Biodegradation Kinetics of Benzene, Toluene, and Phenol as Single and Mixed Substrates for *Pseudomonas putida* F1

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Abstract: Although microbial growth on substrate mixtures is commonly encountered in bioremediation, wastewater treatment, and fermentation, mathematical modeling of mixed substrate kinetics has been limited. We report the kinetics of *Pseudomonas putida* F1 growing on benzene, toluene, phenol, and their mixtures, and compare mathematical models to describe these results. The three aromatics are each able to act as carbon and energy sources for this strain. Biodegradation rates were measured in batch cultivations following a protocol that eliminated mass transfer limitations for the volatile substrates and considered the culture history of the inoculum and the initial substrate to inoculum mass ratio. Toluene and benzene were better growth substrates than phenol, resulting in faster growth and higher yield coefficients. In the concentration ranges tested, toluene and benzene biodegradation kinetics were well described by the Monod model. The Monod model was also used to characterize phenol biodegradation by *P. putida* F1, although a small degree of substrate inhibition was noted. In mixture experiments, the rate of consumption of one substrate was found to be affected by the presence of the others, although the degree of influence varied widely. The substrates are catabolized by the same enzymatic pathway, but purely competitive enzyme kinetics did not capture the substrate interactions well. Toluene significantly inhibited the biodegradation rate of both of the other substrates, and benzene slowed the consumption of phenol (but not of toluene). Phenol had little effect on the biodegradation of either toluene or benzene. Of the models tested, a sum kinetics with interaction parameters (SKIP) model provided the best description of the paired substrate results. This model, with parameters determined from one- and two-substrate experiments, provided an excellent prediction of the biodegradation kinetics for the three-component mixture. *Biotechnol Bioeng* **69:** 385–400, 2000.
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INTRODUCTION

Biodegradation of Pollutant Mixtures

Organic chemical mixtures are prevalent in waste waters 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 impacted by other substituents of the mixture (Egli, 1995; Klečka and Maier, 1988; Meyer et al., 1984; Saéz and Rittmann, 1993). 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 (1982) 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 (McCarty et al., 1984; Schmidt and Alexander, 1985) or induction of required degradative enzymes (Alvarez and Vogel, 1991). More commonly, negative interactions are reported. Reasons for decreased biodegradation rates include competitive inhibition (Bielefeldt and Stensel, 1999; Chang et al., 1993; Oh et al., 1994), toxicity (Haigler et al., 1992), and the formation of toxic intermediates by nonspecific enzymes (Bartels et al., 1984; Klečka and Gibson, 1981).

Although mathematical models of mixed homologous substrate consumption and microbial growth have been proposed (e.g., Bielefeldt and Stensel, 1999; Klečka and Maier, 1988; Kompala et al., 1986; Lendenmann et al., 1996; Ni-

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kolajsen et al., 1991; Tsao and Hanson, 1975; Yoon et al., 1977), 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 (Lendenmann et al., 1996), the growth of a mixed culture on benzene, toluene, ethylbenzene and *o*- and *p*-xylene (BTEX compounds) (Bielefeldt and Stensel, 1999), and the biodegradation of three polycyclic aromatic hydrocarbons (Guha et al., 1999).

Pseudomonas putida F1 and Biodegradation of Monoaromatics

Monoaromatic compounds are an obvious choice for mixture biodegradation studies. These molecules are produced in huge amounts and are used in fuels, as solvents, and as starting materials for the production of plastics, synthetic fibers, and pesticides (Budavari, 1996). Through spills, leakage from tanks, and other releases monoaromatics have become prevalent environmental contaminants, usually in mixtures. Thirty monoaromatics are on the EPA Priority Pollutant List (EPA, 1996), and there are 11 of these compounds in the top 100 chemicals on the Priority List of Hazardous Substances published by the Agency for Toxic Substances and Disease Registry (ATSDR, 1997).

Microorganisms have been isolated that grow on toluene (e.g., Alvarez and Vogel, 1991; Chang et al., 1993), benzene (e.g., Gibson et al., 1968a; Kukor and Olsen, 1991), phenol (e.g., Hutchinson and Robinson, 1988; Murray and Williams, 1974), chlorobenzene (Haigler et al., 1992), and nitrotoluene (Haigler et al., 1994), among others. Most of these microbes are able to grow on two or more aromatic compounds. Of these aromatic hydrocarbon-degrading bacteria, *Pseudomonas putida* F1 is perhaps the best characterized. This strain can use toluene, benzene, ethylbenzene, phenol, and other aromatics as sole carbon and energy sources (Gibson et al., 1990). Like other pseudomonads, many of its induced enzymes are nonspecific and its metabolic pathways contain a high degree of convergence. The convergence of catabolic pathways allows for the efficient utilization of a wide range of growth substrates, while the nonspecificity of the induced enzymes allows for the simultaneous utilization of several similar substrates without an excess of redundant genetic coding for enzyme induction (Hutchinson and Robinson, 1988). *P. putida* F1 has also received attention for its ability to cometabolize the biodegradation of trichloroethene in the presence of toluene and other aromatics (Wackett and Gibson, 1988).

The biodegradation of toluene by *P. putida* F1 has been studied extensively. In the catabolic pathway (Fig. 1), toluene is first oxided to *cis*-toluene dihydrodiol through the action of toluene dioxygenase (TDO) (Finette et al., 1984; Gibson et al., 1990; Yeh et al., 1977). *cis*-Toluene dihydrodiol is dehydrogenated to form 3-methylcatechol, which is

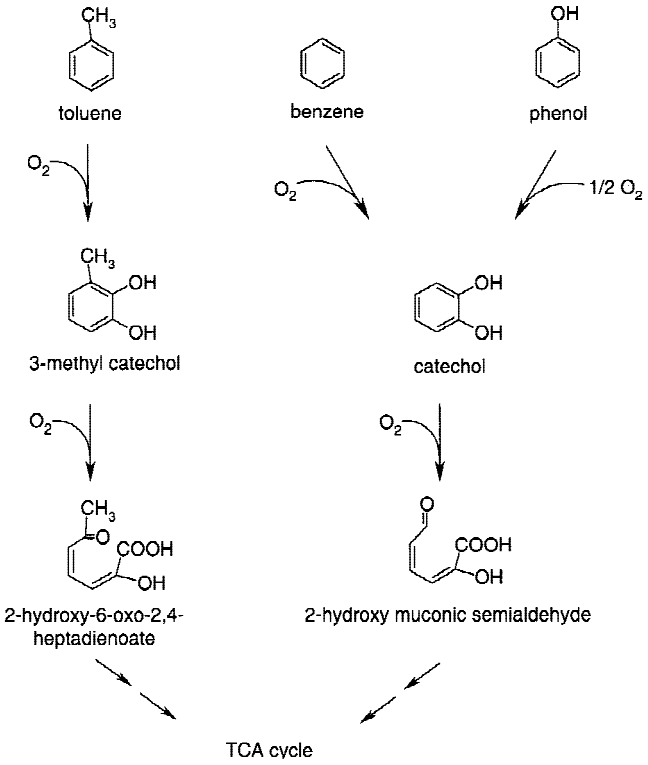

Figure 1. *P. putida* F1 catabolic pathways for toluene, benzene, and phenol.

cleaved at the *meta* position and then converted in three steps to acetaldehyde and pyruvate (Lau et al., 1994; Menn et al., 1991; Zylstra and Gibson, 1989).

In early work on benzene metabolism by *P. putida* F1, a multicomponent benzene dioxygenase was identified (Axcell and Geary, 1985; Gibson et al., 1968a). Further studies detailed similarities between this benzene-hydroxylating enzyme and TDO (Irie et al., 1987; Subramanian et al., 1985; Yeh et al., 1977), and Spain et al. (1989) used TDO mutants to demonstrate that *P. putida* F1 used TDO to grow on benzene. The *P. putida* F1 degradation pathway for benzene (Fig. 1) thus involves the addition of two atoms of oxygen by TDO to form *cis*-benzene dihydrodiol followed by dehydrogenation to catechol, *meta* ring-cleavage, and further transformation to TCA cycle intermediates.

Toluene dioxygenase has also been identified as the enzyme responsible for the initial step in the metabolism of phenol by *P. putida* F1 (Spain and Gibson, 1988; Spain et al., 1989). TDO catalyzes the monohydroxylation of phenol to catechol, which is transformed as described for benzene (Fig. 1).

In the work reported here, we report on the biodegradation kinetics of toluene, benzene, and phenol, individually and in mixtures, by *P. putida* F1. Since these three compounds are homologous substrates that are catabolized by the same enzymes in *P. putida* F1, their mixture is an interesting model system for mixture biodegradation studies. While this pure culture system lacks the complexity of natural soil communities or those in wastewater treatment units,

it is a reasonable starting point for the development and validation of mathematical models for mixtures of more than two homologous substrates. In addition to experimental and modeling results, we describe a protocol for determining biodegradation kinetics in which mass transfer limitations for volatile compounds are eliminated and the effects of inoculum size and history are checked.

MATERIALS AND METHODS

Determination of Biodegradation Kinetics Model Parameters

The determination of growth kinetics model parameters is influenced by many factors, especially parameter identifiability, culture history, and the assay procedure used (Grady et al., 1996; Jannasch and Egli, 1993; Kovárová-Kovar and Egli, 1998). Parameter identifiability was established by establishing a sufficiently low inoculum size and relatively high initial substrate concentration, and culture history effects were examined and found to be minimal (details below). The last factor concerns the mode of cell cultivation (e.g., batch, continuous) that was used to obtain the data to which a model is regressed. Continuous cultivation offers the ability to provide a constant environment to the cells and to maintain cells at low substrate concentrations, and may result in more accurate estimates of affinity constants (e.g., K_s in the Monod model) than batch cultivation. However, estimation of the maximum specific growth rate may be less accurate in continuous cultivations, and results may be affected by wall growth and mutation over the long cultivation periods required by this technique. Furthermore, it may be difficult to maintain continuous cultures under conditions of strong substrate or product inhibition. For the work presented here, we chose to use batch cultures to measure biodegradation kinetics, evaluate models, and determine model parameters. In particular, we wanted to be able to observe substrate inhibition, patterns of substrate consumption, and related physiological changes.

Since each of the three monoaromatics was used as the sole source of carbon and energy and was the limiting growth substrate, all models consisted of two parts: (1) an equation for the specific growth rate, μ, as a function of substrate concentration, and (2) an equation for substrate depletion. Various models for $\mu(S)$ were tested. For phenol, a low volatility chemical, the substrate depletion equation was:

$$\frac{dS}{dt} = -\frac{\mu X}{Y_{X/S}} \tag{1}$$

In this expression, S is substrate concentration, t is time, μ is specific growth rate, $Y_{X/S}$ is the biomass yield, and X is biomass concentration. For the volatile substrates, benzene and toluene, this equation required modification since these compounds were present in both the liquid and gas phases of the bioreactor. Microbial growth rates depended on the liquid phase concentration but the biomass yield depended on the change in the total mass of substrate. The masses of a volatile compound in the gas and liquid phases can be related using Henry's law, yielding:

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

In Eq. 2, m refers to the mass of the volatile substrate in the gas phase (G), liquid phase (L), or in 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 volumes of the gas and liquid phases. Henry's law constants of 8.08×10^{-3} atm $\cdot$ m^3/mol for toluene and 7.31×10^{-3} atm $\cdot$ m^3/mol for benzene at 30°C were used (Montgomery, 1996). Since the temperature was fixed and the volumes of the gas and liquid phases remained essentially unchanged throughout the experiments, the liquid and total substrate concentrations are related by a constant, α. The substrate utilization equation for benzene and toluene was obtained by combining Eq. (2) with a mass balance and contains only liquid-phase substrate concentrations (S_L):

$$\alpha\frac{dS_L}{dt} = -\frac{\mu(S_L)X}{Y_{X/S}} \tag{3}$$

The use of Henry's law in the calculation of α requires that mass transfer from the gas phase is rapid relative to the reaction rate, and this was verified (see below).

The value of the biomass yield on substrate, $Y_{X/S}$, was calculated directly from experimental data (cell mass produced/total mass substrate consumed). Lag times were identified as the time before which less than 2% of the substrate had been consumed. All other model parameters were determined by fitting the proposed model to the experimental data using SimuSolv™ (ver. 3.0). 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. The method of maximum likelihood is superior to ordinary and weighted least-squares analysis for nonlinear systems when the covariance matrix of the measurement errors is known (Robinson, 1985). In this work, knowledge of measurement errors (GC-MS analysis, OD measurements) was good, allowing the method of maximum likelihood to be used. Each model's parameter correlation matrix was determined (using SimuSolv™) and checked for uniqueness of the fitted parameters. For each model, μ_{max} and K_s were found to be unique, with parameter correlation matrix elements <0.85. In addition to maximizing the LLF, plots of residuals were investigated. Models with residual plots showing significant hetereoscedastic errors were judged as less than ideal. The model with the maximum LLF value and most homogeneous error residual plot was chosen. For each final model, the percent variation explained (PVE;

similar to the r^2 value for linear regression) was calculated using the LLF and reported.

Microorganism and Media

P. putida F1 was obtained from David Gibson of the University of Iowa. Cultures grown on toluene vapors were maintained at −70°C in glycerol. For all experiments, a modified Hutner's mineral base (MSB) was used as the carbon-free medium (Cohen-Bazire et al., 1957). Before addition of the carbon source, this solution contained 5.65 g/L Na_2HPO_4, 5.44 g/L KH_2PO_4, 1.00 g/L $(NH_4)_2SO_4$, 0.2 g/L nitrilotriacetic acid, 0.29 g/L $MgSO_4$, 0.07 g/L $CaCl_2 \cdot 2H_2O$, 0.19 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 8.3 mg/L $FeSO_4 \cdot 7H_2O$, 3.1 mg/L EDTA, 13.7 mg/L $ZnSO_4 \cdot 7H_2O$, 1.9 mg/L $MnSO_4 \cdot 7H_2O$, 0.5 mg/L $CuSO_4 \cdot 5H_2O$, 0.3 mg/L $Co(NO_3)_2 \cdot 6H_2O$), and 0.2 mg/L $Na_2B_4O_7 \cdot 10H_2O$. Benzene, toluene, and/or phenol were added as described below. The growth medium was carbon-limited in all experiments.

Chemicals

Benzene (Sigma, St. Louis, MO; HPLC Grade), toluene (Baker, HPLC Grade), and phenol (Sigma, >99.5% pure) were used as the carbon sources. Chloroform and *p*-xylene (both Baxter GC Grade) were used to prepare samples for gas chromatography. 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 and correlated to biomass concentration. McFarland tubidity standards (Koch, 1994) were used to eliminate effects of spectrophotometer sensitivity drift. Since the culture medium changed color during the biodegradation of benzene and phenol, 0.22 μm-filtered samples were used as optical density blanks for those experiments. Deionized water was used as the OD blank for the toluene experiments. The OD-mass correlation was linear over the cell concentration range (up to 300 mg dry cell weight/L) with 1.00 OD_{600} = 1000 mg/L.

Benzene, toluene, and phenol concentrations were measured by gas chromatography. Aqueous samples were extracted (0.75 mL of aqueous sample to 0.75 mL of chloroform containing 25 mg/L *p*-xylene as an internal standard). The chloroform layer was removed and analyzed using an HP 5890 II gas chromatography equipped with a mass selective detector (HP 5971A). Compounds were separated on a 50 m, 0.2 mm HP-5 column. High-purity helium flowed through the column at 1.9 mL/min and 45 psi. The temperatures of the inlet and detector were 200 and 280°C, respectively. The oven temperature was held at an initial value of 35°C for 1.5 min, raised at 50°C/min to 57°C, where it was held for 3 min, then increased at 10°C/min to 100°C, and finally 15°C/min to 160°C. 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 limits of this method for each of the three compounds was 5 μM.

Gas phase concentrations of benzene and toluene were also determined via gas chromatography using the same GC column and conditions. Samples were taken using a 25-μL gas-tight syringe and then injected into the GC-MS. Gas-phase standards were taken from the headspace over aqueous solutions at equilibrium. The detection limits of this method were 50 μM for both benzene and toluene.

Aqueous intermediates that formed during biodegradation experiments were detected by high-pressure liquid chromatography (Beckman, Palo Alto, CA; System Gold). Samples filtered before injection onto a 4.6 × 150 mm C8 column (Rainin Instrument Co.) with a particle size of 5 μm and a pore size of 100 Å. The solvents were sulfuric acid (H_2SO_4; pH 2.5) and acetonitrile (CH_3CN). The solvent flow rate was 1.0 mL/min and the elution profile was 65% H_2SO_4/35% CH_3CN for 1 min, then changed linearly to 40% H_2SO_4/60% CH_3CN over a 6-min period and held at this ratio for 7 min, and finally changed linearly back to 65% H_2SO_4/35% CH_3CN over a 2-min period and held at this ratio for 6 min.

Protocol for Batch Biodegradation Experiments

Biodegradation rate data were obtained from batch cultivations. The inocula for these cultivations were taken from continuous cultivations of *P. putida* F1 maintained at a dilution rate of 0.083 h^{-1} in a 2.0-L New Brunswick BioFlo I continuous culture bioreactor with a working volume of 1.5 L. The feed to this chemostat was MSB plus benzene, toluene, and phenol, each at a concentration that resulted in 35 mg/h fed to the culture. The continuous culture was inoculated from freezer stock before each experiment. The vessel was agitated at 200 RPM and the temperature was controlled at 30°C.

Two 3-L stirred-tank bioreactors (Applikon) were used. Each was fitted with a six-bladed Rushton turbine impeller, and DO (Ingold polarographic) and pH (Ingold) probes. During the runs, the temperature was controlled at 30°C. Although the pH was not controlled, it remained in the range 6.7–6.9. The agitation rate was set at 500 RPM after mass transfer testing (see below).

Since two of the substrates were highly volatile, no air sparging was used; i.e., the bioreactor was run as a closed system after inoculation, with the exception of sampling. The liquid volume used (1.6 L) left a headspace that contained sufficient oxygen for the growth of the cells on the mass of substrate provided. DO levels were monitored and did not fall below 5 mg/L. Since a K_S of 0.048 mg/L for oxygen has been reported for *P. putida* NRRL β-14875 growing on phenol (Şeker et al., 1997), and other sources (e.g., Shuler and Kargi, 1992) state that aerobic bacterial

growth is generally independent of oxygen at concentrations above 0.4 mg/L, we may reasonably assume that oxygen was not the growth rate-limiting substrate in our experiments. Benzene and toluene were added as liquids after the bioreactor and medium had been autoclaved and cooled. The bioreactors were then stirred and maintained at 30°C for at least 12 h to allow equilibrium of the benzene or toluene between the liquid and gas phases. When phenol was a substrate, it was added before autoclaving. The inoculum for each batch culture was taken directly from the chemostat in a syringe. On the basis of preliminary tests (below), the volume of the inoculum was adjusted based on the optical density of the chemostat culture and the substrate concentration in the batch bioreactor to provide an initial substrate concentration/initial biomass concentration ratio (S_0/X_0) of 300 g/g. The S_0/X_0 ratios and ratios of initial substrate concentration to Monod constant (S_0/K_S) were large enough to ensure that "intrinsic" kinetic parameters, as defined by Grady et al. (1996), were obtained. The batch biodegradation experiments were performed in duplicate, with repeated experiments run at separate times to provide an accurate assessment of variability.

Control and Preliminary Experiments

Volatile Losses

Since volatile chemicals were used in some experiments, there was the possibility of volatilization of the compounds during an experiment. To check this, control experiments were performed with sterile blank inocula of MSB instead of cells. Both benzene and toluene were tested using a stirrer speed of 200 RPM. Over an 80-h period, the concentration of each volatile chemical remained unchanged (within the experimental error of the analytical method).

Inoculum Size

The size of the inoculum relative to the substrate concentration is important for accurate determination of biodegradation/growth kinetics since experiments in which inocula are too large do not result in population doublings (Chudoba, 1989). The effect of the inoculum size, expressed as an initial substrate mass to initial biomass ratio (S_0/X_0), was evaluated in batch cultivations with benzene. The S_0/X_0 ratios tested were 30, 176, 391, and 1,270 g/g. For the lowest ratio, the benzene was completely consumed with little increase in biomass. The other inoculum sizes produced good growth, although the S_0/X_0 of 176 g/g resulted in a fairly linear biomass trajectory. Use of smaller inocula resulted in longer lag times. An S_0/X_0 of 300 g/g was chosen for the biodegradation kinetics experiments.

Culture History

Researchers have found that the culture history of an inoculum affects the growth and substrate utilization kinetics (Höfle, 1984; Templeton and Grady, 1988). To screen for this effect, the results of two batch cultivations were compared using benzene as the carbon source. The chemostat was operated at 0.048 h^{-1} to produce inoculum for one culture and at 0.078 h^{-1} for the other. Both batch cultures were inoculated at an S_0/X_0 of 450 g/g. Small differences were evident in the shape of the growth curve but the effect of inoculum history in this range was judged to be minimal (D. C. Mosteller, M.S. thesis, Colorado State University). The dilution rate was set at 0.083 h^{-1} for the remainder of the project.

Elimination of Mass Transfer Limitations

Measurement of intrinsic biodegradation kinetics for volatile compounds can be obscured by slow mass transfer rates of the compound from the vapor to liquid phase. To evaluate this possibility and select conditions under which mass transfer was not rate-limiting, two types of preliminary experiments were performed at each of two agitation speeds (200 and 500 RPM).

The purpose of the first type of experiment was to measure the mass transfer coefficients for benzene and toluene. Two procedures were used: (1) creating a constant headspace concentration of the volatile organic and measuring the aqueous phase concentration of the pollutant as a function of time (200 RPM), or (2) injecting small amounts (~100 mg/L) of benzene or toluene into the liquid in the reactor and passing air through the headspace, creating a gas phase that contained negligible levels of the volatile chemical (500 RPM) (D. C. Mosteller, M.S. thesis, Colorado State University). Mass transfer kinetics were assumed to be described by

$$\frac{dC_L}{dt} = k_L a(C_L^* - C_L) \tag{4}$$

where C_L^* is the liquid equilibrium concentration calculated using the gas concentration data and the Henry's law coefficient, C_L is the liquid concentration of the organic pollutant, k_L is the mass transfer coefficient, and a is the interfacial surface area. In this case, the lumped parameter $k_L a$ was determined by measuring gas and liquid phase concentrations and fitting the data to the integrated form of Eq. (4) using a least-squares method. The $k_L a$ values calculated at 200 RPM for benzene and toluene were 0.66 h^{-1} and 0.82 h^{-1}, respectively, while at 500 RPM the values were 2.1 h^{-1} and 2.6 h^{-1}, respectively.

These $k_L a$ values were then used in biodegradation experiments with different agitation rates to assess whether or not the rate of benzene or toluene mass transfer from gas to liquid phase was the slowest step in the biodegradation process. The substrate concentration was measured in both the gas and liquid phases during these experiments and those data were used to calculate the mass transfer rate and observed disappearance rate. The initial substrate concentration was also varied since a smaller initial substrate con-

centration results in lower biomass levels, which in turn influences the rate of substrate depletion according to Eq. (3). When the initial substrate concentration was 1 mM, tests of this type revealed mass transfer limitations for agitation rates of 200 and 500 RPM for both benzene and toluene. However, no mass transfer limitations were evident for either compound when the initial substrate concentration was 0.5 mM and the impeller rotation was 500 RPM. Thus, this condition was used to produce data for determination of the intrinsic biodegradation kinetics.

RESULTS AND DISCUSSION

Toluene

Data from one of the toluene biodegradation experiments are shown in Figure 2. Growth was apparent after a 7-h lag period and was exactly mirrored by toluene consumption. Toluene (initially 0.42 mM) was completely consumed in less than 15 h. The Monod model

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{\mu_{max} S_L}{K_S + S_L} \tag{5}$$

was used to describe the growth kinetics and Eq. (3) was used to model the consumption (biodegradation) of toluene. In Eq. (5), μ_{max} is the maximum specific growth rate and K_S is the Monod half-saturation constant. The Monod model fit is presented in Figure 2, and the parameter values are listed in Table I. The low standard errors indicate good agreement between the two replicate experiments. With these values, the LLF for the Monod model was 30.2, which translates to a PVE of 98.4%. We tested the importance of cellular death in these *P. putida* F1 experiments on toluene by including a death rate term in Eq. (5) and fitting it in conjunction with

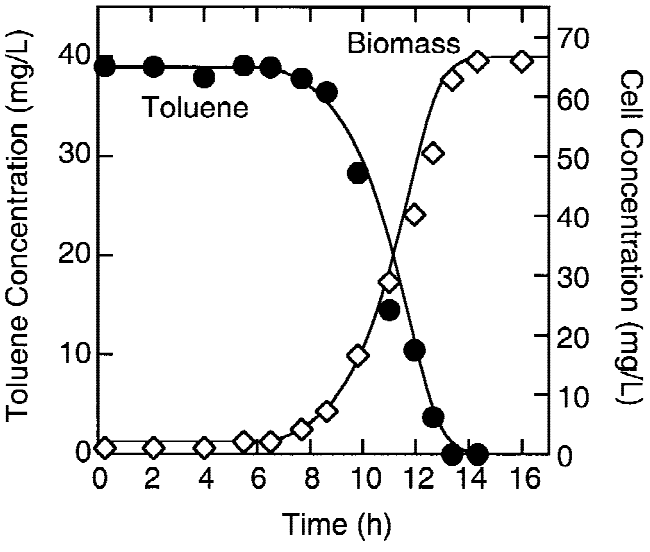

Figure 2. Results from a toluene batch biodegradation experiment with *P. putida* F1. Liquid-phase toluene concentration (●) and biomass concentration (◇) were modeled with the Monod equation (solid lines).

μ_{max} and K_S. The best model fit was found when the death rate was set to zero.

Model constants for biodegradation of toluene by six other pure cultures have been reported (Table I). Substrate inhibition was noted for three of these cultures, and the Andrews model

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{\mu_{max} S_L}{K_S + S_L + \dfrac{S_L^2}{K_i}} \tag{6}$$

was used to model the specific growth rate (K_i is an inhibition constant) (Andrews, 1968). Values of K_i near 44 mg/L were found for *P. putida* strains 54G and O1. For the third culture reported to be substrate inhibited, K_i was nearly 2,000 mg/L; however, the experiments used a maximum toluene concentration of only 30 mg/L at which inhibition would be difficult to detect. Although the initial toluene concentration in our tests with *P. putida* F1 were near the K_i values reported by others (Mirpuri et al., 1997; Oh et al., 1994), we did not observe significant substrate inhibition: the Andrews model was found to fit the data poorly compared to the Monod model (the LLF decreased by 10.1 and the PVE was <90%).

Most of the cultures in Table I have values of the maximum specific growth rate that fall into the relatively narrow range of 0.4–0.55 h^{-1}, with the exception of *P. putida* O1 (0.72 h^{-1}) and *P. putida* F1 (0.86 h^{-1}). Values of K_S, which indicate the ability of a microorganism to grow at low substrate levels, ranged from 0.1 mg/L for *P. putida* R1 to 15 mg/L for *P. putida* O1. Our value of 13.8 mg/L for the half-saturation Monod constant of *P. putida* F1 thus falls near the high end of this range. The theoretical value of the yield coefficient was determined to be 1.23 g biomass/g toluene (Shuler and Kargi, 1992). The $Y_{X/S}$ values in Table I fall into two main groups: those near 100% of theoretical, including *P. putida* F1, and those near 75% of theoretical. *P. putida* O1 is an exception with a $Y_{X/S}$ value about 50% of theoretical.

Phenol

The results of a typical phenol biodegradation experiment are shown in Figure 3. In contrast to toluene biodegradation, the apparent lag time was longer (18 h), phenol consumption was much slower, and biomass production continued for approximately 10 h after phenol was depleted. The Monod model fit to these phenol biodegradation data is shown in Figure 3, and the parameter values are listed in Table II. The overall model fit was good, with an LLF of −66.4 and 93.9% of the variation in the data explained (PVE). The value estimated for the death rate was not significantly different from zero.

Phenol degradation was also modeled using noncompetitive (Andrews) and competitive substrate inhibition models. Of these, the Andrews model described the data slightly better; it had an LLF of −64.2 and the PVE was 95.3%. The

Table I. Biodegradation model parameter values for aerobic toluene biodegradation by pure cultures

Microorganism (cultivation type)	Temperature (°C)	Maximum toluene concentration (mg/L)	μ_{max} (h^{-1})	K_S (mg/L)	K_i (mg/L)	$Y_{X/S}$ (g/g)	Reference
P. putida F1 (batch)	30	43	0.86 ± 0.01	13.8 ± 0.9	—	1.28 ± 0.13	This work
P. putida R1[1] (batch)	25	4	0.504	0.1	—	1.2	Pedersen et al., 1997
P. putida 54G (batch)	24	50	0.42 ± 0.05	3.98 ± 0.78	42.78 ± 3.87	0.90 ± 0.13	Mirpuri et al., 1997
P. putida O1 (batch)	30	70	0.72	15.07	44.43	0.64	Oh et al., 1994
P. fragi B1 (batch)	room	10	0.543 ± 0.076	1.96 ± 0.91	—	1.22 ± 0.1	Chang et al., 1993
P. sp. X1 (batch)	room	10	0.452 ± 0.115	1.88 ± 1.26	—	0.99 ± 0.25	Chang et al., 1993
P. putida ATCC 23973 (batch)	32	30	0.437	6.0	1980[2]	—	Choi et al., 1992

Parameters are for the Monod model unless a value of K_i is given, in which case the Andrews model was used. The theoretical value of $Y_{X/S}$ for growth on toluene is 1.23 g/g.

[1]A value of 0.048 h^{-1} for the specific decay rate was also reported.

[2]The validity of this value is suspect; see text.

Andrews model fit is presented in Figure 3. Although the Andrews model explained more of the variation in the data than did the Monod model, the difference is relatively small ($\Delta LLF = 2.2$), indicating that substrate inhibition had only a minor effect over the concentration range used in these experiments. In addition, an investigation of the parameter correlation matrix indicated a high correlation between μ_{max}, K_i, and K_S for the Andrews model, reducing the validity of the parameter values for that model. Since the maximum concentration in the experiments was less than 60% of K_i, the low level of inhibition observed here is not surprising.

The observation that growth continued after phenol concentrations fell to zero was indicative of the formation (and consumption) of one or more intermediates, as was the development of a brown color in the medium toward the end of the experiment. HPLC analysis of samples from the phenol biodegradation experiment revealed the presence of an intermediate that was formed shortly after phenol began to degrade and later disappeared (Fig. 3). Attempts to identify this intermediate were unsuccessful, so the true concentrations of the intermediate are unknown. The intermediate concentration data plotted in Figure 3 are pseudo-concentrations based upon normalizing the unknown's HPLC chromatogram peak areas to the phenol peak areas and using the molecular weight of catechol, a degradation intermediate of benzene and phenol (Fig. 1). The brown color appeared after this intermediate had been removed from the medium. This observation was similar to that described in other reports, in which it was attributed to the oxidation of phenol or one of its degradation pathway intermediates (Feist and Hegeman, 1969; Gibson et al., 1968b). The color was not due to phenol photooxidation since uninoculated phenol medium did not produce the brown color. In addition, two flasks were identically prepared except that one of the flasks was covered in foil. At the end of the growth phase, both flasks yielded nearly the same optical density, and the medium in both flasks was the same brown color. In a separate experiment, serum bottles with different concentrations of catechol were prepared but not inoculated. After a week, the medium in the serum bottles became brown, with darker colors corresponding to higher initial concentrations of catechol. Therefore, it is likely that the color change near the end of the phenol experiment was due to abiotic catechol oxidation.

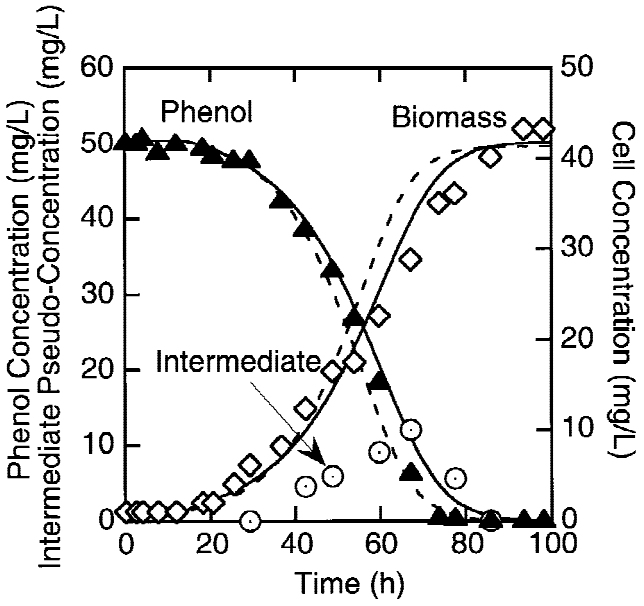

Figure 3. Results from a phenol batch biodegradation experiment with *P. putida* F1. The Monod model (solid lines) and Andrews model (dashed lines) were fit to measured values of phenol concentration (▲) and biomass concentration (◇). Data for an unidentified intermediate (⊙) are pseudo-concentrations based on normalizing the HPLC chromatogram peak areas of the intermediate to the phenol peak areas and using the molecular weight of catechol.

Since the intermediate(s) may have affected growth, we tested models that incorporated competitive and noncompetitive inhibition by the intermediate into the Monod model (data not shown). Of the two, the competitive inhibition model provided a better fit with a PVE (for biomass and phenol) of 97.4%. Although this was an improvement over using the Monod model alone, the difference was not visually apparent. Finally, models were tested that incorporated both substrate and intermediate inhibition (data not

shown). One model described competitive inhibition by two different exclusive inhibitors. The second model represented the effect of one competitive (the intermediate) and one noncompetitive (phenol) inhibitor. Neither model provided a better fit for the data than the other, simpler models. To model these kinetics more accurately requires measurement of all intermediates.

Since phenol has been used widely as a model inhibitory substrate, its biodegradation kinetics have been determined for many microorganisms (Table II). Reported values of the maximum specific growth rates varied from 0.1 to 0.9 h^{-1}; the μ_{max} value for *P. putida* F1 was at the low end of this range. Substrate inhibition was observed in all previously reported studies. In our experiments, this effect was not strong since the substrate concentrations were well below our estimated K_i of 95 mg/L. Although similar K_i values were reported for several other pseudomonads, some species demonstrated inhibition constants as high as 380 mg/L. Reported K_i values for non-pseudomonads were even higher; *C. tropicalis*, with a K_i of 1,200 mg/L, was the most resistant to phenol inhibition. A broad range of Monod constant values (from 1–110 mg/L) has also been reported. However, most K_S values were found to be between 1 and 20 mg/L, and our value for strain F1 is at the high end of this subrange. As was the case with toluene degraders, reported values of the yield coefficient fall into two sets. The larger of these groups (which includes strain F1) has $Y_{X/S}$ values that are 80–95% of theoretical (0.94 g/g). The $Y_{X/S}$ values of the other group were only 55–60% of theoretical.

Benzene

Data from a benzene biodegradation experiment (Fig. 4) reveal that benzene was consumed nearly as rapidly as toluene and with a similarly short lag time (6.5 h). In addition, growth stopped at essentially the same time that benzene was depleted. In Figure 4, the Monod model fit to the data is also shown (parameter values listed in Table III). Although the model satisfactorily described the benzene concentration data, the growth curve is consistently overpredicted. Overall, the model had an LLF of 25.4 and explained 86.6% of the variation in the data. Investigation of the importance of the cell death rate showed that the best fit was provided when this term was set to zero. No substrate inhibition was found with the benzene degradation data; using the Andrews model a lower LLF was found (15.5) and the error residual plot indicated a gross overprediction of the biomass concentration.

The inability of the Monod model to accurately predict the biomass concentration is similar to the results observed for phenol degradation. Since the benzene and phenol catabolic pathways have the same intermediates, the potential for inhibition by an intermediate was also considered for benzene. The brown color noted in the phenol cultures did not appear when benzene was the substrate. Instead, a yellow compound accumulated and was later degraded. Gibson et al. (1968a) made a similar observation and determined that the yellow intermediate was 2-hydroxymuconic semialdehyde. The competitive product inhibition model de-

Table II. Biodegradation model parameter values for aerobic phenol biodegradation by pure cultures

Microorganism (cultivation type)	Temperature (°C)	Max. phenol concentration (mg/L)	μ_{max} (h^{-1})	K_S (mg/L)	K_i (mg/L)	$Y_{X/S}$ (g/g)	Reference
P. putida F1 (batch)	30	54	0.11 ± 0.01	32.0 ± 2.4	—	0.80 ± 0.07	This study
P. putida NRRL β-14875 (continuous)	25	400	0.569	18.54	99.37	0.52	Şeker et al., 1997
P. putida ATCC 17484 (batch)	30	700	0.534	<1.0	470	0.52 ± 0.08	Hill and Robinson, 1975
P. putida Q5 (batch)	10	200	0.119	5.27	377	0.55	Kotturi et al., 1991
Burkholderia cepacia G4 (batch)	23–26	50	0.441	1.22	36.8	0.74	Saéz and Rittmann, 1993
		175	0.441	1.22	138	0.74	
P. putida ATCC 17514 (continuous)	30	500	0.567	2.38	106	—	Yang and Humphrey, 1975
P. putida ATCC 17514 (batch)	28.5	150	0.897	12.2	203.7	0.77	Dikshitulu et al., 1993
P. resinovorans ATCC 14235 (batch)	28.5	150	1.007	13.0	117.7	0.68	Dikshitulu et al., 1993
Trichosporon cutaneum (continuous)	30	900	0.464	1.66	380	0.85[1]	Yang and Humphrey, 1975
Candida tropicalis No. 708 (batch)	30–35	2000	0.22	110	1200	0.5	Shimizu et al., 1973
Nocardia sp. CF222 (continuous)	30	2100	0.29	<1	730	0.88	Mizobuchi et al., 1980

Parameters are for the Monod model unless a value of K_i is given, in which case the Andrews model was used. The theoretical value of $Y_{X/S}$ for growth on phenol is 0.94 g/g.

[1]True yield (Y^T) value, where $\mu/Y_{X/S} = \mu/Y^T + m$. The specific maintenance coefficient (m) was 0.055 mg phenol/mg cells · h.

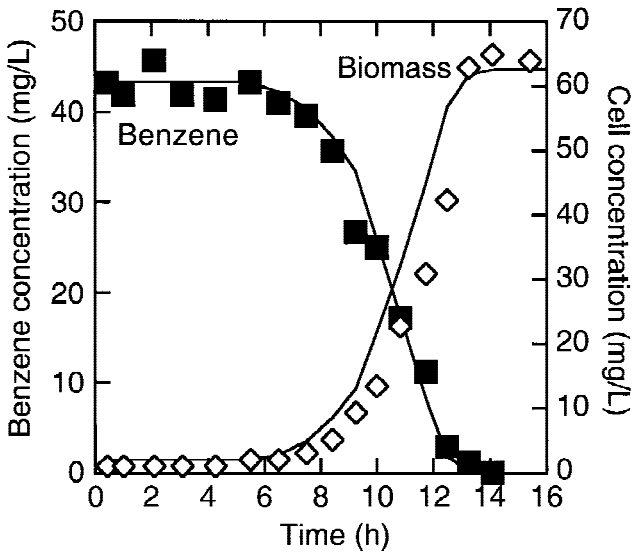

Figure 4. Results from a benzene batch biodegradation experiment with *P. putida* F1. Liquid-phase benzene concentration (■) and biomass concentration (◇) were modeled with the Monod equation (solid lines).

scribed for phenol biodegradation was applied to the benzene data, yielding a slightly better fit (data not shown). Thus, metabolic intermediates may influence the biodegradation of both benzene and phenol.

Although benzene is known to be biodegraded readily under aerobic conditions, only two other reports of kinetic constants for pure cultures were found (Table III). In the concentration ranges tested, substrate inhibition by benzene was not observed for any of the three pseudomonads. The maximum specific growth rate of *P. putida* F1 is about twice that of either of the other strains, while the K_S value of strain F1 growing on benzene is only about 5% of the Monod constants reported for the other strains. F1 is thus able to grow more rapidly at lower benzene concentrations than either *P. fragi* B1 or *P. putida* O1. The yield coefficients for the three strains were quite different, ranging from 100% of theoretical (strain F1) to 54% of theoretical (strain O1).

Comparison of Growth on Different Single Substrates

Our experimental results offer the opportunity to compare the biodegradation kinetics of the three monoaromatic compounds as single substrates. *P. putida* F1 biodegraded toluene slightly more rapidly than benzene; the μ_{max} value for toluene was 18% higher than that for benzene. Qualitatively similar relative biodegradation rates were reported for *P. fragi* B1 (Chang et al., 1993) and *P. putida* O1 (Oh et al., 1994). Both strains grew on toluene faster than benzene, but with μ_{max} ratios of 1.6.

The more striking difference in biodegradation kinetics was between phenol and toluene (or benzene) as the substrate. In this case, the ratio of μ_{max} values is nearly 8 for *P. putida* F1. We know of no other reports in which the biodegradation kinetics of both phenol and one of the BTEX compounds were measured, and thus the generality of this finding cannot be established.

Some insights into the differences in biodegradation kinetics for these three substrates can be gained from a consideration of the relevant metabolic pathways (Fig. 1). These substrates are converted into either catechol (from benzene and phenol) or 3-methyl catechol (from toluene). Since benzene and phenol produce exactly the same intermediate but yield very different biodegradation rates, it is reasonable to conclude that the factor(s) leading to these different biodegradation kinetics are probably associated with either (1) the rates of the reactions that produce catechol or (2) the transport of the substrate into the cytoplasm. The catechol-forming reactions are catalyzed by toluene dioxygenase and *cis*-toluene dihydrodiol dehydrogenase, both of which are part of the coordinately induced *tod* operon (Finette and Gibson, 1988). Differences in the rates of these two reactions for phenol and benzene (or toluene) could be caused by different specific reaction rates (i.e., rates on a per-unit-enzyme basis) and/or by the induction of different enzyme levels. Although some of the physicochemical properties that might influence the reaction rate are significantly different for phenol vs. the other substrates, data from Spain et al. (1989) indicate that the specific reaction rates of toluene and phenol are within a factor of two for nongrowing cells. However, these researchers noted much lower rates (with both substrates) in phenol-grown cells than in toluene-grown cells, suggested different levels of *tod* gene induction. We obtained similar results using a TDO enzyme assay (results not shown).

Differences in substrate transport across the cell membrane could also explain the different biodegradation kinetics. Button (1991) used a two-stage substrate uptake model (transport across the cell membrane followed by metabolism) to demonstrate that differences in transporter content can be as influential as differences in enzymatic reaction rate in determining relative substrate uptake kinetics. Little is known about how aromatic and other hydrophobic compounds are transported into cells; although diffusion is the most-cited mechanism, researchers have identified a membrane protein in *P. putida* F1 that may facilitate toluene transport into the cell (Wang et al., 1995). Different transport rates of phenol and benzene (or toluene) are conceivable for either mechanism.

Toluene–Phenol Mixture

The results of a biodegradation experiment with toluene and phenol are shown in Figure 5. Toluene was consumed before phenol, and phenol biodegradation did not begin until toluene was nearly depleted. Regions corresponding to growth on each substrate are evident in the biomass data. Despite appearances, this is not a case of diauxic growth

Table III. Monod model parameter values for aerobic benzene biodegradation by pure cultures

Microorganism (cultivation type)	Temperature (°C)	Max. benzene concentration (mg/L)	μ_{max} (h^{-1})	K_S (mg/L)	$Y_{X/S}$ (g/g)	Reference
P. putida F1 (batch)	30	43	0.73 ± 0.03	0.12 ± 0.02	1.20 ± 0.05	This study
P. fragi B1 (batch)	room	10	0.335 ± 0.129	3.17 ± 0.82	1.04 ± 0.09	Chang et al., 1993
P. putida O1 (batch)	30	70	0.44	3.36	0.65	Oh et al., 1994

The theoretical value of $Y_{X/S}$ for growth on benzene is 1.21.

since *P. putida* F1 used the same enzymes to metabolize both substrates (Spain et al., 1989).

The most common model type for growth on homologous mixtures is one 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 modeled using Eqs. (1) or (3), as appropriate, with S_i (or $S_{L,i}$) and $Y_{X/S,i}$ instead of S (or S_L) and $Y_{X/S}$. In the simplest form of this model type, Monod expressions are used for each μ_i, yielding a model in which the presence of one substrate does not affect the biodegradation rate of the other. For a binary mixture, this no-interaction sum kinetics model is:

$$\mu = \frac{\mu_{max,1} S_1}{K_{S,1} + S_1} + \frac{\mu_{max,2} S_2}{K_{S,2} + S_2} \quad (7)$$

where the subscripts 1 and 2 refer to each of the two substrates. The curves in Figure 5 are the trajectories predicted by this model using values of the model constants determined from single substrate experiments (Tables I, II). We chose to use the Monod model rather than the Andrews model because of the parameter estimation problems noted earlier and because the differences between the model fits were small. This choice was supported when calculations with an Andrews term in Eq. (7) did not improve the prediction of the mixture data. Although toluene biodegradation was accurately described, phenol biodegradation occurred later and at a lower specific (per cell) rate than predicted by the model. From this, we conclude that substrate interactions were present: toluene inhibited phenol biodegradation (but phenol had little effect on toluene consumption).

Since the same metabolic pathway is used in the catabolism of both toluene and phenol, it is possible that the two substrates compete for the active site on the first enzyme in the pathway, toluene dioxygenase (or that toluene and phenol metabolites compete for another enzyme in the pathway). A sum kinetics model incorporating purely competitive substrate kinetics is (Yoon et al., 1977):

$$\mu = \frac{\mu_{max,1} S_1}{K_{S,1} + S_1 + \left(\frac{K_{S,1}}{K_{S,2}}\right) S_2} + \frac{\mu_{max,2} S_2}{K_{S,2} + S_2 + \left(\frac{K_{S,2}}{K_{S,1}}\right) S_1} \quad (8)$$

The output curves from this model are shown in Fig. 6. The model predicted cell growth well, but the curves of toluene and phenol degradation are inaccurate. The model prediction for phenol degradation represented the data better than

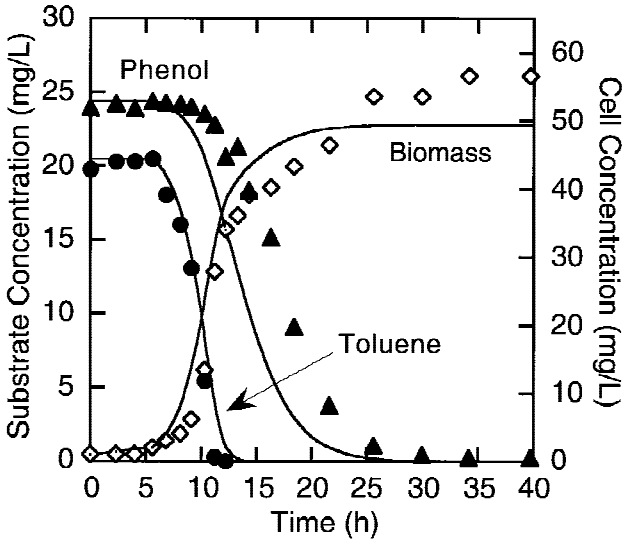

Figure 5. Results from a batch cultivation of *P. putida* F1 on a toluene-phenol mixture and model predictions from the no-interaction sum kinetics models. Symbols indicate measurements of liquid-phase toluene (●), phenol (▲), and biomass concentrations (◇); lines are model predictions.

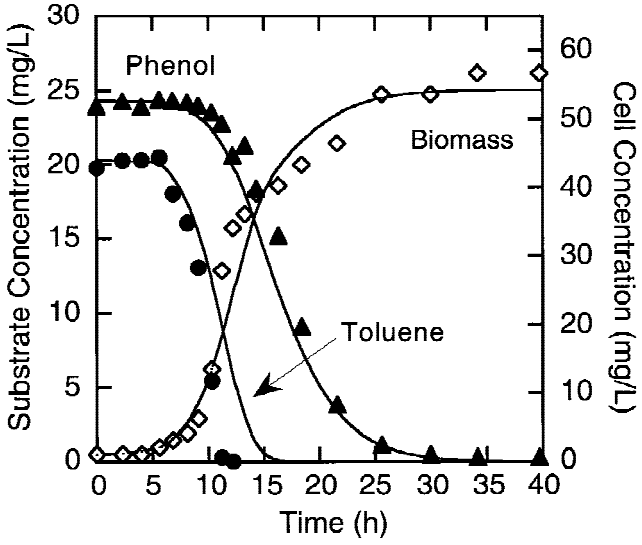

Figure 6. Comparison of prediction (lines) from the sum kinetics model with purely competitive substrate interactions vs. measured results (symbols) from a batch cultivation of *P. putida* F1 on a toluene–phenol mixture. The measured data are those shown in Figure 5.

the model without interactions, supporting the hypothesis that toluene inhibited phenol degradation. However, toluene was consumed more rapidly than predicted.

Another form of interaction between an enzyme and two substrates is noncompetitive inhibition, in which a nonreactive complex is formed when both substrates are simultaneously bound to the enzyme (Segel, 1975). The cell growth model based on this type of interaction is:

$$\mu = \frac{\mu_{\max,1} S_1}{(K_{S,1} + S_1)\left(1 + \frac{S_2}{K_{S,2}}\right)} + \frac{\mu_{\max,2} S_2}{(K_{S,2} + S_2)\left(1 + \frac{S_1}{K_{S,1}}\right)} \quad (9)$$

This model predicted the biomass and toluene data well but overpredicted the phenol consumption rate.

Uncompetitive enzyme inhibition is similar to noncompetitive inhibition except that one of the compounds (the inhibitor) can bind only to the enzyme-substrate complex and not to the free enzyme (Segel, 1975). A cell growth model based on uncompetitive substrate interaction is:

$$\mu = \frac{\mu_{\max,1} S_1}{K_{S,1} + S_1\left(1 + \frac{S_2}{K_{S,2}}\right)} + \frac{\mu_{\max,2} S_2}{K_{S,2} + S_2\left(1 + \frac{S_1}{K_{S,1}}\right)} \quad (10)$$

This model yielded LLF values and error residual plots similar to those for Eq. (9).

Based on the outcomes of these modeling efforts, we concluded first that there are interactions between these substrates that cannot be described by sum kinetics models using only parameters determined in single substrate experiments, and second that the interaction is not competitive, noncompetitive, or uncompetitive enzyme inhibition. An alternative model was formulated by incorporating an unspecified type of interaction into the sum kinetics framework:

$$\mu = \frac{\mu_{\max,1} S_1}{K_{S,1} + S_1 + I_{2,1} S_2} + \frac{\mu_{\max,2} S_2}{K_{S,2} + S_2 + I_{1,2} S_1} \quad (11)$$

The interaction parameter I_{ij} indicates the degree to which substrate *i* affects the biodegradation of substrate *j* (larger values indicate stronger inhibition). Equations (1) (for each substrate) and (11) form a sum kinetics with interaction parameters (SKIP) model, first proposed by Yoon et al. (1977).

The SKIP model was fit to both toluene–phenol mixture datasets to determine the interaction parameters $I_{T,P}$ and $I_{P,T}$ (Table IV). With these parameters, the SKIP model accurately described the biodegradation data (Fig. 7), demonstrating that the SKIP model can be used to fit unspecified types of inhibition between two substrates. The large value of $I_{T,P}$, indicating a high degree of inhibition of toluene on phenol biodegradation, is expected from earlier observations (Figs. 5, 6). Similarly, the low value of $I_{P,T}$ is reasonable given the negligible impact phenol appeared to have on toluene consumption. Since the SKIP model reduces to the purely competitive inhibition model if $I_{i,j} = K_{Sj}/K_{S,i}$, comparison of these quantities indicates whether the kinetics are statistically different than competitive inhibition. For the toluene–phenol data, the inhibition parameter values are statistically different at the 95% significance level, with two-sided *p*-values of 0.038 for $I_{P,T}$ vs. $(K_{S,T}/K_{S,P})$ and 0.043 for $I_{T,P}$ vs. $(K_{S,P}/K_{S,T})$. Thus, the inhibition between toluene and phenol is not competitive. Of the five mixture biodegradation models tested, the SKIP model best matched the experimental data (Table V). While the competitive inhibition and SKIP models have similar PVE values, the SKIP model is clearly better based on a comparison of the LLF values and residual error plots. In particular, the competitive inhibition model fit the toluene data poorly, as indicated by significant heterscedasticity in the errors (not present for the SKIP model).

Table IV. Interaction parameters (with standard errors) for the SKIP model

Parameter	Value (−)
$I_{T,P}$	55 ± 5
$I_{P,T}$	0.01 ± 0.002
$I_{T,B}$	5 ± 0.3
$I_{B,T}$	0.01 ± 0.003
$I_{B,P}$	18.5 ± 1.5
$I_{P,B}$	0.01 ± 0.002

Toluene–Benzene Mixture

The results of a toluene–benzene mixture experiment are shown in Figure 8. Although *P. putida* F1 consumed both of these substrates simultaneously during most of the cultivation, toluene biodegradation began before that of benzene,

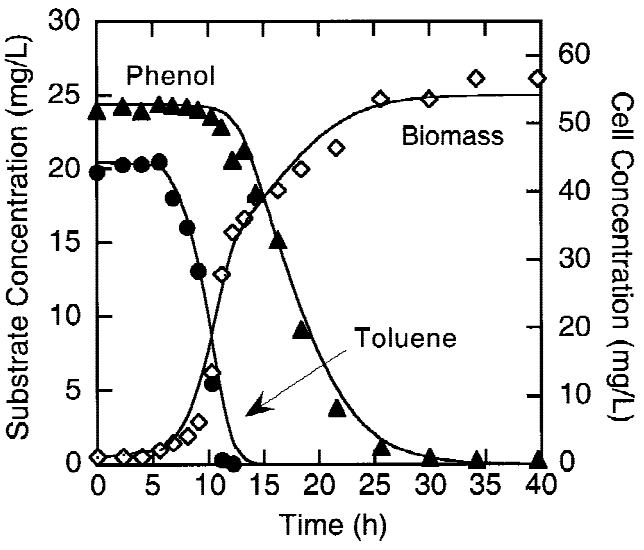

Figure 7. Comparison of fitted model output (lines) from the SKIP model vs. measured results (symbols) from a batch cultivation of *P. putida* F1 on a toluene–phenol mixture. The measured data are those shown in Figure 5.

Table V. Comparison of model representations of experimental data using the log of the likelihood function (LLF) and percent variation explained (PVE)

Model	Toluene–Phenol		Toluene–Benzene		Benzene–Phenol	
	LLF	PVE*	LLF	PVE*	LLF	PVE*
No interaction (Eq. 7)	−25.8	80.2 (74.3)	—	— (—)	−8.6	92.1 (96.8)
Competitive inhibition (Eq. 8)	−7.1	96.6 (93.1)	−37.6	72.6 (78.0)	−54.9	82.9 (82.0)
Noncompetitive inhibition (Eq. 9)	−68.7	90.1 (89.9)	−37.5	72.2 (81.5)	−90.6	56.1 (49.9)
Uncompetitive inhibition (Eq. 10)	−78.3	87.0 (84.8)	−46.1	42.5 (53.3)	−60.8	78.4 (77.0)
SKIP (Eq. 11)	11.2	98.1 (95.8)	−29.3	95.7 (92.1)	−4.4	94.2 (89.0)

"—" Indicates instances in which the model does not represent data sufficiently well to permit calculation of LLF or PVE.
*Values shown in parentheses do not include biomass concentration data.

and toluene was depleted first. The five models discussed previously were applied to the data from these experiments. Values of the $I_{i,j}$ SKIP model parameters are listed in Table IV. The output of the competitive inhibition and SKIP models are presented in Figure 8, and the goodness-of-fit data (LLF and PVE) for all models are listed in Table V. Analogous to the case of the toluene–phenol mixture, Eqs. (7–10) predicted earlier and faster biodegradation of benzene and underpredicted the rate and time of toluene consumption. Again, the presence of toluene inhibited the degradation of the second substrate (benzene), while the presence of benzene had little effect on toluene consumption. The SKIP model provided the best match to the data (Table V). The inhibition parameter values of the SKIP and competitive inhibition models were different at the 95% significance level, with two-sided *p*-values of 0.032 for $I_{T,B}$ vs. ($K_{S,B}/K_{S,T}$) and 0.0091 for $I_{B,T}$ vs. ($K_{S,T}/K_{S,B}$). The interaction between toluene and benzene as substrates for *P. putida* F1 is thus different than competitive inhibition.

Benzene–Phenol Mixture

The results of the benzene–phenol mixture experiments (Fig. 9) were similar to those of the toluene–phenol mixture. Benzene was degraded first, and phenol consumption did not begin until benzene concentrations were near zero. When the five models were applied to these datasets, the SKIP model was again able to describe the trajectories of benzene, phenol, and biomass better than the other models (Table V). Application of the competitive, noncompetitive, and uncompetitive models led to predictions of phenol consumption that were faster than that observed, and to slower than observed benzene degradation. Statistical comparison of the inhibition parameters of the SKIP and competitive inhibition models revealed that $I_{B,P}$ and ($K_{S,P}/K_{S,B}$) were different at the 95% significance level, with a two-sided *p*-value of 0.043. However, the parameters $I_{P,B}$ and ($K_{S,B}/K_{S,P}$) were not different at this significance level, suggesting that phenol may competitively inhibit benzene degradation,

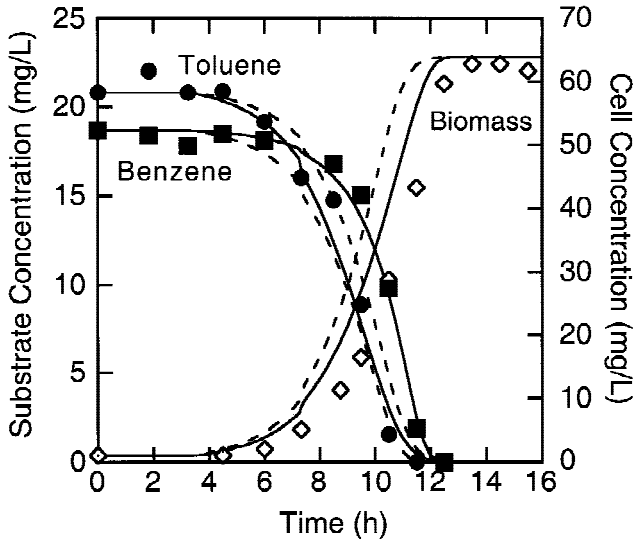

Figure 8. Results from a batch cultivation of *P. putida* F1 on a toluene-benzene mixture and comparison of model results. Symbols indicate measurements of liquid-phase toluene (●), benzene (■), and biomass concentrations (◇); dashed lines are predictions from the sum kinetics model with purely competitive substrate interactions and solid lines are outputs from the fitted SKIP model.

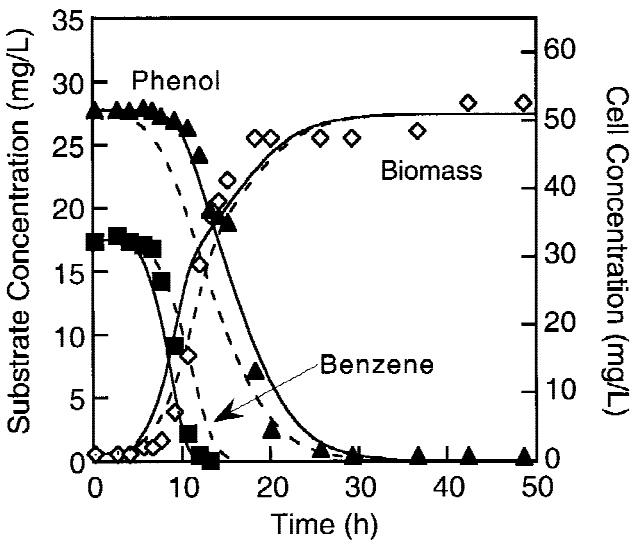

Figure 9. Results from a batch cultivation of *P. putida* F1 on a benzene–phenol mixture and comparison of model results. Symbols indicate measurements of liquid-phase benzene (■), phenol (▲), and biomass concentrations (◇); dashed lines are predictions from the sum kinetics model with purely competitive substrate interactions and solid lines are outputs from the fitted SKIP model.

while the effect of benzene on phenol consumption is more complex. The LLF and PVE values for the sum kinetics and SKIP models were similar (Table III). A plot of the residuals revealed significant heteroscedasticity in the biomass errors for the sum kinetics model. In contrast, errors for the SKIP model were homoscedastic, indicating that this was the better model.

Toluene–Benzene–Phenol Mixture

The concentration-time data for *P. putida* F1 growth on the full three-substrate mixture are shown in Figure 10. Toluene consumption started first and this compound was the first to be depleted. Although benzene consumption began later, both compounds were degraded simultaneously from 9–12 h. Significant phenol consumption did not begin until the toluene concentration was nearly zero and the benzene concentration was low.

The SKIP model form for this three compound mixture is

$$\mu = \left[\frac{\mu_{\max,T} S_T}{K_{S,T} + S_T + I_{B,T} S_B + I_{P,T} S_P}\right] + \left[\frac{\mu_{\max,B} S_B}{K_{S,B} + S_B + I_{T,B} S_T + I_{P,B} S_P}\right] + \left[\frac{\mu_{\max,P} S_P}{K_{S,P} + S_P + I_{T,P} S_T + I_{B,P} S_B}\right] \quad (12)$$

where the subscripts T, B, and P denote parameters for toluene, benzene, and phenol biodegradation, respectively. Using the values of $\mu_{\max,i}$, $K_{S,i}$, and $Y_{Y/S,i}$, determined from single-substrate experiments and values of $I_{i,j}$ determined from the dual substrate experiments, this model was used to predict the consumption of the three substrates. This prediction, shown in Figure 10, matched both sets of measured data well, with a percent variation explained (PVE) value of 96.7%.

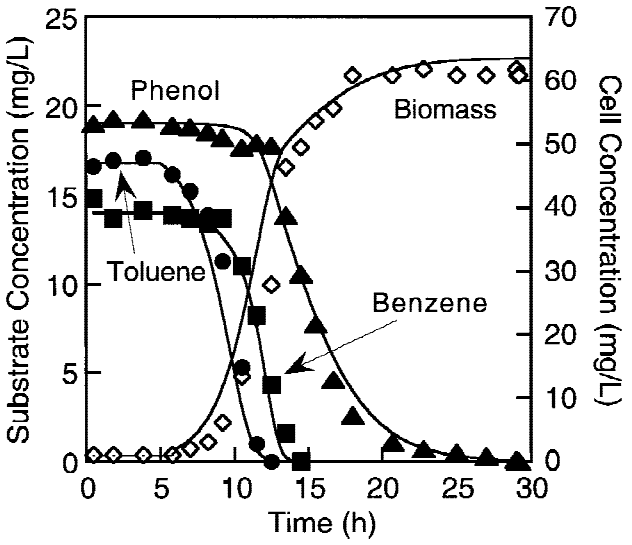

Figure 10. Results from a batch cultivation of *P. putida* F1 on a toluene–benzene-phenol mixture and model predictions from the SKIP model. Symbols indicate measurements of liquid-phase toluene (●), benzene (■), phenol (▲), and biomass concentrations (◇); lines are model predictions.

Biodegradation Kinetics for Mixtures

Biodegradation kinetics for mixtures of homologous growth substrates are often modeled using no-interaction sum kinetics or purely competitive inhibition kinetics (Table VI). In fact, these models are the only mixture biodegradation models included in biochemical engineering texts (in addition to product kinetics models, which are inappropriate for homologous substrates). The applicability of these models seems intuitive and reasonable, particularly for similar compounds (e.g., benzene and toluene) that can be catabolized by the same pathways. However, there are many cases in which the interactions between homologous substrates are *not* purely competitive, even for similar compounds. For example, in experiments with *P. putida* O1 and a mixed culture, Oh et al. (1994) found that benzene consumption was inhibited by the presence of toluene to a much greater extent than predicted by the ratio of $K_{S,i}$ values, and toluene consumption was far less inhibited by the presence of benzene than would be the case in purely competitive inhibition. Similarly, using mixtures of pentachlorophenol and trichlorophenol, and pentachlorophenol and phenol, Klečka and Maier (1988) determined that the preferred substrate (pentachlorophenol) exerted a much greater inhibition on the other substrate than predicted by purely competitive inhibition. In many other reports, unusual substrate interactions in mixtures have been reported but not modeled (Arvin et al., 1989; Millette et al., 1995; Smith et al., 1991).

Until the nature of each of these various unknown interactions is understood, mechanistic mathematical models cannot be proposed. In the interim, at least three modeling approaches have appeared in the literature:

1) Use of either an empirical interaction parameter or function. The interaction parameter approach was proposed by Yoon et al. (1977) and used by Klečka and Maier (1988) and Oh et al. (1994), in addition to the present work. The interaction function approach was used by Papanastasiou and Maier (1982). Their function had the advantage of not requiring additional fitting parameters; however, it is not clear how it could be extended to larger substrate mixtures.
2) Variations of the phenomenological model proposed by Lendenmann et al. (1996). For example, Kovárová-Kovar et al. (1997) expressed the contributions of each mixture component in terms of either the Gibbs free energy of combustion, the moles of oxygen required for combustion, or the carbon content of that compound. Each of these reflects differences among substrates that may be important in determining the consumption rates in mixtures.
3) Cybernetic modeling, in which cells are assumed to follow a strategy of optimal allocation of resources

Table VI. Selected literature reports of mixture biodegradation kinetics

Substrates	Culture	Biodegradation kinetics	Reference
Benzene, toluene	*P.* sp. B1	Purely competitive	Chang et al., 1993
Toluene, *p*-xylene	*P.* sp. X1	Purely competitive	Chang et al., 1993
Glucose, fructose	*Candida tropicalis*	Purely competitive	Yoon et al., 1977
Naphthalene, phenanthrene, pyrene	Mixed culture	Purely competitive	Guha et al., 1999
Benzene, toluene, ethylbenzene, *o*-xylene	Mixed cultures	Purely competitive or no interaction	Bielefeldt and Stensel, 1999
Glucose, galactose, fructose	*Escherichia coli* DSM 1329	No interaction	Lendenmann et al., 1996
Pentachlorophenol, phenol	Pure culture (unidentified)	Interaction parameters (not purely competitive)	Klečka and Maier, 1988
Benzene, toluene	*P. putida* O1	Interaction parameters (not purely competitive)	Oh et al., 1994
Glucose, 2-4-dichlorophenoxyacetic acid	Mixed culture	Interaction function (not purely competitive)	Papanastasiou and Maier, 1982

(Ramkrishna, 1982). Kompala et al. (1986) used this approach to predict diauxic and triauxic growth with single-substrate cultivation data. More recently, the cybernetic approach was extended to include metabolic intermediates, thereby allowing preferential and simultaneous substrate consumption patterns to be described (Ramakrishna et al., 1996). However, since cybernetic models are based on optimal control of metabolic pathways that are different for each substrate, their description of our results would require the use of three hypothetical metabolic pathways rather than the single one that exists.

An important feature of mixed-substrate kinetic models is their ability to be applied to mixtures containing any number of compounds. As Kovárová-Kovar and Egli (1998) point out, many proposed models do not satisfy this requirement. In this regard, it is noteworthy that the kinetics of the three-substrate experiment were well predicted by the SKIP model. The quality of this prediction indicates that only binary interactions are important in this mixture and suggests that the SKIP model can be extended to larger mixtures. Since the interaction parameters ($I_{i,j}$) must be determined experimentally, additional research is required to test this hypothesis. However, for the simplification in which the SKIP model reduces to the competitive inhibition model (i.e., if $I_{i,j} = K_{S,j}/K_{S,i}$), our calculations show that the overall specific growth rate predicted by Eq. (4) never exceeds the largest maximum specific growth rate of any individual substrate, no matter how many components in the mixture. Thus, the competitive inhibition and SKIP models should be applicable to mixtures with any number of substrates.

Studies with *P. putida* F1 mutants have demonstrated that the catabolism of benzene, toluene, and phenol proceed via the toluene dioxygenase pathway (Spain and Gibson, 1988). If the same enzymes are used in the transformation of each substrate, purely competitive inhibition kinetics would certainly be expected. Potential reasons for deviations from such kinetics include: 1) interactions at the level of substrate transport into the cytoplasm, 2) interactions with regulatory compounds, and 3) the presence of a previously unidentified catabolic pathway or pathway branch. The work of Button and colleagues (e.g., (Button, 1998)) on the relationships between nutrient uptake and microbial growth has led them to propose a model ("janusian kinetics"), which includes both transmembrane substrate transport and enzymatic reaction (Button, 1991). Given the apparent success of this concept for single substrates and the fact that the unusual interactions noted here are difficult to explain by focusing only on the enzymatic steps, our observations may be explained by considering transport-level interactions.

CONCLUSIONS

Over the concentration ranges tested, the biodegradation kinetics of *P. putida* F1 follow Monod kinetics for toluene and benzene. The Andrews model was found to describe the biodegradation of phenol only slightly better than the Monod model in the low concentration range used. Growth on toluene was slightly faster than that on benzene, and both of these compounds were much better substrates for *P. putida* F1 than was phenol. In batch cultivations of *P. putida* F1 with substrate mixtures of benzene, toluene, and/or phenol, the resulting biodegradation and growth kinetics were best described by the SKIP model. Although all substrates are metabolized by the same enzymes, the inhibitory interactions between substrates were not purely competitive in nature. Toluene was the preferred substrate in the sense that its presence exerted the largest degree of inhibition on the consumption of the other substrates. Model parameters from single- and dual-substrate mixture experiments were sufficient to accurately predict the outcome of the three-substrate mixture using the SKIP model. Although we do not currently have an explanation for these nonideal kinetics, we note that they are not unique, even for substrates as similar as benzene and toluene. Furthermore, since they were obtained in this apparently simple system, our findings raise the question of how to model more complex systems with multiple metabolic pathways.

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