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Kinetics and interactions of BTEX compounds during degradation by a bacterial consortium

Jennifer V. Littlejohns, Andrew J. Daugulis *

Department of Chemical Engineering, Queen's University, Kingston, Ontario K7L 3N6, Canada

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ABSTRACT

A model to describe the biodegradation of benzene, toluene, ethylbenzene and *o*-xylene (BTEX) and growth of a bacterial consortium was systematically developed from a series of aerobic batch degradation experiments. The bacterial consortium was enriched from petroleum contaminated soil on a mixture of BTEX components and was identified to contain 7 unique species of *Pseudomonas*. Parameter estimates are reported for both conventional Monod parameters obtained from single substrate degradation experiments and interaction parameters obtained from dual substrate experiments. Key interactions identified include both the inhibition and enhancement of biodegradation rates for mixed substrates relative to single substrate experiments. Enhancement interactions have been qualitatively observed by other authors to occur to BTEX mixtures, but have not been quantified previous to the current study. Observations include the inhibition of benzene degradation in the presence of toluene and in the presence of ethylbenzene. As well, enhanced degradation of benzene was observed in the presence of *o*-xylene and toluene degradation was enhanced in the presence of benzene. A sum kinetics with interaction parameters (SKIP) model was found to accurately describe these interactions. In addition, it was found that *o*-xylene was cometabolized in the presence of toluene and/or benzene and a mathematical model was used to describe this interaction. The SKIP and cometabolism models were combined to predict both BTEX degradation as well as biomass production for this consortium when all BTEX components are present simultaneously.

1. Introduction

Benzene, toluene, ethylbenzene and *o*-xylene, collectively known as BTEX, are toxic compounds commonly emitted into the environment due to their ubiquitous presence in fuel and petroleum products. Biological processes are becoming increasingly popular for the elimination of these compounds from air [1], water [2] and soil environments [3] with the goal of achieving regulatory levels. Relative to thermochemical destruction methods, biological processes have inherent green benefits and potential cost savings. In addition, biological processes have the ability to effectively mineralize BTEX in low concentrations.

In order to properly design and model biodegradation processes it is necessary to determine the degradation kinetics of these compounds by bacterial communities. Degradation of combinations of BTEX components by pure bacterial strains has been studied, such as by bacterial cultures of *Rhodococcus rhodochrous* [4], several strains of *Pseudomonas putida* [5] and *Alcaligenes xylosoxidans* [6]. However, in order to efficiently degrade all BTEX components simultaneously a bacterial consortium is required, particularly for the removal of *o*-xylene, which has been found to be markedly persistent compared to other BTEX compounds [4,7].

The degradation of more than one growth limiting substrate by a bacterial population is not straightforward, as many different substrate interactions have been identified for combinations of BTEX components that can alter degradation rates relative to the absence of other substrates [4]. Such interactions can involve the enhancement or inhibition of degradation of substrates when in mixtures [8,9,10]. Inhibition of BTEX degradation by a bacterial consortium has previously been modeled using purely competitive inhibition kinetics [11], however enhancement interactions that have been qualitatively observed to occur during BTEX degradation by a consortium [8,12] have not been modeled to date. Moreover, an important stimulation interaction that has been observed to occur to *o*-xylene in the presence of other BTEX compounds is cometabolism [7] and models in order to describe the degradation of xylenes due to this interaction have been applied successfully [13].

* Corresponding author. Tel.: +1 613 533 2784; fax: +1 613 533 6637.
E-mail address: andrew.daugulis@chee.queensu.ca (A.J. Daugulis).

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Systematic approaches for determining substrate interactions during degradation by pure bacterial strains have been used for a mixture of three aromatic hydrocarbons [14] and three PAHs [15] and an identified and quantified bacterial consortia for a mixture of three aromatic hydrocarbons [16]. The present study successfully quantifies the kinetic parameters and substrate interactions for all four BTEX components during degradation by a bacterial consortium. As noted above, the only substrate interaction modeled for BTEX components to date has been competitive inhibition, and the current study serves to quantify additional interactions that have been observed only qualitatively. An appropriate model to describe interactions between all four BTEX components is systematically determined using dual substrate experimental data. The model structure is then verified by means of comparison to the experimental data for the degradation of all four components simultaneously. In addition, the current study investigates the use of the semi-empirical Monod model to predict biomass growth for a bacterial consortium with minimal knowledge of consortium composition. The developed model is shown to fit experimental data accurately.

2. Theory

For batch degradation biomass growth can be described by Eq. (1) [17], which can describe biomass growth due to a single or multiple substrates.

$$\frac{dX}{dt} = \mu X \tag{1}$$

Depletion of growth associated substrates in a batch degradation for a given substrate, i, can be described using Eq. (2).

$$\frac{dS_i}{dt} = -\frac{\mu_i X}{Y_{X/S_i}} \tag{2}$$

These equations hold true when maintenance requirements are negligible, which is typically assumed during the period of kinetic measurement of rapidly growing cells, as the metabolism of substrate is primarily growth associated [18]. It should be noted that the yield coefficient (Eq. (2)) must consider the consumption of compounds from both gas and liquid phases due to the volatility of BTEX, assuming that transfer between gas and liquid phases is rapid.

There are several models used to describe the specific growth rate for use in Eqs. (1) and (2). The most common model for the biodegradation of a single growth substrate, the Monod model, is shown as Eq. (3) [17].

$$\mu_i = \frac{\mu_{\max_i} S_i}{K_{S_i} + S_i} \tag{3}$$

Single substrate degradation experiments can be used to estimate the kinetic parameters $\mu_{\max}$ and K_s for each substrate. One method of estimating the kinetic parameters $\mu_{\max}$ and K_s involves fitting Eq. (3) to experimentally obtained specific growth rates as a function of substrate concentration for single substrate experiments.

Due to the toxic nature of BTEX and the possibility of substrate inhibition, a modified Monod model, the Andrews model, shown as Eq. (4) [19] may provide a better fit to experimental data obtained from single substrate experiments.

$$\mu_i = \frac{\mu_{\max_i} S_i}{K_{S_i} + S_i + S_i^2/K_I} \tag{4}$$

Again, experimentally obtained specific growth rates can be plotted as a function of substrate concentrations and fit to Eq. (4) to estimate the three kinetic parameters, $\mu_{\max}$, K_s and K_I.

The kinetic parameters, $\mu_{\max}$, K_s and possibly K_I, determined from single component degradation processes can be retained and used in specific growth rate models in which more than one growth limiting substrate is present. However, as stated previously, there is increased complexity in modeling multiple substrate degradation due to substrate interactions. Different models to describe the specific growth rate during the degradation of multiple interacting substrates have been developed in analogy to enzyme kinetics. The analogy can be made between enzyme kinetics and cellular kinetics because, if a reaction is enzyme catalyzed, then the inhibition of enzyme activity results in the inhibition of microbial growth by the same pattern [17]. The models used to account for these interactions can be used in substrate degradation equations (Eq. (2)). A common interaction for BTEX compounds is competitive inhibition, which can be seen in Eq. (5) [20]. During competitive inhibition, substrates compete for binding sites in order to be metabolized by the bacterial population.

$$\mu_i = \frac{\mu_{\max_i} S_i}{K_{S_i}(1 + (S_I/K_{S_I})) + S_i} \tag{5}$$

Another inhibition interaction is non-competitive inhibition wherein a nonreactive complex is formed when both substrates simultaneously are bound to one enzyme. This is shown as Eq. (6) [20].

$$\mu_i = \frac{\mu_{\max_i} S_i}{(K_{S_i} + S_i)(1 + (S_I/K_{S_I}))} \tag{6}$$

Uncompetitive inhibition is another interaction that can occur when multiple substrates are present, which is shown in Eq. (7) [20]. Uncompetitive inhibition is a situation in which one substrate can bind to only a substrate enzyme complex, not just the free enzyme.

$$\mu_i = \frac{\mu_{\max_i} S_i}{K_{S_i} + S_i(1 + (S_I/K_{S_I}))} \tag{7}$$

Finally, a model that accounts for substrate interactions without directly specifying the type of interaction is shown as Eq. (8) [21]. This model contains an interaction parameter that is treated as an unknown.

$$\mu_i = \frac{\mu_{\max_i} S_i}{K_{S_i} + S_i + I_{2_i} S_I} \tag{8}$$

Eq. (8) is called SKIP for sum kinetics with interaction parameters (SKIP) [14], which will become more evident as sum kinetics is described below.

In order to describe the growth rate of biomass when mixed growth substrates are present, sum kinetics, shown in Eq. (9) [21], can be used as an expression for specific growth rate, which can then be substituted into Eq. (1). Sum kinetics considers the contribution of each substrate present in a system to biomass growth.

$$\mu = \mu_1 + \mu_2 + \ldots + \mu_n = \frac{\mu_{\max_1} S_1}{K_{S_1} + S_1} + \frac{\mu_{\max_2} S_2}{K_{S_2} + S_2} + \ldots + \frac{\mu_{\max_n} S_n}{K_{S_n} + S_n} \tag{9}$$

The sum kinetics equation shown in Eq. (9) is for a situation in which there are no substrate interactions and simple Monod equations are summed. Furthermore, specific growth rate equations accounting for interactions, which have been described above, can be used in sum kinetics equations if interactions are found to occur among mixed substrates [11,14]. There is a limitation in using Eq. (9) substituted into Eq. (1) to describe biomass growth for a bacterial consortium, as a biomass formation equation should consider the biomass concentration able to

degrade each particular substrate [22]. However, sum kinetics equations have been used to describe biomass growth for pure strains of bacteria degrading mixtures of substrates [14], in which the fraction of pure strain degrading each substrate is not considered. The measured biomass concentration is multiplied by each term accounting for the biomass growth due to one substrate in the sum kinetics equation, implying that the entire biomass population will metabolize each substrate. Due to the previous success in using sum kinetics to predict biomass growth, as well as the semi-empirical nature of these kinetic equations [17], sum kinetics equations will be used to predict growth of a consortium of bacteria, with minimal knowledge of the consortium composition, in the current study.

By determining which of these models provide the most accurate fit to experimental data for a particular pair of substrates, the presence of any substrate interactions, as well as the nature of the interactions can be determined. Once the interactions between each pair of substrates is determined, a specific growth rate equation taking into account the interactions of each compound present can be determined for mixed substrates with more than two compounds present. An example of such an equation for a SKIP model with four interacting components is shown in Eq. (10).

$$\mu_i = \frac{\mu_{\max_i} S_i}{K_{S_i} + S_i + I_{2_i} S_2 + I_{3_i} S_3 + I_{4_i} S_4} \tag{10}$$

Again, models considering interactions between more than two substrates can be used in sum kinetics form to describe the growth of biomass.

For the case of cometabolism, the specific growth rate due to the degradation of a component is zero, as the substrate is not metabolized for energy purposes. However, an expression is needed to describe the disappearance of a cometabolized compound. Cometabolization has been described by Chang et al. [13] using Eq. (11), wherein the disappearance of the non-growth substrate is described by the disappearance of the growth substrate.

$$\frac{dS_N}{dt} = -\left(T_g^c\left(\frac{dS_G}{dt}\left(\frac{1}{X}\right)\right)\right)\left(\frac{S_N}{K_{S_N} + S_N}\right)X \tag{11}$$

Using these established interaction equations, the present study identifies and quantifies interactions that occur during the degradation of BTEX components while providing a method of estimating biomass growth for a bacterial consortium.

3. Materials and methods

3.1. Growth medium and chemicals

The carbon-free growth medium formulation included the following components in water [23]; 7 g/L $(NH_4)_2SO_4$, 0.75 g/L $MgSO_4{\cdot}7H_2O$, 6.6 g/L K_2HPO_4, 8.42 g/L KH_2PO_4 and 1 mL/L trace elements. Stock trace element solution was prepared as follows: 16.2 g/L $FeCl_3{\cdot}6H_2O$, 9.44 g/L $CaHPO_4$, 0.15 g/L $CuSO_4{\cdot}5H_2O$, and 40 g/L citric acid. All nutrients used in the growth medium were obtained from either Sigma–Aldrich (Canada) or Fisher Scientific (Canada). Carbon sources as benzene (99%, min., assay) and *o*-xylene (98%, HPLC grade) were obtained from Sigma–Aldrich and toluene and ethylbenzene were obtained from Fisher Scientific. Tryptic Soy Broth (TSB) for inoculum preparation was obtained from DIFCO (Canada).

3.2. Microorganisms

The bacterial consortium was previously enriched from petroleum contaminated soil obtained from a refinery in Sarnia, Ontario in a continuous reactor with BTEX as the only available carbon source. Gaseous BTEX was continuously fed into the reactor at a rate of 50 mg BTEX/Lh with approximately equal amounts of each component. The continuous addition and removal of growth medium was undertaken to achieve an aqueous dilution rate of 0.1 h^{-1} in order to effectively washout the bacteria that were not metabolizing the BTEX. This enrichment was carried out for 12 days. The consortium was then cryogenically preserved at −86 °C after preserving in 10% (v/v) dimethyl sulfoxide until inoculation. Prior to inoculation the cryogenically preserved consortium was incubated for 24 h with sterile TSB as a carbon source in order to increase cell density. The culture was then centrifuged and the resulting biomass pellet was rinsed several times and resuspended in medium. The mixture was then used for inoculation.

In order to determine the type of bacteria present within the consortium, a denatured gradient gel electrophoresis (DGGE) was completed by Microbial Insights, TN, USA.

3.3. Kinetic experiments

In total, 12 batch degradations were completed; 4 single component batch biodegradations for each of benzene, toluene, ethylbenzene and *o*-xylene, 6 dual substrate batch degradation for each combination of BTEX components, one batch degradation with all BTEX components present and one control batch degradation without the addition of biomass in order to verify there were no losses due to volatilization during sampling. Each batch degradation was carried out in a sterilized 250 ml air-sealed bottle containing 100 ml of media with a self sealing Teflon septum on the cap for sampling. A sufficient amount of headspace was provided to avoid oxygen limiting conditions. The compounds for each experimental run were added in amounts to obtain an approximate total of 80 mgBTEX/L in the liquid phase, considering partitioning of the compounds between the gas and liquid phases. Each bottle, with the exception of the control, was inoculated by the same bacterial culture at approximately the same time to give a final concentration of approximately 20 mg/L biomass in the liquid phase in order to make the initial substrate to biomass ratio high to obtain intrinsic and unique parameter estimates of kinetics [24]. After inoculation, each bottle was put on a rotary shaker and maintained at 30 °C. Each bottle was periodically removed from the shaker and the headspace was sampled using a gas-tight syringe and the aqueous phase was sampled using a chemical resistant liquid sampling syringe.

3.4. Analytical methods

Gas chromatography was used to measure BTEX concentrations directly in the gas phase, allowing calculation of aqueous phase concentrations using Henry's Law. This indirect method of determining the aqueous phase concentration has been shown to provide accurate kinetic measurements without being mass transfer limited [10,13]. Samples were analyzed on a Perkin–Elmer AutoSystem Gas Chromatograph, which was fitted with a flame ionizing detector and a fused silica capillary column (DB-5, 0.53 mm I.D., 30 m length, 1 μm film thickness) that was designed to be well suited for the analysis of volatile components, particularly BTX compounds. Helium was used as the carrier gas, flowing at 15 mL/min and the injector and detector temperatures were set at 140 °C and 290 °C, respectively. The column had an initial temperature of 75 °C, a final temperature of 140 °C and a temperature increase rate of 25 °C/min. Output from the gas chromatograph was recorded on a personal computer equipped with Millenium^{32} (Workstation Version 3.05.01, Waters Corp., USA) software to perform peak integration and analysis.

Biomass concentrations were determined using optical density measurements, which were evaluated using a Biochrom Ultraspec 3000 UV/Visible Spectrophotometer (Biochrom, Ltd., UK) at 600 nm. A 10 ml syringe was used to extract 1 ml from each airtight bottle and serial dilutions were preformed if necessary to ensure the sample was in the linear range of the instrument.

3.5. Parameter estimation and determination of model adequacy

In order to estimate kinetic parameters for single substrate experiments, nonlinear curve fitting was preformed on Eq. (3) fit to specific growth rate vs. substrate concentration experimental data. For each experiment, as many data points as possible were taken over the entire substrate range in order to obtain unique parameter estimates [25]. The software performing the nonlinear curve fitting was JMP™, which uses a Gauss-Newton iteration sequence. The parameter estimates were retained based on the following criteria; residual analysis, confidence intervals for the estimated parameters and Lack of Fit (LOF) testing. Additionally, linear regression was performed in JMP™ in order to estimate yield coefficients for each BTEX component. The adequacy of these parameter estimates were determined by viewing residual vs. predicted plots, mean square regression ratio tests, R^2 values and parameter significance to the 95% interval. The Andrews model (Eq. (4)) was also fit to single substrate experimental data and the interaction constant estimated in order see if it would provide a better fit.

Using the kinetic parameters determined from single substrate experiments, models for dual substrate degradation were determined by substituting the different possible specific growth rate equations into Eq. (2). All possible specific growth rate equations (Eqs. (5–8)) were fit to the experimental substrate degradation data in order to identify the type of substrate interaction occurring for each combination of substrates. Also, using sum kinetics, biomass growth equations (Eq. (1)) were solved for different combinations of possible substrate interactions. These equations were solved using MATLAB® 6.1 (The MathWorks, Inc., USA). ODE15S was the intrinsic ordinary differential equation solver used in MATLAB® 6.1. These resulting model outputs were visually compared to dual substrate experimental data to determine the corresponding interaction or inhibition type that provided the most accurate fit. Visual inspection was used

to estimate initial guesses for interaction parameters for the case of the SKIP model, as well as the growth substrate transformation capacity for the case of cometabolism. Finally, the kinetic parameters and substrate interactions that were determined from single and dual substrate experiments were validated by combining them to model the degradation of all four components of BTEX, which was compared to experimental data to verify model accuracy.

4. Results and discussion

4.1. Microorganisms

It was determined that the bacterial consortium consisted of 7 distinct species of *Pseudomonas*, 5 with an excellent similarity index and 2 with a good similarity index. Each species had to constitute greater than 1–2% of the total bacterial population in order to represent a band on the DGGE. A blast search on the DGGE sequences showed that 2 of the *Pseudomonas* species present are likely *Pseudomonas putida* and *Pseudomonas fluorescens*.

4.2. Single substrate experiments

Substrate degradation profiles and the corresponding biomass growth as a function of time for single substrate experiments can be seen in Fig. 1a, b, c and d for benzene, toluene, ethylbenzene and *o*-xylene, respectively. All experimental data reveal a typical saturation type shape that is characteristic of the Monod model [22], with the exception *o*-xylene in Fig. 1d. Unlike benzene, toluene and ethylbenzene, the degradation of *o*-xylene reveals that it is not metabolized by the consortium when being the only carbon source present.

It should be noted that the lag period is not predicted by any of the unstructured kinetic models used in this study. All plots show the presence of a lag phase during these experiments, however, the models were fit only to the post lag phase data. The solution to the substrate degradation equation and biomass growth equation for benzene, toluene and ethylbenzene using the Monod model can also be seen plotted with experimental data in Fig. 1a, b and c, respectively. These results reveal that the Monod model describes the experimental data accurately and a summary of the Monod kinetic parameter estimates for single substrate biodegradation experiments is shown in Table 1. The likelihood intervals for the parameter estimates, also shown in Table 1, indicate that the estimated Monod parameters are significant for benzene, toluene and ethylbenzene, however, there were no statistically significant parameters estimated for *o*-xylene due to degradation not occurring. LOF tests were completed for each Monod model fit to experimental data. In order to get an estimate of the pure error, replicates completed at the beginning of each data set during the lag period were used. There was no significant lack of fit for the case of benzene, toluene and ethylbenzene, confirming that the Monod model adequately describes the observed trends for single substrate degradation. In addition, residual analysis was completed and no significant trend was found. These statistics show that the estimated parameters and Monod model are adequate to describe individual substrate degradation and biomass growth for benzene, toluene and ethylbenzene. The results for yield coefficients for benzene, toluene and ethylbenzene are also displayed in Table 1. Again, residual plots were determined to have no trend, the mean square regression ratios revealed that the variance described by the model is significant relative to inherent variance, and all yield coefficient parameter estimates were deemed to be significant.

When fitting the Andrew's model (Eq. (4)) to experimental data, the value of K_I was very large for all three cases, causing the Andrews model to reduce to the Monod model (data not shown).

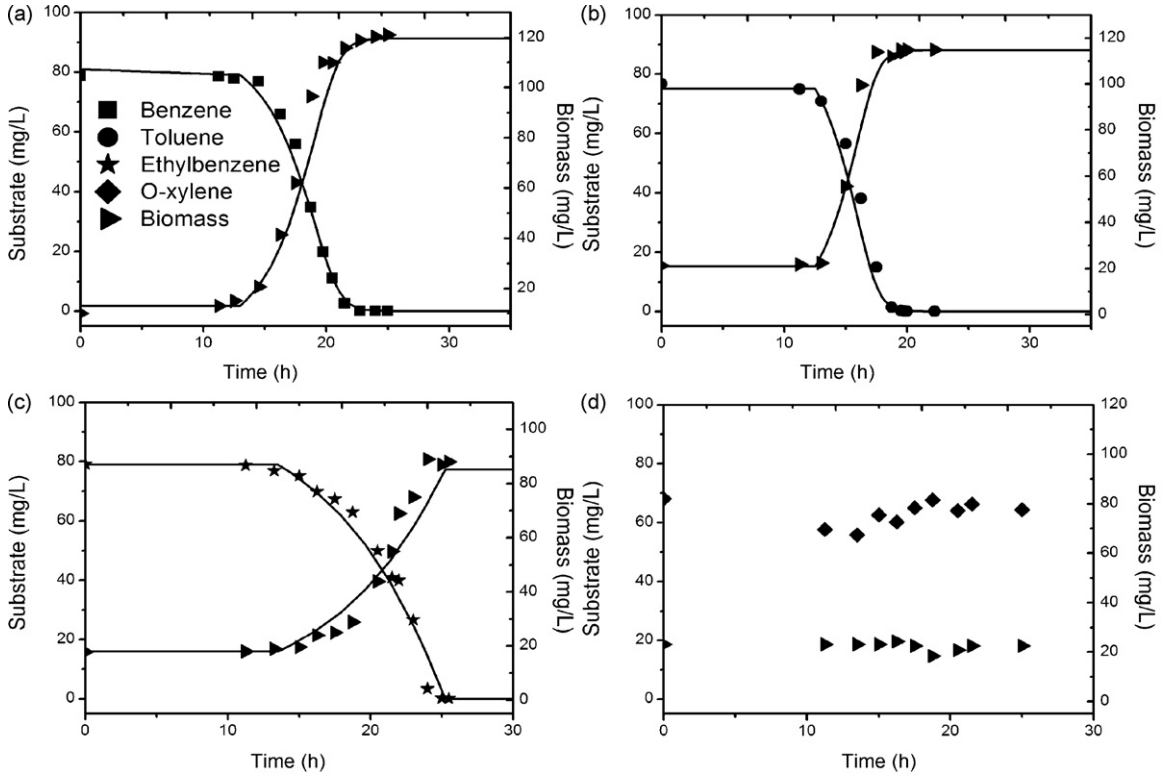

Fig. 1. (a) Benzene and biomass concentrations in aqueous phase for single substrate experiment (shapes) and Monod model fit (lines). (b) Toluene and biomass concentrations in aqueous phase for single substrate experiment (shapes) and Monod model fit (lines). (c) Ethylbenzene and biomass concentrations in aqueous phase for single substrate experiment (shapes) and Monod model fit (lines). (d) *o*-Xylene and biomass concentrations in aqueous phase for single substrate experiment (shapes) and Monod model fit (lines).

Table 1
Parameter estimates for single substrate experiments

Compound	μ_{max} (1/h)	μ_{max} Likelihood interval	K_S (mg/L)	K_S Likelihood interval	$Y_{x/s}$ (mg/mg)	$Y_{x/s} - R^2$
Benzene	0.44	0.39–0.50	27.57	19.51–38.58	1.35	0.991
Toluene	0.60	0.52–0.68	34.12	25.04–46.24	1.25	0.981
Ethylbenzene	0.13	0.11–0.16	0.36	0.11–2.12	0.85	0.879

Therefore, substrate inhibition was not a factor for any single substrate experiments over the range of substrate concentrations investigated and only the Monod parameters were retained.

4.3. Dual substrate experiments

Dual substrate experiments for combinations of BTEX components revealed interactions that have not been quantified previously by other authors for BTEX components. Specific growth rate models that account for competitive inhibition, noncompetitive inhibition and uncompetitive inhibition among dual substrates (Eqs. (5–7)) were determined using kinetic parameters from single substrate experiments and were substituted into substrate depletion and biomass growth equations. These models did not provide an accurate fit to experimental data for any combination of BTEX components (data not shown). Therefore, the SKIP model was used in order to describe any observed substrate interactions and will be described in more detail below. Those dual substrate experiments that are combinations of substrates that can be used as sole carbon sources will be discussed first and dual experiments containing *o*-xylene will be discussed later in this section. Experimental data for combinations of substrates that can be used as sole carbon sources, can be seen in Fig. 2a, b and c for benzene/toluene, benzene/ethylbenzene and toluene/ethylbenzene, respectively.

Relative to single substrate experiments (Fig. 1a and b), the simultaneous degradation of benzene and toluene, shown in Fig. 2a, resulted in a slightly inhibitory effect of toluene on benzene degradation as an interaction parameter, $I_{T,B}$, of 2 provided the most accurate fit to experimental data, whereas the presence of benzene had an enhancing effect on toluene degradation as an interaction parameter, $I_{B,T}$, of −0.4 provided the most accurate fit. The SKIP model using these parameters is plotted along with experimental data in Fig. 2a. The SKIP model provides an accurate fit for benzene/toluene degradation; however, the rate of growth of biomass is slightly over predicted at lower substrate levels. A lag of biomass growth behind the disappearance of the parent substrates has previously been noted by Chang, et al. [13] and can be attributed to the possibility of intermediate formation during the degradation of the original substrates being the rate limiting step

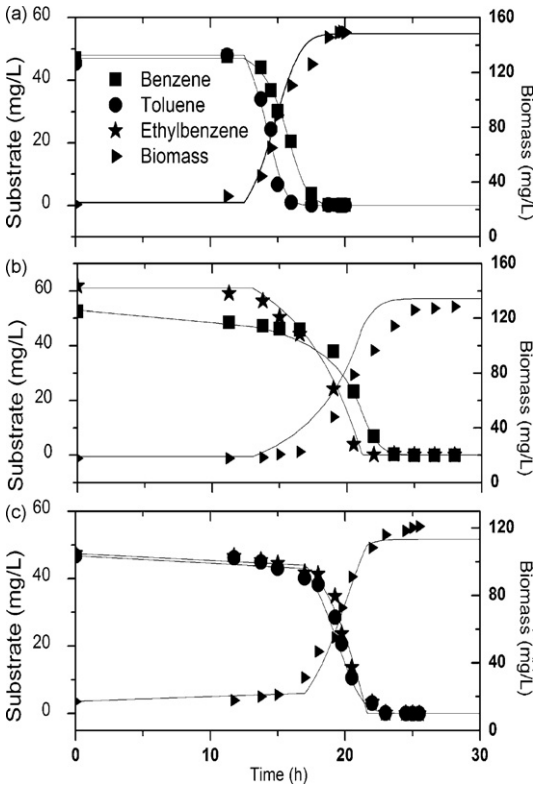

Fig. 2. (a) Dual degradation of benzene and toluene in aqueous phase experimental data (shapes) and SKIP model (lines). (b) Dual degradation of benzene and ethylbenzene in aqueous phase experimental data (shapes) and SKIP model (lines). (c) Dual degradation of toluene and ethylbenzene in aqueous phase experimental data (shapes) and SKIP model (lines).

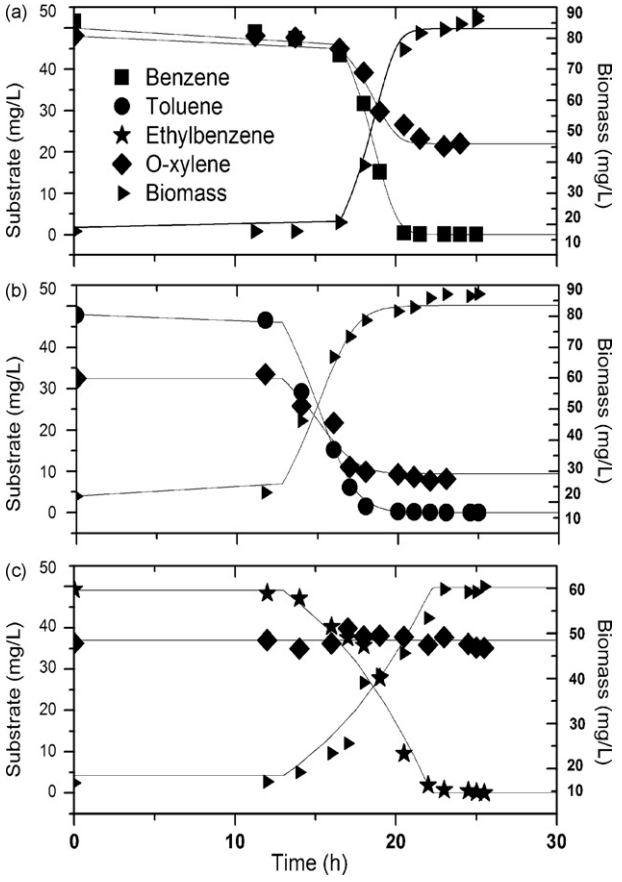

Fig. 3. (a) Dual degradation of benzene and *o*-xylene in aqueous phase experimental data (shapes) and SKIP/cometabolism model (lines). (b) Dual degradation of toluene and *o*-xylene in aqueous phase experimental data (shapes) and SKIP/cometabolism model (lines) (c) Dual degradation of ethylbenzene and *o*-xylene in aqueous phase experimental data (shapes) and Monod model (lines).

in biomass formation, as disappearance of the parent compound is not directly proportional to biomass growth.

The dual substrate degradation of benzene and ethylbenzene, shown in Fig. 2b, revealed that, relative to single substrate experiments (Fig. 1a and c), the presence of ethylbenzene had an inhibitory effect on benzene degradation as an interaction parameter, $I_{E,B}$, of 4 provided the most accurate fit to experimental data and benzene had no effect on ethylbenzene degradation as an interaction parameter, $I_{B,E}$, of 0 provided the most accurate fit. The SKIP model is plotted in Fig. 2b along with experimental data. It should be noted that, because there was no effect of benzene on ethylbenzene degradation, the interaction parameter for ethylbenzene set to zero reduces to a simple no-interaction Monod model. Fig. 2b also revealed that biomass growth continues after the disappearance of the parent substrate compounds. Again, this can be possibly attributed to the build up of intermediates that result in delayed biomass growth. The model predicts a slightly higher yield than the experimental data.

The dual degradation of toluene and ethylbenzene, shown in Fig. 2c, revealed that the degradation data for both compounds were similar to those from single degradation experiments (Fig. 1b and c) as interaction parameters, $I_{E,T}$ and $I_{T,E}$, of 0 provided the most accurate fit to experimental data for both substrates. This is equivalent to a no-interaction Monod model, which is plotted along with experimental data in Fig. 2c. The biomass prediction, while showing a correct growth rate, slightly under predicts the overall biomass yield.

Dual substrate experiments containing *o*-xylene, which cannot be used as a sole carbon source, can be seen in Fig. 3a, b and c for *o*-xylene/benzene, *o*-xylene/toluene and *o*-xylene/ethylbenzene, respectively. These results show that *o*-xylene was cometabolized in the presence of both benzene and toluene. Therefore, Eq. (11) was used to describe the disappearance of *o*-xylene in both of these cases. In addition, relative to single substrate experiments (Fig. 1a and d), the degradation rate of benzene was enhanced in the presence of *o*-xylene as an interaction parameter, $I_{X,B}$, of −0.7 provided the most accurate fit to experimental data, whereas the presence of *o*-xylene had no effect on toluene degradation as an interaction parameter, $I_{X,T}$, of 0 provided the most accurate fit to experimental data. The SKIP model for the degradation of benzene in the presence of *o*-xylene and toluene in the presence of *o*-xylene can be seen plotted with experimental data in Fig. 3a and b, respectively. Again, it should be noted that the interaction parameter for toluene in the presence of *o*-xylene was set to zero, which reduces to a no-interaction Monod model. The cometabolism and SKIP models fit experimental substrate degradation data accurately; however, the biomass growth model slightly under predicts the overall biomass yield.

The degradation of ethylbenzene with *o*-xylene revealed that *o*-xylene was not cometabolized in the presence of ethylbenzene as a growth substrate. As expected, a simple, no-interaction Monod model described ethylbenzene degradation in the presence of *o*-xylene as an interaction parameter, $I_{X,E}$, of 0 provided the most accurate fit to experimental data, and results are shown in Fig. 3c.

It was found that the SKIP model proved to be the most accurate model type in order to describe interactions found in dual substrate experimental data and Eq. (11) was successful at describing cometabolism of *o*-xylene in the presence of both benzene and toluene. The estimated interaction parameters for each substrate combination are summarized in Table 2.

4.4. Quaternary substrate experiments

The determined kinetic parameters and substrate interactions from single and dual substrate experiments were validated by combining them in order to model the degradation of all four BTEX

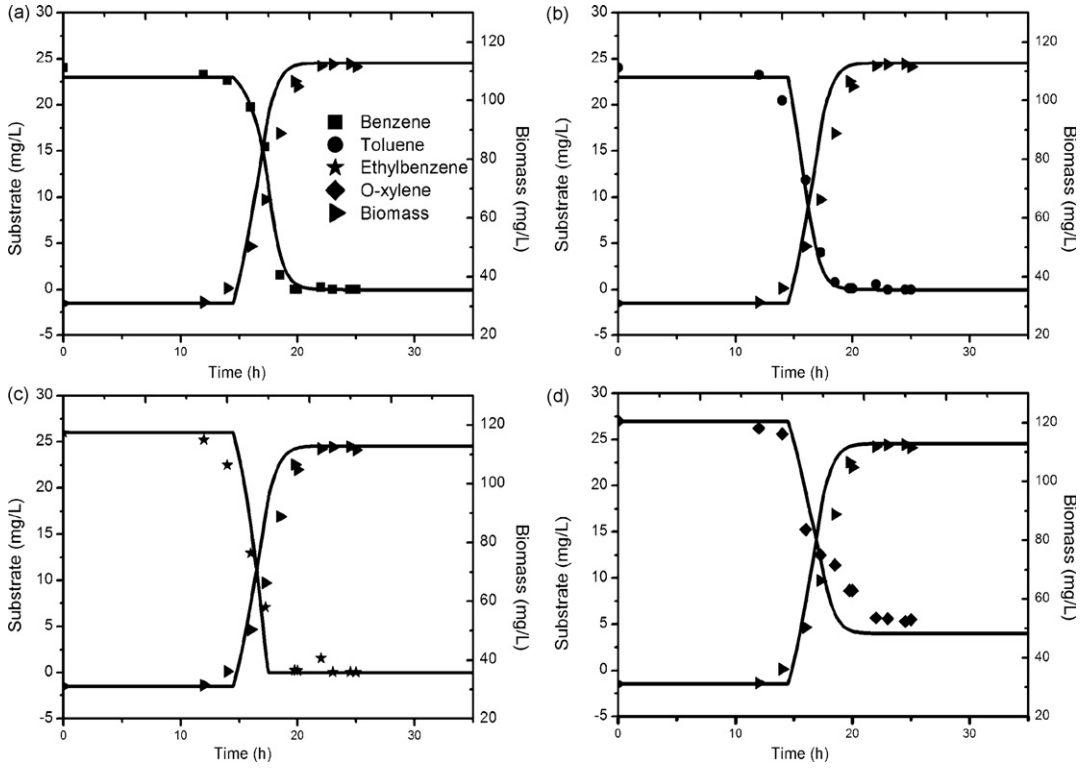

Fig. 4. (a) Benzene and biomass concentrations in the aqueous phase in the presence of TEX compounds (shapes) and model (lines). (b) Toluene and biomass concentrations in the aqueous phase in the presence of BEX compounds (shapes) and model (lines). (c) Ethylbenzene and biomass concentrations in the aqueous phase in the presence of BTX compounds (shapes) and model (lines). (d) *o*-Xylene and biomass concentrations in the aqueous phase in the presence of BTE compounds (shapes) and model (lines).

Table 2
Parameter estimates for dual substrate experiments

Substrates	Model type	Interaction/inhibition parameters
Benzene/toluene	SKIP	$I_{T,B} = 2$, $I_{B,T} = -0.4$
Benzene/ethylbenzene	SKIP	$I_{E,B} = 4$
Benzene/xylene	Cometabolism, SKIP	$I_{X,B} = -0.7$ $T_g^c = 0.5$
Toluene/ethylbenzene	Monod	–
Toluene/xylene	Cometabolism, Monod	$T_g^c = 0.5$
Ethylbenzene/xylene	Monod	–

components simultaneously. Accounting for each interaction in a substrate mixture has been completed for purely competitive kinetics [11,21], as well as for SKIP kinetics [14]. However, the range of interactions observed in the present study has not previously been modeled for BTEX compounds. As stated previously, the specific growth rate during the degradation of mixed components can be described using Equation 10 for SKIP kinetics, which was the model found to best describe substrate interactions between BTEX components during dual substrate experiments. For those substrates that were found not to have an impact on the degradation of a second substrate the interaction term for the later substrate is simply set to zero, which is the equivalent of using a no-interaction Monod model. For *o*-xylene, which was shown to be cometabolized by both benzene and toluene, degradation due to the presence of both growth substrates was summed.

Both the experimental data and corresponding SKIP/cometabolism models are shown for the simultaneous degradation of all four BTEX components in Fig. 4a , b, c and d for benzene, toluene, ethylbenzene and *o*-xylene, respectively. It can be seen that the resulting kinetics parameters, substrate interactions and interaction parameters determined from single and dual substrate experiments combine to provide an adequate prediction of the experimental data for the degradation of all four BTEX components, however, the total amount of *o*-xylene degraded is slightly over predicted. The control experiment confirmed that BTEX losses from the experimental runs were negligible as substrate concentrations did not decrease over the period of time during which degradation tests were performed (data not shown).

As mentioned previously, several studies have investigated the degradation of combinations of BTEX components. A summary of a number of these findings, along with kinetic parameter estimates and inhibition constants or interaction parameters, can be seen in Table 3. Comparing the kinetic parameters found in this study to

Table 3
Kinetic parameters obtained from degradation of BTEX components

Compounds	Model type	Parameters	Microorganism	Author
Benzene	Monod	$K_s = 0.12 \pm 0.02$ mg/L	*P. Putida* F1	Reardon, et al. [14]
	–	$\mu_{max} = 0.73 \pm 0.03$ 1/h	–	–
	–	$Y_{x/s} = 1.20 \pm 0.05$ g/g	–	–
Toluene	Monod	$K_s = 13.8 \pm 0.9$ mg/L	*P. Putida* F1	Reardon, et al. [14]
	–	$\mu_{max} = 0.86 \pm 0.01$ 1/h	–	–
	–	$Y_{x/s} = 1.28 \pm 0.01$ g/g	–	–
Toluene	Monod	$K_s = 12.22$ mg/L	Consortium	Oh, et al. [10]
	–	$\mu_{max} = 0.68$ 1/h	–	–
	–	$Y_{x/s} = 0.71$ g/g	–	–
BT	SKIP	$I_{T,B} = 5 \pm 0.3$	*P. Putida* F1	Reardon, et al. [14]
	–	$I_{B,T} = 0.01 \pm 0.003$	–	–
BTEX	Competitive inhibition	$K_{s,B} = 0.08 \pm 0.003$ mg/L	Consortium	Bielefeldt and Stensel [11]
	–	$K_{s,T} = 0.20 \pm 0.14$ mg/L	–	–
	–	$K_{s,E} = 0.21 \pm 0.13$ mg/L	–	–
	–	$K_{s,X} = 0.18 \pm 0.18$ mg/L	–	–
BT	Competitive inhibition	$\mu_{max,B} = 0.34 \pm 0.0004$ 1/h	*Pseudomonas fragi*	Chang, et al. [13]
	–	$K_{s,B} = 3.17 \pm 0.82$ mg/L	–	–
	–	$Y_{x/s,B} = 1.04 \pm 0.09$ g/g	–	–
	–	$\mu_{max,T} = 0.54 \pm 0.0004$ 1/h	–	–
	–	$K_{s,T} = 1.96 \pm 0.91$ mg/L	–	–
	–	$Y_{x/s,T} = 1.22 \pm 0.1$ g/g	–	–
	–	$K_{I,B} = 3.10 \pm 0.12$ mg/L	–	–
	–	$K_{I,T} = 1.71$ mg/L	–	–
Tp-X	Cometabolism of *p*-xylene	$T_g^c - 0.45$ mg/mg	*Pseudomonas fragi*	Chang, et al. [13]
BTEo-X	SKIP, cometabolism	$\mu_{max,B} = 0.44$ 1/h	Consortium	Current study
	–	$K_{s,B} = 27.57$ mg/L	–	–
	–	$Y_{x/s,B} = 1.35$ g/g	–	–
	–	$\mu_{max,T} = 0.60$ 1/h	–	–
	–	$K_{s,T} = 34.12$ mg/L	–	–
	–	$Y_{x/s,T} = 1.25$ g/g	–	–
	–	$\mu_{max,E} = 0.13$ 1/h	–	–
	–	$K_{s,E} = 0.36$ mg/L	–	–
	–	$Y_{x/s,E} = 0.85$ g/g	–	–
	–	$I_{T,B} = 2$	–	–
	–	$I_{B,T} = -0.4$	–	–
	–	$I_{E,B} = 4$	–	–
	–	$I_{X,B} = -0.7$	–	–
	–	$T_g^c - 0.5$	–	–
	–	$T_g^c - 0.5$	–	–

the parameters found by other authors, it can be seen that yield coefficients and specific growth rates are of similar magnitude. The half saturation constants found in this study are much larger than those found in other studies, although a large range of values have been reported in the literature [14]. For dual substrate experiments, interaction parameters for the SKIP model are similar in this study to other reports, yet the amount of substrate cometabolized relative to growth substrate degraded was slightly higher in the current study. This can be attributed to the difference in diversity and type of bacterial populations between studies.

With the exception of cometabolism, competitive inhibition interactions between BTEX components are common and are inclusive of those that have been quantified by other authors [10,11,13]. The enhancement interactions observed during the present study have not been quantified previously by other authors, although, enhancement interactions occurring among BTEX components have been qualitatively observed, including the enhancement of benzene degradation in the presence of *o*-xylene and toluene [8] and the presence of toluene enhancing benzene and *p*-xylene degradation [12]. The mechanism of the presence of a substrate enhancing the degradation of a second substrate could possibly be due to the induction of required catabolic enzymes [12].

An inherent limitation of this overall cometabolism/SKIP model and estimated kinetic parameters lies in the fact that it is consortium specific. If the concentration of BTEX in the aqueous phase fluctuates, which is a possibility in many industrial [26] and remediation [27] scenarios that involve the biodegradation of BTEX components, the consortium may change composition over time. However, due to the convergence of typical metabolic pathways of BTEX components it is possible that the current model may be applicable to a change within the bacterial population. The applicability of the current model to changes in the enrichment process is a topic needing further investigation. In addition, the model provides more accurate predictions of substrate degradation than it does biomass growth. However, prediction of biomass growth is, overall, quite accurate in comparison to attempts by other authors to model mixed populations using SKIP models [16] and does not require quantification of proportions of pure species that comprise the consortium. Overall, the current study provides a method of obtaining an empirical equation to estimate biomass growth of a bacterial consortium. This method has not been demonstrated to date by other authors using an unquantified mixed population of bacteria.

5. Conclusions

This study found that during the degradation of individual BTEX components by a bacterial consortium, benzene, toluene and ethylbenzene could be used as a sole carbon source, whereas *o*-xylene could not. When combinations of BTEX components were present, relative to single substrate degradation, several interactions were identified including enhancement, inhibition and cometabolism. This array of interactions has not been quantified for BTEX components prior to the current study. It was found that the model that provided the most accurate description of the interactions was sum kinetics with interaction parameters (SKIP) model. In addition, the cometabolization of *o*-xylene was modeled mathematically. Finally, the SKIP and cometabolism models were validated by successfully modeling growth of a bacterial consortium and substrate degradation when all four BTEX components were present simultaneously. A method for obtaining an empirical equation to predict biomass growth of an unquantified bacterial consortium has also been provided.

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Appendix A. Nomenclature

$I_{2,1}$	interaction parameter for effect of substrate 2 on substrate *i*
K_S	half saturation constant (mg/L)
K_I	inhibition constant (mg/L)
S	substrate concentration (mg/L)
T_g^c	growth substrate transformation capacity (mg_N/mg_G)
μ	specific growth rate of biomass (h^{-1})
μ_{max}	maximum specific degradation rate (h^{-1})
X	biomass concentration (dry weight) (mg/L)
$Y_{X/S}$	biomass yield (mg/mg)

Subscripts

1	first species present in mixed substrate experiments
2	second species present in mixed substrate experiments
3	third species present in mixed substrate experiments
4	fourth species present in mixed substrate experiments
B	Benzene
E	Ethylbenzene
G	growth substrate
i	species *i*, one of B, T, E or o-X components
I	interacting species for mixed substrate experiments
n	number of substrates in an experimental run
N	non-growth substrate
T	Toluene
X	*o*-Xylene

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