Biodegradation **12:** 465–475, 2001.

A two-step model for the kinetics of BTX degradation and intermediate formation by *Pseudomonas putida* F1

Haibo Yu[1], Byung J. Kim[2] & Bruce E. Rittmann[3]
[1]*McKinsey & Company, Inc., 600 Campus Drive, Florham Park, NJ 07932, USA;* [2]*US Army Engineer Research and Development Center, Champaign, IL 61821, USA;* [3]*Department of Civil and Environmental Engineering, Northwestern University, Evanston, IL 60208-3109 USA*

Accepted 21 December 2001

Key words: BTX, intermediates, kinetics, modeling, *Pseudomonas putida* F1, substrate interactions

Abstract

A two-step model is developed for the aerobic biodegradation of benzene, toluene, and *p*-xylene (BTX) by *Pseudomonas putida* F1. The model contains three unique features. First, an initial dioxygenation step transforms BTX into their catechol intermediates, but does not support biomass growth. Second, the benzene or toluene intermediates are mineralized, which supports biomass synthesis. Third, BTX exhibit competitive inhibition on each other's transformation, while toluene and benzene noncompetitively inhibit the mineralization of their catechol intermediate. A suite of batch and chemostat experiments is used to systematically measure the kinetic parameters for the two-step transformations and the substrate interactions.

Introduction and background

Although biodegradation of most organic compounds involves a series of reactions, Monod and Haldane-Andrews kinetics are based on the assumption that an initial reaction involving the substrate controls the rate of cell synthesis. For this assumption to be correct, the first reaction must be much slower than are all the following reactions so that no intermediates accumulate. Biodegradation of benzene, toluene, and xylenes (BTX) is an example of multi-step biodegradation that sometimes can be described adequately by the single-step assumption. In some cases, Monod kinetics was adequate for benzene and toluene (Arcangeli 1994; Oh et al. 1994; Chang et al. 1993), while self-inhibition (Haldane-Andrews) kinetics better represented the degradation of toluene in other cases (Mirpuri et al. 1997; Oh et al. 1994).

Despite some success of the single-step approach for modeling BTX biodegradation, recent studies of BTX degradation suggest that the intermediates, rather than the substrates, control the growth of cells (Chang et al. 1993; Yu et al. 2001). Typical phenomena showing intermediate-supported growth are (1) biomass production lags behind the removal of the original substrate and (2) biomass continues to grow even after all the original substrates are completely removed. Both phenomena were observed in a study of BTX biodegradation by two *Pseudomonas* isolates (Chang et al. 1993). Of particular importance are the well-controlled batch and continuous experiments of Yu et al. (2001), who showed that intermediates were controlling the biodegradation of BTX by *Pseudomonas putida* F1. They identified the accumulation of intermediates using chromatography and chemical oxygen demand (COD) analyses. Transformation of BTX to their catechol intermediates did not support biomass growth. Instead, biomass growth occurred only in response to degradation of the intermediates, catechol for benzene and 3-methylcatechol for toluene.

Chemical pollutants rarely exist alone in the environment. For example, BTX coexist at many contaminated sites (Rittmann et al. 1994; NRC 2000). The presence of several contaminants provides an opportunity for substrate interactions, such as inhibition. Inhibition can be competitive, when two substrates compete for access to the same active site on the enzyme; or noncompetitive, when the inhibitor does not compete for

the active site, but interacts with the enzyme in such a way that the enzyme's reactivity toward its target substrate is hindered (Rittmann & McCarty 2001). For the simultaneous biodegradation of benzene and toluene, their interaction is competitive inhibition (Oh et al. 1994; Chang et al. 1993), because they compete for the active sites on the initial dioxygenase. On the other hand, toluene or benzene can noncompetitively inhibit other reactions, such as those responsible for transformation of metabolic intermediates.

This study uses a systematic approach to quantify the kinetics of BTX degradation by *Pseudomonas putida* F1 when the formation and mineralization of intermediates are important and when inhibitory substrate interactions occur among benzene, toluene, *p*-xylene, and their intermediates. Batch and chemostat experiments provide experimental results that define the different phenomena. Mathematical modeling is used to estimate the kinetic parameters that represent each of the phenomena.

Table 1. Initial condition for the batch experiments with a single substrate.

Test Name	Substrate name	Concentration (mg/l)	Biomass (mg/l)
T1	Toluene	39	11
T2		35	23
T3		15	12
T4		20	20
T5		24	18
T6		8.1	20
T7		39	18
B1	Benzene	31	11
B2		36	18
B3		22	9
B4		18	12
P1	*p*-Xylene	10	52
P2		3	23
P3		2.3	65
P4		12	44

Experimental methods

The reactors used in the study included open-system batch reactors, closed-system batch reactors, and a closed-system chemostat. The reactor systems are described in detail in Yu et al. (2001). In brief, the open-system batch reactors were 1-liter flasks. The closed-system batch reactors were glass bottles with Teflon-faced septa at the center of the sealing caps. The closed chemostat had a liquid volume of 1.45 liter and was supplied with mineral media and saturated substrate stock continuously. In order to maintain strictly aerobic conditions, oxygen was supplied in excess in all the experiments. *P. putida* F1 was inoculated and maintained a pure culture in all experiments (Yu et al. 2001).

Analyses carried out in this study include concentrations of suspended biomass (in mg-biomass/l), BTX in gas and liquid phases (in mg-BTX/l), and intermediates in chemical oxygen demand (mg-COD/l). Details of the analytical methods are described by Yu et al. (2001).

Three biomass-decay experiments were conducted using the open-system batch reactors with no feeding of BTX. The decrease in biomass from an initial concentration of 150–200 mg l^{-1} was used to estimate the endogenous decay coefficient.

The chemostat experiments used either toluene or benzene as the sole substrate. For each substrate, a series of steady states was developed. Biomass, toluene or benzene concentration, and chemical oxygen demand (COD) in the liquid were measured to observe the arrival of steady states. The substrate influent concentration was always maintained near 70 mg l^{-1}, while the dilution rate was varied systematically from 1 to 8 day^{-1}. Biomass and toluene or benzene concentrations in the liquid were analyzed at each steady state. The headspace was analyzed for toluene or benzene. The chemostat results were used to estimate parameters on the utilization of toluene and benzene and the growth of bacteria due to the mineralization of their intermediates.

The kinetic experiments in the closed-system batch reactors were divided into two groups. In the first group of experiments, only one substrate was used, i.e., toluene, benzene, or *p*-xylene. In the second group of experiments, two components were used as substrates, i.e., toluene and benzene, or toluene and *p*-xylene. Toluene was always used as the primary substrate, and benzene and *p*-xylene were used as the secondary substrates. Tables 1 and 2 list the initial conditions of the first and second groups of batch experiments, respectively. The concentrations of BTX shown in the two tables are equilibrium concentrations in the liquid phase. The closed batch experiments were used to evaluate the kinetics of substrate interactions with each other and their intermediates.

Table 2. Initial conditions for the batch experiments with dual substrates.

Test Name	Primary substrate	Concentration (mg/l)	Secondary substrate	Condition (mg/l)	Biomass (mg/l)
TB1	Toluene	13	Benzene	4.0	8.1
TB2		15		3.9	9.1
TB3		45		7.7	16
TB4		42		8.1	16
TP1	Toluene	35	*p*-Xylene	15	13
TP2		21		4.7	7.6
TP3		9.5		4.3	9.7

Mass balance equations

The following assumptions were made in developing the mathematical equations for biomass growth and BTX utilization:

1. Biodegradation of BTX is a two-step process (Yu et al. 2001).
2. The first step produces intermediates: catechol, 3-methylcatechol, and 3,6-dimethylcatechol from benzene, toluene, and *p*-xylene, respectively (Yu et al. 2001). No biomass growth occurs from the first step, because these dioxygenase reactions release no electrons or organic carbon
3. In the second step of benzene and toluene degradation, catechol and 3-methylcatechol are completely mineralized to CO_2 and H_2O through reactions that yield electrons, energy, and carbon for growth (Yu et al. 2001).
4. The intermediate of *p*-xylene, 3,6-dimethylcatechol, does not support biomass growth and is not degraded by the biomass. Thus, *p*-xylene transformation to 3,6-dimethylcatechol is cometabolic. The electrons used to degrade *p*-xylene come from the primary electron donor (toluene) and/or cell decay (Sáez & Rittmann 1993; Criddle 1993)
5. During toluene or benzene biodegradation, toluene or benzene may inhibit the degradation of its intermediate. If present, the inhibition is noncompetitive.
6. When toluene and benzene or toluene and *p*-xylene are degraded as dual substrates, they competitively inhibit each other in their first reaction step.
7. In a closed system, the BTX in the gas and liquid phases are in equilibrium described by Henry's law.

When no growth substrate is present in a batch reactor, the biomass mass balance is:

$$\frac{dX_a}{dt} = -bX_a \tag{1}$$

where b is the endogenous-decay coefficient (T^{-1}) and X_a is the concentration of active biomass ($M_x L^{-3}$).

Toluene or benzene as the sole substrate

In a batch reactor, the mass balance equations for toluene ($S_{1,T}$), its intermediate ($S_{2,T}$), and biomass (X_a) are:

$$\frac{dS_{1,T}}{dt} = -\frac{q_{1,\max,T} S_{1,T}}{K_{1,T} + S_{1,T}} X_a \frac{V_l}{V_l + V_g H_T} \tag{2}$$

$$\frac{dS_{2,T}}{dt} = \alpha_{12,T} \frac{q_{1,\max,T} S_{1,T}}{K_{1,T} + S_{1,T}} X_a - \frac{q_{2,\max,T}}{1 + \frac{S_{1,T}}{K_{I,12,T}}} \times \frac{S_{2,T}}{K_{2,T} + S_{2,T}} X_a \tag{3}$$

$$\frac{dX_a}{dt} = \frac{q_{2,\max,T} Y_{2,T}}{1 + \frac{S_{1,T}}{K_{I,12,T}}} \frac{S_{2,T}}{K_{2,T} + S_{2,T}} X_a - bX_a \tag{4}$$

In Equations (2)–(4), subscript 1 refers to variables for toluene, while subscript 2 is for its intermediate (3-methylcatechol). $S_{1,T}$ is the concentration of toluene ($M_{\text{tol}} L^{-3}$), $q_{1,\max,T}$ is the maximum specific rate of toluene utilization ($M_{\text{tol}} M_X^{-1} T^{-1}$), and $K_{1,T}$ is the half-maximum-rate concentration of toluene ($M_{\text{tol}} L^{-3}$). In Equation 2), V_l is the liquid volume (L^3), V_g is the headspace volume (L^3), and H_T is the "dimensionless" Henry's constant of toluene

($L^3_{\text{liq}}L^{-3}_{\text{gas}}$. In Equation (3), $\alpha_{12,T}$ is the stoichiometric coefficient ($M_{\text{mcat}}M^{-1}_{\text{tol}}$) for the production of the intermediate (3-methylcatechol) from toluene, $S_{2,T}$ is the concentration of the intermediate ($M_{\text{mcat}}L^{-3}$), and $K_{2,T}$ is the half-maximum-rate concentration of the intermediate ($M_{\text{mcat}}L^{-3}$). In Equation (4), $Y_{2,T}$ is the biomass true yield for the intermediate ($M_X M^{-1}_{\text{mcat}}$). The parameter $K_{I,12,T}$ in Equations (3) and (4) is the non-competitive inhibition constant for toluene to its intermediate ($M_{\text{tol}}L^{-3}$). The values of $K_{I,12,T}$ indicates if and how much toluene inhibits the degradation of its intermediates. If the value of $K_{I,12,T}$ is very large for the concentration of toluene present, the ratio of $S_{1,T}/K_{I,12,T}$ is always very small, and the inhibition effect is small or negligible. On the other hand, a relatively small value for $K_{I,12,T}$ means the inhibition effect is significant.

For the closed-system chemostat, the steady-state equations are modified from the batch equations by adding terms for advection of mass into and out of the reactor to their right-hand sides and by deleting the dynamic accumulation term on the left-hand side:

$$0 = (S_{1,\text{in},T} - S_{1,T})D - \frac{q_{1,\max,T}S_{1,T}}{K_{1,T} + S_{1,T}}X_a \quad (5)$$

$$0 = (S_{2,\text{in},T} - S_{2,T})D + \alpha_{12,T}\frac{q_{1,\max,T}S_{1,T}}{K_{1,T} + S_{1,T}}X_a - \frac{1_{2,\max,T}}{1 + \frac{S_{1,T}}{K_{I,12,T}}}\frac{S_{2,T}}{K_{2,T} + S_{2,T}}X_a \quad (6)$$

$$0 = (X_{a,\text{in}} - X_a)D + \frac{q_{2,\max,T}Y_{2,T}}{1 + \frac{S_{1,T}}{K_{I,12,T}}}\frac{S_{2,T}}{K_{2,T} + S_{2,T}} \times X_a - bX_a \quad (7)$$

where subscript 'in' refers to the concentrations in the liquid influent, and D is the liquid dilution rate (T^{-1}), which equals the ratio of liquid flowrate to the liquid volume in the reactor.

The following steps are used to obtain a solution to Equations (5) to (7). In this study, $S_{2,\text{in},T}$ and $X_{a,\text{in}}$ always equaled zero. Since $S_{1,T}$ in the steady-state chemostat always was below the detection limit of GC/FID assay, it was assumed to be zero. Then, Equation (5) is substituted into Equation (6) to yield:

$$(\alpha_{12,T}S_{2,\text{in},T} - S_{2,T})D = \frac{q_{2,\max,T}S_{2,T}}{K_{2,T} + S_{2,T}}X_a \quad (8)$$

Equation (8) is then substituted into Equation (7). The result is the following equation, which relates the biomass concentration to the influent toluene concentration, the effluent intermediate concentration, and the operating condition (D):

$$(D + b)X_a = Y_{2,T}(\alpha_{12,T}S_{1,\text{in},T} - S_{2,T})D \quad (9)$$

Equations for benzene are the same as Equations (2) to (9), except the variables used are related to benzene (subscript B) instead of to toluene (subscript T).

Toluene and benzene as dual substrates

When toluene and benzene are degraded simultaneously in batch reactors, both contribute to the overall electron and energy flows in the cells. Thus, their mass balance equations are:

$$\frac{dS_{1,B}}{dt} = -\frac{q_{1,\max,B}S_{1,B}}{K_{1,B}\left(1 + \frac{S_{1,T}}{K_{TB}}\right) + S_{1,B}}X_a\frac{V_l}{V_l + V_g H_B} \quad (10)$$

$$\frac{dS_{2,B}}{dt} = \alpha_{12,B}\frac{q_{1,\max,B}S_{1,B}}{K_{1,B}\left(1 + \frac{S_{1,T}}{K_{TB}}\right) + S_{1,B}X_a} - \frac{q_{2,\max,B}}{1 + \frac{S_{1,B}}{K_{I,12,B}}}\frac{S_{2,B}}{K_{2,B} + S_{2,B}}X_a \quad (11)$$

$$\frac{dS_{1,T}}{dt} = -\frac{q_{1,\max,T}S_{1,T}}{K_{1,T}\left(1 + \frac{S_{1,B}}{K_{BT}}\right) + S_{1,T}}X_a\frac{V_l}{V_l + V_g H_T} \quad (12)$$

$$\frac{dS_{2,T}}{dt} = \alpha_{12,T}\frac{q_{1,\max,T}S_{1,T}}{K_{1,T}\left(1 + \frac{S_{1,B}}{K_{BT}}\right) + S_{1,T}}X_a - \frac{q_{2,\max,T}}{1 + \frac{S_{1,T}}{K_{I,12,T}}}\frac{S_{2,T}}{K_{2,T} + S_{2,T}}X_a \quad (13)$$

$$\frac{dX_a}{dt} = \frac{q_{2,\max,B}Y_{2,B}}{1 + \frac{S_{1,B}}{K_{I,12,B}}}\frac{S_{2,B}}{K_{2,B} + S_{2,B}}X_a + \frac{q_{2,\max,T}Y_{2,T}}{1 + \frac{S_{I,T}}{K}_{I,12,T}}\frac{S_{2,T}}{K_{2,T} + S_{2,T}}X_a - bX_a \quad (14)$$

In Equations (10) to (14), K_{BT} and K_{TB} are the competitive inhibition constant of benzene to toluene and toluene to benzene, respectively.

p-xylene as the sole or secondary substrate

Since *p*-xylene is degraded cometabolically, it does not support biomass growth. Thus, sustainable biodegradation of *p*-xylene requires the presence of another electron-donor substrate (e.g., toluene). The mass-balance equations for toluene are the same as Equations (12) to (13), except that K_{BT} is substituted by the competitive inhibition constant of *p*-xylene to toluene, K_{PT} $(M_{pxl}L^{-3})$. The biomass equation is the same as Equation (4), which assumes that the cometabolic transformation of *p*-xylene diverts a negligible fraction of the electron flows. The mass balance of *p*-xylene is developed based on Assumption 4 and expressed as:

$$\frac{dS_P}{dt} = -\left(\beta_{1,T}\frac{q_{2,\max,T}}{1+\frac{S_{1,T}}{K_{I,12,T}}}\frac{S_{2,T}}{K_{2,T}+S_{2,T}}+\beta_{2,T}b\right) \times\frac{S_P}{K_P\left(1+\frac{S_{1,T}}{K_{TP}}\right)+S_P}X_a\frac{V_l}{V_l+V_gH_P} \quad (15)$$

where S_P is the concentration of *p*-xylene $(M_{pxl}L^{-3})$, K_P is the half-maximum-rate concentration for *p*-xylene $(M_{pxl}L^{-3})$, $\beta_{1,T}$ is the amount of *p*-xylene transformed per unit of toluene intermediate consumed $(M_{pxl}M_{\text{mcat}}^{-1})$, $\beta_{2,T}$ $(M_{pxl}M_X^{-1})$ is the amount of *p*-xylene transformed per unit of biomass oxidized $(M_{pxl}M_X^{-1})$, and K_{TP} is the competitive inhibition constant of toluene to *p*-xylene $(M_{\text{tol}}L^{-1})$.

When toluene is not present, electrons from toluene intermediate degradation and inhibition from toluene are absent, and Equation (15) simplifies to:

$$\frac{dS_P}{dt} = -\beta_{2,T}b\frac{S_P}{K_P+S_P}X_a\frac{V_l}{V_l+V_gH_P} \quad (16)$$

Estimating the kinetic parameters

All the kinetic parameters shown in Equations (1) and (16) were systematically estimated from the batch and chemostat experiments described earlier. Table 3 indicates which experiments were used to estimate each parameter. These systems of nonlinear differential equations, together with their initial conditions, were solved using the built-in numerical differential equation functions in *Mathematica*.

First, the decay coefficient (b) was obtained by fitting the results from the decay experiments to Equation (1). A semi-log plot of biomass vs. time yielded a slope that is b.

Second, the parameters for toluene or benzene intermediates ($q_{2,\max}$ and K_2) were obtained by fitting the chemostat results to Equation (8). Then, the biomass true yields from intermediates of benzene or toluene were obtained from Equation (9).

Third, the parameters obtained for the intermediates were applied to the equations for the single-substrate batch experiments (T1 to T7, B1 to B4) to obtain the parameters for the first-step reactions.

Fourth, the competitive inhibition constants between toluene and benzene were obtained from batch experiments using both as substrates (TB1 to TB4).

Fifth, the parameters for *p*-xylene were obtained from batch experiments with and without the presence of a growth substrate. The contribution of electrons from biomass decay (β_2) and effect of *p*-xylene concentration (K_P) were first obtained from batch experiments without the presence of any donor substrate (P1 to P4). Then, the contribution of electrons from a donor substrate, toluene, and the competitive inhibition constants between toluene and *p*-xylene were obtained from batch experiments using toluene and *p*-xylene together (TP1 to TP3).

Results and discussion

Decay coefficient

A plot of $\text{Ln}(X_a/X_{a0})$ vs. time from the biomass-decay experiments (not shown) gave $b = 0.06 \pm 0.01\ \text{day}^{-1}$.

Biomass true yield (Y_2) for the intermediates of toluene and benzene

Figures 1 and 2 shows the plots of $(D+b)X_a$ vs. $(\alpha_{12}\times S_{1,\text{in}} - S_2)D$ for the steady-state chemostat experiments using toluene and benzene as the sole donor substrate, respectively. The units of concentrations for toluene/ benzene and their intermediates are mg/l and mgCOD/l, respectively. From stoichiometry, the value of α_{12} equals 2.78 gCOD-S_2/g-toluene and 2.67 gCOD-S_2/g benzene for toluene and benzene, respectively. The slopes of the fitted lines equal the biomass true yields for benzene and toluene intermediates, which are 0.38 mg-X_a/mgCOD-S_2 and 0.32 mg-

Table 3. The equations and experiments used to fit the kinetic parameters.

Substrates	Parameters	Experiment names	Equation numbers
None	b	Decay	1
Benzene	$T_{2,B}$	Chemostat	9
	$q_{2,\max,B}$, $K_{2,B}$	Chemostat	8
	$q_{1,\max,B}$, $K_{1,B}$, $K_{I,12,B}$	B1–4	2–4
Toluene	$T_{2,T}$	Chemostat	9
	$q_{2,\max,T}$	Chemostat	8
	$q_{1,\max,T}$, $K_{1,T}$, $K_{I,12,T}$	T1–7	2–4
p-Xylene	$\beta_{2,T}$, K_P	P1–4	1, 16
Toluene/benzene	K_{TB}, K_{BT}	TB1–4	10–14
Toluene/*p*-xylene	$\beta_{1,T}$, K_{TP}, K_{PT}	TP1–3	4, 12–13, 15

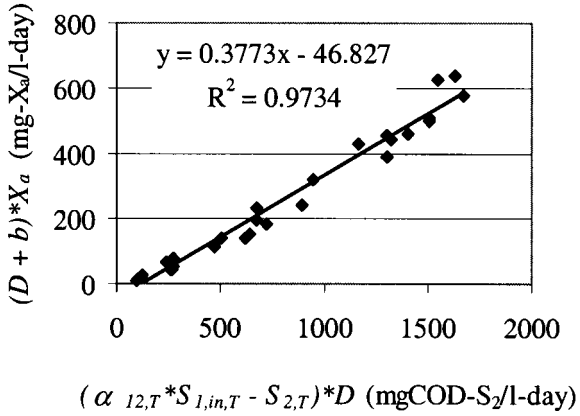

Figure 1. Fitting Equation (9) with the steady state results for toluene in the chemostat.

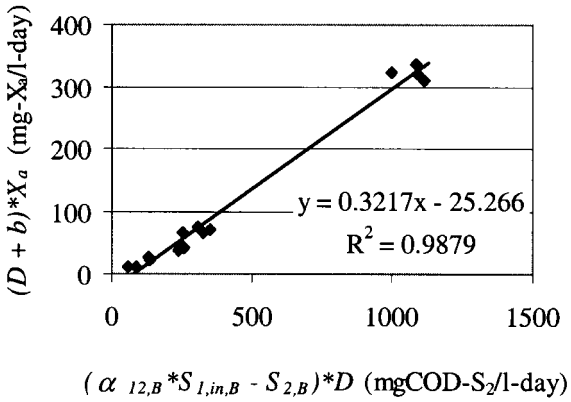

Figure 2. Fitting Equation (9) with the steady state results for benzene in the chemostat.

X_a/mgCOD-S_2, respectively. Since most studies of BTX degradation treated the degradation as a single-step reaction, direct comparison of these values with literature values is not directly relevant. However, these values can be converted to the yields from the original substrates - toluene and benzene – and equal 1.06 mg- X_a/mg-toluene and 0.85 mg-X_a/mg-benzene. The converted values are higher than the ones reported by Oh et al. (1994) (0.64 mg-X_a/mg-toluene and 0.65 mg-X_a/mg-benzene), but slightly lower than the ones reported by Chang et al. (1993) (1.22 mg-X_a/mg-toluene and 1.04 mg-X_a/mg-benzene). Both studies by Oh et al. (1994) and Chang et al. (1993) used *P. putida* strains.

Kinetic parameters $q_{2,\max}$ and K_2 for the intermediates of toluene and benzene

To fit for parameters $q_{2,\max}$ and K_2, Equation (8) was rearranged to the following form:

$$\frac{Y_{2,\text{obs}} S_2}{D} = \frac{K_2}{q_{2,\max}} + \frac{S_2}{q_{2,\max}} \tag{17}$$

where $Y_{2,\text{obs}}$ is the observed biomass yield (mg-X_a/mgCOD-S_2) in the steady-state chemostat and can be expressed as:

$$Y_{2,\text{obs}} = \frac{X_a}{\alpha_{12} S_{1,\text{in}} - S_2} \tag{18}$$

Figures 3 and 4 shows the plots of $Y_{2,\text{obs}} \times S_2/D$ vs. S_2 for the steady-state chemostat experiments with toluene and benzene, respectively. The inverses of the slopes shown in these figures is $q_{2,\max}$, and the values equal 29 mgCOD-S_2/mg-X_a-day and 27 mgCOD-

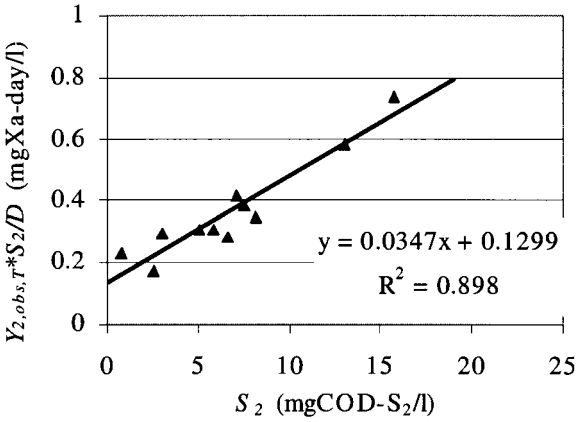

Figure 3. Fitting Equation (17) with the steady state results for toluene in the chemostat.

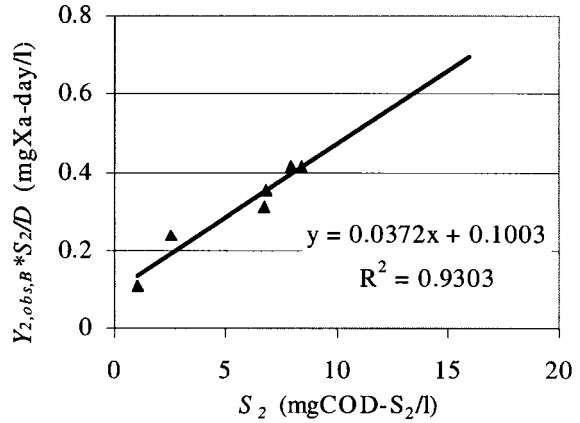

Figure 4. Fitting Equation (17) with the steady state results for benzene in the chemostat.

S_2/mg-X_a-day for the intermediates of toluene and benzene, respectively. From the intercepts of the fitted model and the values of $q_{2,\max}$ the values of K_2 are 3.7 mgCOD-S_2/l and 2.7 mgCOD-S_2/l for toluene and benzene, respectively. Since most studies of BTX degradation treated the degradation as a single-step reaction, direct comparison of these values with literature values is not appropriate.

Kinetic parameters $q_{1,\max}$ and K_1 for toluene and benzene and $K_{I,12}$ for toluene

The parameters for the first-step reactions of toluene and benzene degradation were fitted using data from batch experiments and numerical solutions of Equations (2) to (4). Equation (2) shows that, at the beginning of the batch experiments (i.e., when $S_1 > K_1$), the removal rate of toluene or benzene (dS_1/dt) is much more sensitive to the value of $q_{1,\max}$ than to the value of K_1. Thus, the value of $q_{1,\max}$ was obtained from a best fit between the numerical solutions and measurements of S_1 for the first several hours of experiments.

The value of K_1 has its most significant effects on toluene/benzene removal and biomass growth at low S_1 concentrations. Once an accurate value of $q_{1,\max}$ was found, the value of K_1 was estimated from the best fit of numerical solutions and measurements of S_1 at low S_1 concentrations, i.e., after the first several hours. In some cases, it was difficult to measure accurately the change of small S_1 values. Thus, the values of K_1 exhibit variability among experiments.

The batch experiments using toluene or benzene as the sole substrate showed inhibition to biomass growth when toluene or benzene was present. Thus, the assumption that toluene or benzene inhibited the degradation of its intermediate and biomass growth was valid. Because Equation 4 shows that the biomass growth rate is sensitive to the non-competitive inhibition constant for toluene to its intermediate, $K_{I,12,T}$, the value of $K_{I,12,T}$ was obtained from the fit between modeling and experimental results of biomass concentrations in the batch reactors.

Since we had to fit the limited amount of experimental data into a system of three nonlinear differential equations to obtain the estimations of three parameters, we chose not to use the standard optimization methods of parameters in systems of differential equations. Guha and Jaffé (1996) have reported that in such situations many sets of parameters can give almost equally good fit to the experimental data. The set of parameters that has the "best fit" may not be the most realistic one. Therefore, in this study, we used a systematic fitting method based on the characteristics of toluene/benzene degradation, biomass growth, and the model equations used. The following is an example of obtaining the values of first-step reaction parameters $q_{1,\max,T}$, $K_{1,T}$, and $K_{I,12,T}$ from batch experiment T1, whose experimental results are shown in Figure 5. The initial concentrations of biomass and toluene at equilibrium for T1 were 11.2 mg-X_a/l and 38.5 mg-toluene/l, respectively.

A *Mathematica* program solved Equations (2) to (4) numerically (fourth-order Runge–Kutta algorithm) with the initial conditions of T1 and trial kinetic parameters of the first- and second-step reactions for toluene. The values for the second-step parameters already were known from the chemostat experiments. The ranges of values for the first-step parameters were first roughly estimated by trial-and-error. For experiment

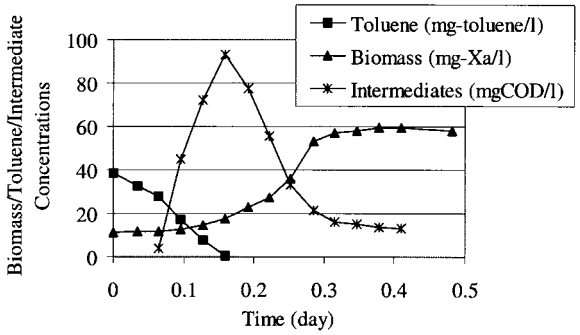

Figure 5. Closed-system batch experiment of aerobic toluene degradation.

T1, the ranges for $q_{1,\max,T}$, $K_{1,T}$ and $K_{I,12,T}$ were 23 to 30 mg-toluene/mg-X_a-day, 0.2 to 3 mg-toluene/l, and 1 to 5 mg-toluene/l, respectively.

Then, a large number of sets of $q_{1,\max,T}$, $K_{1,T}$, and $K_{I,12,T}$ values (within the ranges) were input into the program to generate a set of numerical results of $S_{1,T}$, $S_{2,T}$, and X_a for each set of $q_{1,\max,T}$, $K_{1,T}$, and $K_{I,12,T}$ values. For each set of numerical results, the relative least squares (RLS) criterion was computed for $S_{1,T}$ and X_a. The RLS criterion is defined as (Sáez and Rittmann 1992):

$$\sum_{i=1}^{n}\left(\frac{y_i - y_i^*}{y_i}\right)^2 \tag{19}$$

where n is the number of experimental data points, and y_i and y_i^* are the measured and model-generated values for variable y, respectively. Since the intermediate COD ($S_{2,T}$) was only measured for one batch experiment (T1) in this study, its values were not used for parameter fitting.

For experiment T1, the values of $q_{1,\max,T}$, $K_{1,T}$, and $K_{I,12,T}$ were stepwise varied with step sizes of 0.3 mg-toluene/mg-X_a-day, 0.2 mg-toluene/l, and 0.2 mg-toluene/l, respectively. This method resulted in 7,560 sets of parameters, each of which was imported by the *Mathematica* program to solve the equations and compute the RLS values.

Since the concentrations of toluene at equilibrium and biomass were used in the fitting, an overall criterion, F, was used to select the best-fit set of parameters:

$$F = \sqrt{\sum_{i=1}^{m}\left(\frac{S_{1,T,i} - S_{1,T,i}^*}{S_{1,T,i}}\right)^2 + \sum_{i=1}^{n}\left(\frac{X_{a,i} - X_{a,i}^*}{X_{a,i}}\right)^2} \tag{20}$$

where m and n are the number of toluene and biomass data points used in the fitting, respectively. Since biomass growth continued after toluene was depleted, m was always smaller than n. In experiment T1, the values of m and n used were 6 and 10, respectively. The set of values of $q_{1,\max,T}$, $K_{1,T}$, and $K_{I,12,T}$ that had the smallest F was chosen as the best fit. For T1, the three values were 26 mg-toluene/mg-X_a-day, 0.4 mg-toluene/l, and 3.4 mg-toluene/l, respectively. The F value for this set of parameters was 0.311.

To see the sensitivity of the parameter sets, we defined an F boundary giving a "satisfactory" fit to the experimental data. For example, if every data point and its model prediction had a 10% relative error, then Equation (20) gives an F value of equals $0.1\sqrt{m+n}$. For experiment T1, this boundary of F was 0.4, and 108 sets of parameters fell within this boundary. The average $q_{1,\max,T}$, $K_{1,T}$, and $K_{I,12,T}$ values for all these 108 "satisfactory" sets were 27 mg-toluene/mg-X_a-day, 0.76 mg-toluene/l, and 3.2 mg-toluene/l, respectively.

The same fitting method was applied to the other batch experiments using toluene or benzene as the sole substrate. Table 4 lists the averages and standard deviations of fitted values of $q_{1,\max,T}$, $K_{1,T}$, and $K_{I,12,T}$ for toluene and benzene (108 sets for each substrate) from the experiments. Table 4 also shows the averages of $q_{1,\max}$, K_1, and $K_{I,12}$ calculated from all the tests. The values of $q_{1,\max}$ and K_1 for toluene and benzene obtained in this study are within the ranges of values reported by other researchers (Arcangeli 1994; Chang et al. 1993; Oh et al. 1994; Mirpuri et al. 1997). Since most studies treated toluene and benzene degradation as single-step reactions, no direct comparison of $K_{1,12}$ values is possible.

Figure 6 compares the model-generated and measured concentrations of toluene and biomass. The model predictions from parameters with the minimum F and with the "averages" values overlap each other completely. The model-generated results capture the major trends in toluene and biomass. Some systematic deviation occurs as the biomass approaches its maximum plateau. This suggests that the model may slightly over-estimate the rate of intermediate degradation for low intermediate concentrations.

Kinetic parameters of $\beta_{2,T}$ and K_P for p-xylene removal by biomass

When an electron-donor substrate is absent, cometabolic degradation of *p*-xylene (Equation (16)) con-

Table 4. Fitted values of $q_{1,\max}$, K_1, and $K_{I,12}$ for toluene and benzene.

Test Name	$q_{1,\max}$ (mg-VOC/mg-X_a-day		K_1 (mg-toluene/l) or (mg-benzene/l)		$K_{I,12}$ (mg-toluene/l) or (mg-benzene/l)	
	Average	Std. dev.	Average	Std. dev.	Average	Std. dev.
T1	27	1.2	0.76	0.43	3.2	0.74
T2	26	0.87	0.73	0.25	3.0	1.2
T3	27	0.63	0.43	0.23	3.7	0.94
T4	26	0.95	1.6	0.30	3.5	0.94
T5	24	0.29	1.9	0.13	3.8	0.82
T6	26	1.1	1.8	0.20	3.5	0.96
T7	28	0.15	0.26	0.10	4.4	0.59
Average	26	1.3	1.1	0.68	3.6	0.44
B1	24	0.87	3.9	0.52	7.1	0.91
B2	28	0.95	2.8	0.41	4.8	0.94
B3	27	0.93	1.2	0.31	7.3	0.94
B4	23	1.5	8.8	1.0	7.8	0.78
Average	25	2.3	4.2	3.3	6.8	1.4

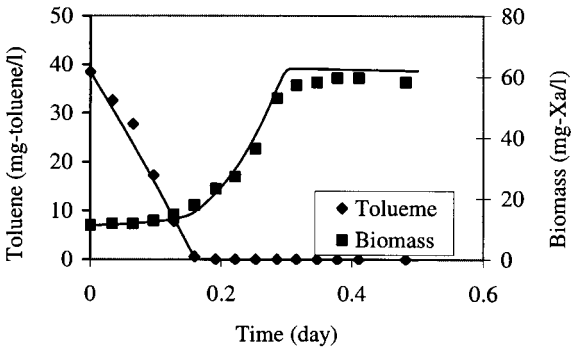

Figure 6. Comparison of model-generated and measured concentrations of toluene biomass for batch experiment T1.

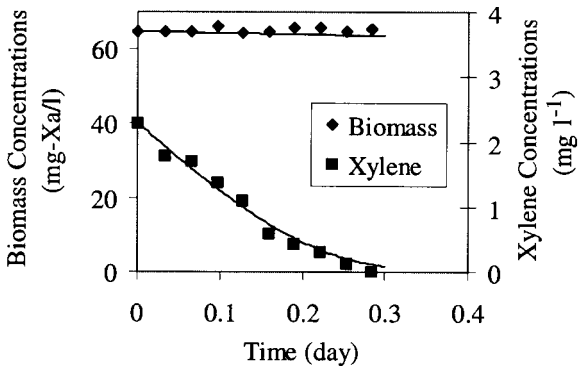

Figure 7. Comparison of experimental and model-generated data for p-xylene experiment P3. The lines are model-generated results.

sumes electrons released from biomass decay (Equation (1)). Thus, the values of $B_{2,T}$ and K_P can be obtained from fitting experimental data to a model that consists of Equations (1) and (16). The same approach used for fitting the values of $q_{1,\max}$, K_1, and $K_{I,12}$ for toluene and benzene was used. Since biomass concentration in the liquid was not related to the values of $\beta_{2,T}$ *and* K_P (Equation (17)), only the RLS for p-xylene concentrations was used in the criterion F. The boundary for F used also was narrowed to $0.05\sqrt{m}$. Figure 7 shows comparison of the best-fit model-generated and experimental data of a typical run. The results from all the tests using p-xylene as the sole substrate are shown in Table 5. Since each mole of p-xylene requires 4 moles of electrons for its first-step reaction, the fraction of decay-released electrons that is used by p-xylene can be calculated from the value for β_2:

$$4.1\frac{g-\text{xylene}}{g-X_a}\frac{113g-X_a}{20\,\text{mole}-e^-}\frac{4\,\text{mole}-e^-}{106g-\text{xylene}}=87\%$$

Competitive inhibition constants between toluene and benzene, K_{BT} *and* K_{TB}

The competitive inhibition constants between toluene and benzene, K_{BT} and K_{TB}, were obtained from similar fitting methods used to obtain the first-step kinetic

Table 5. Fitted values of K_P and β_2 for p-xylene.

Test name	K_P (mg-xylene/l)		β_2 (mg-xylene/mg-X_a)	
	Average	Std. dev.	Average	Std. dev.
P1	0.60	0.19	4.1	0.24
P2	0.59	0.19	3.9	0.39
P3	0.37	0.04	4.3	0.15
P4	0.48	0.12	4.0	0.23
Average	0.51	0.11	4.1	0.18

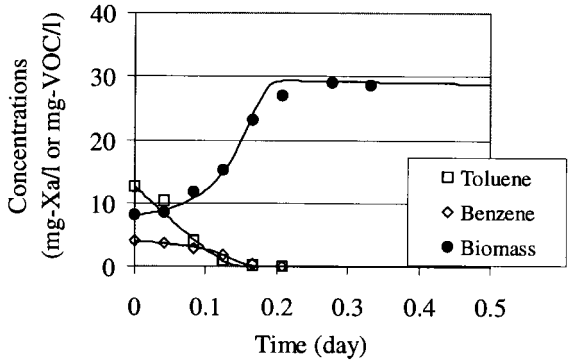

Figure 8. Comparison of experimental and model-generated data from toluene-benzene experiment, TB1. The lines are model-generated results using average parameter values.

parameters. A *Mathematica* program was developed using Equations (10)–(14). The changes in concentrations of biomass, toluene, and benzene were fitted to the model to obtain the best-fit values of K_{BT} and K_{TB}, which were the only unknowns in the model. Figure 8 shows the results for one of the batch experiments using toluene and benzene as dual substrates. Table 6 lists the fitted parameters for all dual-substrate batch experiments.

The small values of K_{BT} and K_{TB} mean that toluene and benzene strongly inhibit each other during the

Table 6. Fitted values of K_{BT} and K_{TB} between toluene and benzene.

Test name	K_{BT} (mg-benzene/l)		K_{TB} (mg-toluene/l)	
	Average	Std. dev.	Average	Std. dev.
TB1	0.51	0.06	0.48	0.09
TB2	0.49	0.05	0.49	0.06
TB3	0.52	0.10	0.50	0.06
TB4	0.52	0.04	0.49	0.06
Average	0.51	0.01	0.49	0.01

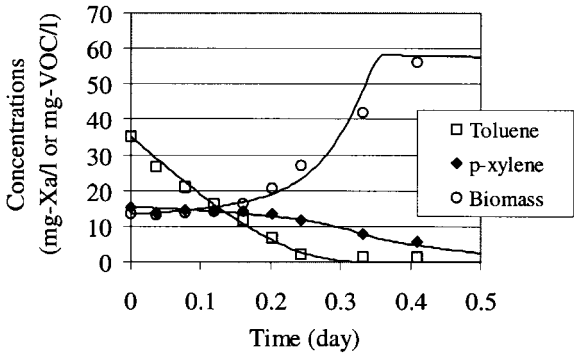

Figure 9. Comparison of experimental and model-generated data from toluene-xylene experiment. TP1. The lines are model-generated results using average parameter values.

first step of reaction. In addition, the values of K_{BT} and K_{TB} are close to each other, which reinforces that their interaction is truly competitive.

Competitive inhibition constants K_{PT} and K_{TP}, and β_1 for toluene

The competitive inhibition constants between toluene and p-xylene, K_{PT}, and K_{TP} were obtained from similar fitting methods. The equations used here include Equations (4), (12) to (13), and (15). The changes in concentrations of biomass, toluene, and p-xylene were fitted to the model to obtain the best-fit values of β_1, K_{PT}, and K_{TP}, which were the only unknowns in the model. Figure 9 shows the results for one of the batch experiments using toluene and p-xylene as dual substrates. Table 7 lists the fitted parameters for the dual-substrate batch experiments. Since complete oxidation of one mole of toluene intermediate, 3-methylecatechol, generates 32 moles of electrons, the fraction of electrons used by p-xylene is:

$$0.21\frac{g-\text{xylene}}{g\text{COD}-S_{2,T}}\frac{256g\text{COD}}{32\,\text{mole}-e^-}\frac{4\,\text{mole}-e^-}{106g-\text{xylene}}$$

$$=6.3\%$$

The results in Table 7 show that the inhibition for p-xylene to toluene was much stronger than vice versa. Although toluene inhibits p-xylene degradation only at large concentrations, p-xylene slows toluene destruction throughout the experiment. Due to the small changes of p-xylene concentration during the period of high toluene concentration, the values of K_{TP} have relatively large standard deviations.

Table 7. Fitted values of β_1, K_{PT}, and K_{TP} for toluene and p-xylene.

Test Name	β_1 (mg-xyl/mgCOD-S_2)		K_{PT} (mg-xylene/l)		K_{TP} (mg-toluene/l)	
	Average	Std. dev.	Average	Std. dev.	Average	Std. dev.
TP1	0.21	0.05	0.84	0.09	25	8.6
TP2	0.20	0.01	0.99	0.09	25	8.7
TP3	0.21	0.06	0.77	0.05	25	8.6
Average	0.21	0.01	0.87	0.11	25	0.22

Conclusions

All features of toluene, benzene, and p-xylene biodegradation could be modeled well as a two-step process. The first step does not support the biomass growth and does not generate electrons or carbon for cell synthesis. Biomass growth is supported by the second-step reactions, which are the oxidations of catechol intermediates of toluene and benzene. p-xylene is cometabolically transformed and supports no biomass synthesis. Toluene or benzene inhibits the degradation of its intermediate non-competitively. When toluene and benzene or toluene and p-xylene are degraded simultaneously, they inhibited each other's degradation competitively.

A suite of different batch and chemostat experiments yielded the experimental results needed to obtain all of the kinetic parameters associated with the fully aerobic biodegradation of toluene, benzene, or p-xylene as a single substrate and with toluene-benzene or toluene-xylene as dual substrates. The best values of all the kinetic parameters are summarized in Table 8.

The values in Table 8 do not necessarily represent the "optimal" parameters in a strictly statistical sense. Nonetheless, they clearly show that the two-step model accurately described all aspects of the biodegradation of toluene, benzene, and p-xylene with reasonable parameter values in all cases.

Table 8. Summary of kinetic parameters for BTX degradation.

	Toluene	Benzene	p-Xylene
Y_2 (mg-X_a/mgCOD)	0.38	0.32	
$q_{2,\max}$ (mgCOD/mg-X_a-day)	29	27	
K_2 (mg-COD/l)	3.7	2.7	
$q_{1,\max}$ (mg-VOC/mg-X_a-day)	26	25	
K_1 (mg-VOC/l)	1.1	4.2	
$K_{I,12}$ (mg-VOC/l)	3.6	6.8	
β_2 (mg-xylene/mg-X_a)			4.1
K_P (mg-xylene/l)			0.51
K_{BT} (mg-benzene/l)		0.51	
K_{TB} (mg-toluene/l)	0.49		
β_1 (mg-xylene/mgCOD)			0.21
K_{PT} (mg-xylene/l)			0.87
K_{TP} (mg-toluene/l)	25		
b (day^{-1})	0.06		

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