters (Table 1), the SKIP model was fitted to each set of binary mixture data sets using values of μ_m, K_S, and $Y_{X/S}$ determined from the single-substrate experiments. The fitted SKIP model accurately describes the biodegradation data for all three binary mixtures (Figures 2–4), demonstrating that the SKIP model can be used to fit unspecified types of inhibition between two substrates.

The ability of the SKIP model to predict the outcome of the 3-substrate mixture was also examined. As shown in Figure 5, the consumption of toluene began first, followed by benzene, and these two chemicals were then degraded simultaneously. Significant phenol consumption did not begin until the toluene concentration was nearly zero and the benzene concentration was low. A three-term version of Equation 8 successfully predicted this pattern using parameters determined independently from the one- and two-substrate mixture experiments (*37*).

Biodegradation of chemical mixtures by pure cultures of* Burkholderia *sp. strain JS150. A second study, using *Burkholderia* sp. strain JS150, was performed to investigate mixed-substrate biodegradation by a bacterium that employs different catabolic pathways to degrade the mixture components. The Monod model was found to fit the biodegradation data well for both toluene and phenol (Table 1) (*38*). During growth of strain JS150 on phenol, the release of several metabolites into the medium was noted, and one was identified as 2-hydroxymuconic semialdehyde (*38*). However, these metabolites did not inhibit phenol consumption.

When strain JS150 was grown on an equimolar solution of toluene and phenol,

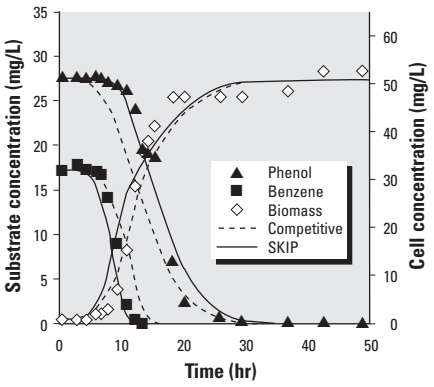

Figure 3. Experimental data and model output for batch biodegradation of a benzene–phenol mixture by *P. putida* F1. Symbols indicate measurements of liquid-phase benzene (■), phenol (▲), and biomass concentrations (◇). Dashed lines are predictions from the competitive inhibition model, and solid lines are curve fits for the SKIP model. Reprinted from Reardon et al. (*37*) with permission from John Wiley & Sons, Inc.

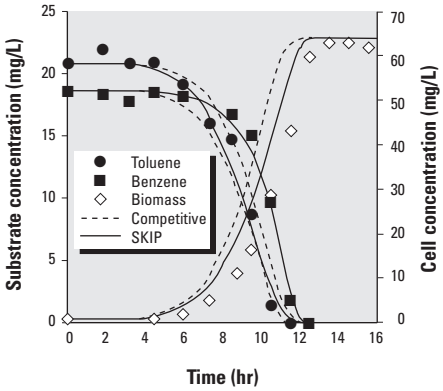

Figure 4. Experimental data and model output for batch biodegradation of a toluene–benzene mixture by *P. putida* F1. Symbols indicate measurements of liquid-phase toluene (●), benzene (■), and biomass concentrations (◇). Dashed lines are predictions from the competitive inhibition model, and solid lines are curve fits for the SKIP model. Reprinted from Reardon et al. (*37*) with permission from John Wiley & Sons, Inc.

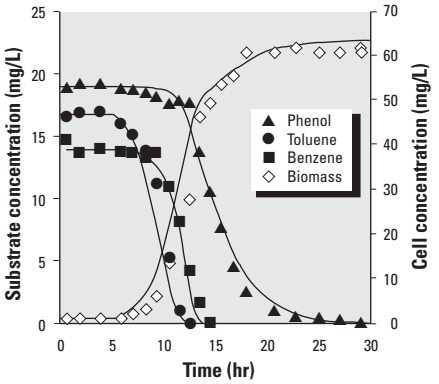

Figure 5. Experimental data and SKIP model predictions for batch biodegradation of a toluene–benzene–phenol mixture by *P. putida* F1. Symbols indicate measurements of liquid-phase toluene (●), benzene (■), phenol (▲), and biomass concentrations (◇); lines are model predictions. Reprinted from Reardon et al. (*37*) with permission from John Wiley & Sons, Inc.

toluene consumption began first. However, phenol was degraded while toluene was present in the medium, in contrast to the experiments with *P. putida* F1. The use of the no-interaction mixtures model (Equation 6) revealed that the presence of each substrate had an inhibitory effect on the biodegradation of the other. Competitive (Equation 7), noncompetitive, and uncompetitive inhibition models were also tested, although not mechanistically supported because strain JS150 uses multiple biodegradation pathways (*12*). The predictions from all three models were poor. Finally, the SKIP model was applied, with $I_{T,P}$ and $I_{P,T}$ values obtained by fitting Equation 8 to the data. The model fits were very good (PVE = 97.3%), and the values of the interaction parameters indicate that toluene inhibited phenol degradation much more than the reverse.

Biodegradation of single chemicals by mixed cultures. Biodegradation models for mixed cultures often treat the microorganisms as a single lumped quantity (e.g., total biomass). However, this was shown to be inadequate in the case of a 1:10 mixture of strain JS150 and *P. putida* F1 growing on phenol (*39*), suggesting that interactions between these two species were important. Further evidence for complex interactions was obtained by cultivating 1:1 mixtures of these species on phenol and measuring the sizes of the two populations using the FISH protocol (Figure 6). The resulting kinetics did not follow a model derived from the concept of pure-and-simple competition, in which the only interaction is competition for a growth-limiting substrate. Instead, *P. putida* F1 grew much more than the model predicted, and strain JS150 grew less than predicted by this simple competition model. Further investigation demonstrated that strain JS150 released a metabolite, probably 2-hydroxymuconic semialdehyde, which strain F1 was able to use as a growth substrate, and thus the interaction between the two species included commensalism in addition to competition (*39*). Further complexity was added by including *Bacillus subtilis* American Type Culture Collection 7003, a species unable to grow on phenol, in the mixed culture. When medium containing phenol was inoculated with a 1:1:1 ratio of the three microorganisms, *B. subtilis* grew to a greater extent than did species JS150, presumably by competing for metabolic intermediates (*40*).

Purely competitive interactions were also insufficient to describe the dynamics between strains JS150 and F1 when similar experiments were conducted with toluene (Figure 7). In this case, *P. putida* F1 grew more slowly and to a lesser extent than predicted by the pure-and-simple model. Using spent medium tests, this was determined to be the result of inhibition by an unidentified chemical released by species JS150 (*39*). Thus, amensalism occurred along with competition when these species grew together on toluene.

Biodegradation of a chemical mixture by a mixed culture. Finally, the biodegradation and growth kinetics of the 1:1 mixed culture of strains JS150 and F1 were examined for an equimolar mixture of toluene and phenol. The experimental results for the aromatic hydrocarbons and both microbial populations are shown in Figure 8, along with the predictions of a model based on the SKIP representation of substrate consumption (Equation 8) and the pure-and-simple kinetics representation of microbial growth. As was the case when this mixed culture was grown on either toluene or phenol alone, the model predictions were poor. *P. putida* F1 grew faster and to a greater extent than predicted by the model, and the growth of strain JS150 was less than predicted. Given the conflicting impacts on strain F1 in the phenol-only and toluene-only cultivations noted above, it is interesting to note that the phenol pattern dominated in this mixed substrate experiment. In addition the concentrations of both substrates reached nondetect levels sooner than predicted by the model, indicating that the mixed culture is able to degrade the mixture faster than either pure culture alone.

Discussion

The results presented here clearly illustrate that the biodegradation kinetics of chemical mixtures can be complex and difficult to describe mathematically, even when the chemicals serve as homologous substrates for pure cultures of microorganisms. Although these kinetics can in some cases be described by relatively simple no-interaction (*16*) or competitive inhibition (*9,18,20*) models, we have demonstrated that such models are inadequate for *P. putida* F1 growing on mixtures of toluene, benzene, and phenol and for *Burkholderia* sp. JS150 growing on mixtures of toluene and phenol. Furthermore, the biodegradation kinetics of a mixed culture growing on 1-butanol, 2-butoxyethanol, and *N,N*-dimethylethanolamine also were not well predicted by competitive inhibition (*42*). These findings led us to develop the SKIP model, in which a fitting parameter, $I_{i,j}$ was introduced to describe the influence of chemical *i* on the rate of biodegradation of chemical *j*. Using $I_{i,j}$ values obtained from the two-chemical experiments, we demonstrated the ability of the model to predict the outcome of the three-chemical biodegradation experiments. The SKIP framework has also been used as the basis of a model in

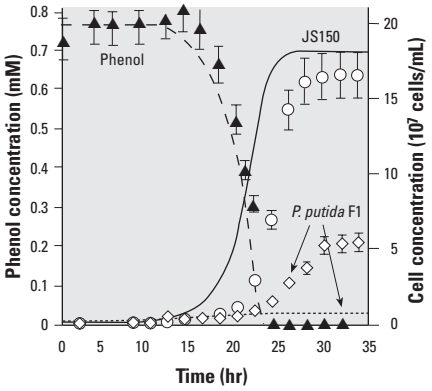

Figure 6. Experimental data and model predictions for batch biodegradation of phenol inoculated with a 1:1 mixture of strains JS150:*P. putida* F1. Symbols represent the experimental data values for phenol (▲), strain JS150 (○), and strain F1 (◇). Lines depict the pure-and-simple competition model output. Error bars represent one standard deviation based on replicate analyses of each sample. Reprinted from Bull Rogers et al. (*39*) with permission from John Wiley & Sons, Inc.

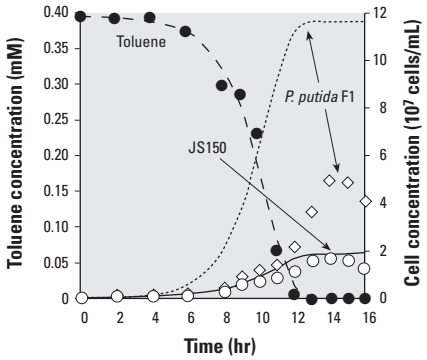

Figure 7. Experimental data and model predictions for batch biodegradation of toluene inoculated with a 1:1 mixture of strains JS150:*P. putida* F1. Symbols represent the experimental data values for toluene (●), strain JS150 (○), and strain F1 (◇). Lines are the pure-and-simple competition model output. Reprinted from Bull Rogers et al. (*39*) with permission from John Wiley & Sons, Inc.

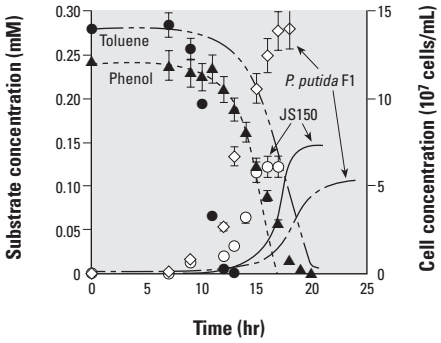

Figure 8. Experimental data and model predictions for batch biodegradation of a toluene–phenol mixture by the 1:1 binary mixture of strains JS150:*P. putida* F1. Symbols represent the experimental data values for toluene (●), phenol (▲), strain JS150 (○), and strain F1 (◇). Lines are predictions from a pure-and-simple competition model between the species and the SKIP model for substrate interactions. Reprinted from Bull Rogers et al. (*39*) with permission from John Wiley & Sons, Inc.

which a 13-chemical mixture was divided into four groups and $I_{i,j}$ values determined for interactions between groups. In cases without substrate inhibition, this modified SKIP model accurately predicted the experimental outcomes (*43*).

Although the differences between the predictions of the SKIP and other models are highly significant in a statistical sense, their impacts do not necessarily appear large in the batch experiments presented here. The novelty of the SKIP model is the inclusion of inhibition terms that are different than those in purely competitive, uncompetitive, or noncompetitive inhibition. In the case of the toluene–phenol mixture, toluene inhibits phenol consumption to a much greater extent than predicted by the other models, and phenol inhibition of toluene degradation is much less. In a batch experiment, the main outcomes of this inhibition are a prolonged lag phase before phenol consumption begins and a faster toluene degradation rate. Because toluene is rapidly consumed in this batch cultivation, the impacts on phenol degradation are relatively small. However, in a continuous-flow bioreactor, the differences among these various models would be much more noticeable. Because phenol is not consumed until toluene concentrations fall below some low level (only accurately represented by the SKIP model), the hydraulic residence times and sizes of continuous bioreactors treating toluene–phenol mixtures would be substantially underpredicted unless the SKIP model were used. Because aquifers can also be represented as continuous-flow bioreactors, the result of using the SKIP versus another model would be similar but expressed in terms of the size of the contaminant plume and the length of time required for remediation.

Despite the success of the SKIP model in the cases presented here, the need to include the fitting parameter $I_{i,j}$ with no clear mechanistic basis is unsatisfying. This is particularly true in the case of *P. putida* F1, where the same set of enzymes appears to be involved in the biodegradation of toluene, benzene, and phenol. We have investigated this phenomenon further using two-dimensional polyacrylamide electrophoresis of soluble proteins (*44*). Although this proteomic study has not been completed, our current evidence points to differences in the cell membrane composition as one of factors involved in these unexpected kinetics. Based on the identification of acyl carrier protein as one of the proteins with transient synthesis during biodegradation of toluene–phenol mixtures, we performed analyses of the phospholipid fatty acid content of *P. putida* F1 cells. The predominant phospholipid fatty acid of cells growing on toluene was *cis*-7-hexadecenoic acid (16:1w7c), whereas cells growing on phenol had high levels of cyclopropylheptadecanoic acid (cy17:0) in their membranes. The membranes of cells growing on toluene–phenol mixtures shifted from 16:1w7c to cy17:0 after degradation of toluene in the medium was complete (*45*). Based on these findings, we have developed the hypothesis that the inhibition of phenol biodegradation in the presence of toluene is caused by very slow transport of phenol into the cell when the membrane has adapted to the more hydrophobic environment. Then, when toluene is depleted from the medium, the membrane composition shifts to a form through which phenol can more readily diffuse. A model based on this hypothesis has been shown to predict toluene–phenol mixture results very well using only data from single-substrate experiments. We are continuing our investigations into this hypothesis and will also consider the implications of other proteins that are differentially expressed by cells growing on toluene versus phenol.

We have also shown here that the interactions between microbial species in a mixed culture are both significant for the biodegradation kinetics and difficult to predict. In particular, we noted a large effect of the carbon source on the nature of the microbial interactions, with commensalism occurring when the cells grew on phenol and amensalism observed when toluene was the growth substrate. We also noted that the presence of a secondary degrader (*B. subtilis*) had an additional impact on the biodegradations by introducing a new type of interaction. Although the mechanism of these interactions could be determined after they were observed, it seems unlikely that they could be predicted from pure culture experiments without prior knowledge of all possible metabolites produced by each species. Finally, it is interesting to consider the question of whether microbial species interactions become less important as the mixed cultures become more diverse. For example, although the SKIP model alone did not describe the kinetics of the binary *P. putida* F1/*Burkholderia* sp. JS150 culture when "total biomass" was used in the model, it was very accurate in describing the biodegradation kinetics of a larger (estimated 10–20 species) mixed culture growing on a mixture of 13 organic chemicals (*43*).

Conclusions. Although the biodegradation kinetics of mixed microbial cultures growing on mixtures of organic contaminants are often assumed to be simple extensions of pure-culture/single-substrate kinetics, we have demonstrated that they are not. In the case of pure cultures growing on aromatic chemical mixtures, neither a no-interaction nor a competitive inhibition model accurately predicted the mixture kinetics. To overcome this difficulty, we developed the SKIP model, which used model parameters from single- and dual-substrate mixture experiments to accurately predict the outcome of the 3-substrate mixture experiment. When we conducted similar experiments with a binary mixed culture rather than pure cultures, we found that interactions between the species had a significant impact on the biodegradation kinetics, and that the nature of these interactions depended on the growth substrate(s).

These findings reveal the significant challenges that face efforts to model real-world biodegradation kinetics, in which mixed substrates and mixed cultures are the rule. Predictive modeling of these systems will be difficult and time-consuming if one must determine all pairwise chemical interactions (e.g., as required by the SKIP model) and all species interactions (with corresponding concentrations of inhibitors and metabolic intermediates). Options to these traditional approaches may be developed through a fundamental understanding of the effects involved [e.g., as hinted at by the proteomic results in (*44,45*)] and by alternative modeling approaches such as that presented by Liao et al. in this volume (*46*).

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