Toluene Degradation Kinetics for Planktonic and Biofilm-Grown Cells of *Pseudomonas putida* 54G

Raj Mirpuri,* Warren Jones, James D. Bryers

Center for Biofilm Engineering, Montana State University, Bozeman, Montana 59717-3980; Telephone: 406-994-6129; fax: 406-994-6098; e-mail: warren_j@erc.montana.edu

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Abstract: Toluene degradation kinetics by biofilm and planktonic cells of *Pseudomonas putida* 54G were compared in this study. Batch degradation of ^{14}C toluene was used to evaluate kinetic parameters for planktonic cells. The kinetic parameters determined for toluene degradation were: specific growth rate, μ_{max} = 10.08 ± 1.2/day; half-saturation constant, K_S = 3.98 ± 1.28 mg/L; substrate inhibition constant, K_I = 42.78 ± 3.87 mg/L. Biofilm cells, grown on ceramic rings in vapor phase bioreactors, were removed and suspended in batch cultures to calculate ^{14}C toluene degradation rates. Specific activities measured for planktonic and biofilm cells were similar based on toluene degrading cells and total biomass. Long-term toluene exposure reduced specific activities that were based on total biomass for both biofilm and planktonic cells. These results suggest that long-term toluene exposure caused a large portion of the biomass to become inactive, even though the biofilm was not substrate limited. Conversely, specific activities based on numbers of toluene-culturable cells were comparable for both biofilm and planktonically grown cultures. Planktonic cell kinetics are often used in bioreactor models to model substrate degradation and growth of bacteria in biofilms, a procedure we found to be appropriate for this organism. For superior bioreactor design, however, changes in cellular activity that occur during biofilm development should be investigated under conditions relevant to reactor operation before predictive models for bioreactor systems are developed. *Biotechnol Bioeng* **53:** 535–546, 1997.

Keywords: toluene; biofilm kinetics; planktonic cell kinetics; specific activity; injury; *Pseudomonas putida* 54G; vapor phase bioreactor

INTRODUCTION

Air emissions of volatile organic compounds (VOCs) have been the subject of stringent environmental regulations during the last decade. Applications of vapor phase bioreactors (VPBRs) to treat contaminated vapor streams have increased considerably because they offer a low-cost and low-maintenance alternative to other air pollution control technologies. VPBRs use biofilms (attached bacteria) to degrade volatile contaminants, and performance of these reactor systems is the ultimate result of a complex interaction between transport of rate-limiting nutrients to bacteria within the biofilm and reactions within the biofilm. In contrast to planktonic (free) cells, biofilms attach to surfaces, producing extracellular polymers and metabolizing nutrients that are transported to the biofilm. Results from laboratory-scale VPBRs have been published for the removal of VOCs such as methanol (Shareefdeen et al., 1993), phenol (Zilli et al., 1993), benzene (Baltzis and Shareefdeen, 1993), toluene (Baltzis and Shareefdeen, 1993; Kirchner et al., 1989) dichloromethane (Diks and Ottengraf, 1991a,b) propionaldehyde (Kirchner et al., 1989), and trimethylamine (Partidário and Carrondo, 1993). Scale-up of VPBRs can best be achieved by use of predictive models that depend on accurate estimates of kinetic and stoichiometric coefficients. The majority of VPBR models reported in the literature use planktonic cell kinetics to predict biofilm growth rates under the assumption that kinetic values are valid for biofilm cells (Baltzis and Shareefdeen, 1993; Diks and Ottengraf, 1991a,b; Partidário and Carrondo, 1993; Shareefdeen et al., 1993). Effects of surface interaction and extracellular polymer substance (EPS) production might lead to differences in free and attached cell kinetics.

Hamilton and Characklis (1989) reported that activities of cells in biofilms were likely to differ qualitatively from those of planktonic bacteria and, in assessing relative activities, suggested that it is necessary to identify and evaluate important cellular processes involved in bacterial growth. For an accurate understanding of molecular mechanisms associated with the analysis of kinetic and stoichiometric coefficients, they suggested that the following should be considered: the role of EPS associated physicochemical and biological variables within the biofilm and in situ measurements of fundamental cellular processes supported by assays of relevant metabolic activities.

Differences in the activity of biofilm and planktonic cells are probably system dependent. This topic was considered in some detail by Fletcher and Marshall (1983) who concluded that increases and decreases in activity were observed for surface-attached cells relative to planktonic cells. Karel et al. (1985) reported that under certain conditions metabolic activity of adhered cells was different from

* *Present address:* Basys Technologies, 1345 Northland Dr., Mendota Heights, MN 55120-1141.

Correspondence to: W. Jones

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planktonic cells but there was no consistent method of predicting how the behavior would be different. Because in many cases no difference in activity was observed, they suggested that activity data obtained for planktonic bacteria could be used to model the behavior of attached cells.

Hamilton (1987) reported that attached cells were generally assumed to be at an advantage, but experimental evidence for this advantage or a related increase in metabolic activity was inconclusive. In an exhaustive review on the effect of interfaces on microbial activity, van Loosdrecht et al. (1990) similarly concluded that although a qualitative consensus existed to support the theory that attachment to surfaces influenced bacterial metabolism, experimental observations had been inconsistent. They observed that attached bacterial growth and substrate metabolism depended on the nature of both the organism and the substratum and on the concentration of the substrate. Any resulting differences between adhered and free cells could be attributed to a modification of the surroundings of the cells due to the presence of surfaces.

Bakke et al. (1984, 1989) studied the activity of biofilm cells of a monoculture of *Pseudomonas aeruginosa* and compared kinetic parameters with those obtained by Robinson et al. (1984) for planktonic cells of the same bacterium. They concluded that kinetics derived for planktonic cells closely represented biofilm cells. Keen and Prosser (1987) reported that attachment of *Nitrobacter* cells to surfaces increased their growth rates by 25%. McFeters et al. (1990) concluded that sand-associated biofilm bacteria adapted more quickly and had a greater degradative activity for nitrilotriacetate than planktonic cells. Jefferey and Paul (1986) reported that specific growth rates decreased when bacteria attached onto surfaces because of loss of cell surface available for substrate uptake. Harms and Zehnder (1994) concluded that free and attached cells of *Sphingomonas* sp. strain HH19k exhibited different activities during dibenzofuran uptake. Deretic et al. (1990, 1994) and Martin et al. (1994) recently hypothesized that planktonic-biofilm transformation is controlled by a σ factor, akin to control of sporulation in Gram-positive bacteria, that might lead to a phenotypic change in a large cassette of genes for biofilm bacteria, resulting in a phenotypically distinct expression of the genome.

Based on these studies, differences in substrate utilization kinetics of free and attached bacteria seem to be system and organism dependent. Although mathematical models to evaluate steady-state biofilm kinetics have been documented (Harremoës, 1978; LaMotta, 1976; Rittmann and McCarty, 1980; Skowlund, 1990; Skowlund and Kirmse, 1989), very few studies on biofilm-mediated degradation kinetics of VOCs exist. Arcangeli and Arvin (1992) studied the biodegradation of toluene (sole carbon and energy source) by biofilm cells. Cells were grown in annular reactors that provided ideal conditions for biofilm growth. They determined that maximal toluene removal in biofilms occurred within 20–30 h of attachment to the substratum. Concomitant with a reduction in toluene removal with time, they estimated that the active biomass was only about 5% of the biofilm biomass. Similar results were obtained by Diks and Ottengraf (1991b) for the degradation of dichloromethane in a VPBR. In both studies, mass transfer limitation was not a factor.

With such conflicting information available, we need to understand the relation between VOC degradation by free and attached cells. Clearly, cellular processes involved during VOC removal by free and attached bacteria could be different from the degradation of benign substrates such as glucose or acetate. Bacterial injury, defined as a physiological or genetic response to a sublethal environmental effect (McFeters, 1989) due to toluene degradation, was observed by H. F. Ridgway (Personal Communication) in cultures of *P. putida* 54G using methods as described here. Leddy et al. (1995) reported the formation of *tol*-mutants of *P. putida* 54G that lose their toluene degradation capability on exposure to toluene.

Before kinetics of toluene degradation by free and attached cells are compared, a practical basis for this comparison should be chosen. Specific activity, defined as the rate of substrate removal per unit biomass or cells, provides an absolute scale for evaluation of substrate uptake rates of free and attached cells. Also, the sue of a pure culture biofilm provides the opportunity to elucidate the fundamental processes leading to accumulation and growth without the confounding factors of population dynamics and species interaction.

We hypothesize that suspended and biofilm-grown cells of *P. putida* 54G exhibit similar toluene degradative capabilities when kinetic parameters are expressed on the appropriate cellular basis, and that the specific activity for biofilm and planktonic cells decreases during long-term toluene exposure. The increased exposure to VOCs injuries *P. putida* 54G, which in turn increases the inactive biomass fraction. In the present investigation, kinetics of toluene degradation by planktonic and biofilm-grown cells of *P. putida* 54G grown on toluene as a sole source of carbon and energy are compared.

MATERIAL AND METHODS

Bacterial Strain

P. putida 54G, a toluene-degrading bacterium, was isolated from a gasoline contaminated aquifer at Seal Beach, CA. The isolate was capable of growth on HCMM2 mineral salts media (containing only inorganic compounds) in the presence of vapor phase toluene and on complex carbon sources such as R2A medium (Reasoner and Geldreich, 1985). HCMM2 mineral salts medium was used as the liquid inorganic nutrient medium as described elsewhere (Ridgway et al., 1990).

Enumeration of Viable Cells

Growth media employed in this investigation were R2A plates and toluene vapors supplied continuously to cells on

HCMM2 plates in sealed containers incubated at room temperatures, designated as HT plates. *P. putida* 54G cells grown on toluene as the sole source of carbon and energy were enumerated for viable cells on R2A and HT plates, with results reported as colony forming units per milliliter (cfu/mL). The difference between R2A (nonselective) and HT (selective) plate counts was used to determine injured cells according to (McFeters, 1989)

$$\text{injury \%} = \left(\frac{\text{nonselective counts} - \text{selective counts}}{\text{nonselective counts}}\right) \times 100. \quad (1)$$

It should be noted that this technique does not differentiate between phenotypic and genotypic loss of culturability on a selective medium.

Protein Assay

Levels of suspended cell biomass were quantified using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). Bovine serum albumin served as a standard.

Toluene Sampling

Gas samples (250 μL headspace) were analyzed for toluene using an HP 5890 Series II gas chromatograph (GC) equipped with a flame ionization detector and an Alltech 0.1% A1-1000 Graphpac 80/100, 1.8 m × 3.2 mm o.d. × 2.2 mm i.d. stainless steel column. The column was operated at a constant temperature of 140°C. Toluene concentration in the liquid was determined by combining 1 mL of the aqueous samples with 0.5 mL hexane and injecting 2 μL of the hexane phase into a HP 5890 Series II GC equipped with a photoionization detector and a DB-624 J & W Scientific megabore capillary column, 30 m × 0.53 mm i.d., with temperature ramped from 140 to 200°C, Helium was used as the carrier gas in both columns at a flow rate of 30 mL/min.

Ion Chromatography

Nitrate, phosphate, and sulfate ion concentrations in HCMM2 medium were determined using an ion chromatograph. Aqueous samples were filtered and diluted in autoclaved water (double distilled). Aliquots (15 μL) of the diluted sample were injected into a Dionex ion chromatograph (model AI-450, Danes Co., San Francisco, CA) equipped with a pulse electrochemical detector (model DX-300) and a Dionex Ionpac AS4A-SC column (2 mm, column temperature = ambient).

Biofilm Thickness Measurement

Biofilm thickness was measured using a Bio-Rad MRC600 confocal laser (Kr/Ar) system with an Olympus BH2 microscope and an MS plan 10× objective. Ceramic rings were stained with 10 mg/L propidium iodide and mounted on a sample holder filled with liquid HCMM2 medium.

Kinetic Measurements

Three different studies were conducted. The relevant information including physiological state of the bacteria, the parameters analyzed, and the duration of study are summarized in Table I.

Planktonic Cell Kinetics

In the planktonic cell study, liquid HCMM2 medium was combined in 26-mL Supelco glass vials with ^{14}C toluene (56.2 mCi/mmol, Sigma Chemical Company, St. Louis, MO) and *P. putida* 54G cells that were harvested in the early stationary phase after planktonic growth on toluene vapors. Vials were sealed using Teflon-lined rubber septa and an aluminum cap. The total liquid volume in each vial was 14 mL, which allowed sufficient headspace such that oxygen was not stoichiometrically limiting. Labeled toluene was dissolved in autoclaved water (double distilled) and

Table 1. List of parameters analyzed, duration of study, and physiological state of bacteria during studies.

Experiment	Maximum duration of toluene exposure	Physiological state	Analyzed for
Biofilm cell kinetics	22–25 days	Biofilm cells	^{14}C toluene degradation Cell nos. on R2A and HT plates Biomass = protein analysis × 2 Biofilm thickness by SCLM
Suspended cell kinetics	1 day	Suspended cells	^{14}C toluene degradation Cell nos. on R2A and HT plates Biomass = ^{14}C biomass produced
Long-term toluene exposure	29 days	Suspended cells	^{14}C toluene degradation Cell nos. on R2A and HT plates Biomass = protein analysis × 2

SCLM, scanning confocal laser microscopy.

combined with HPLC-grade toluene (Fisher Scientific Company, Fair Lawn, NJ) in the glass vials to final concentrations ranging from 1 to 50 mg/L. Vials were incubated in a water bath at 24°C with stirring. Controls were prepared without bacteria to determine abiotic losses and were used to determine actual toluene removal. Toluene partitioning to headspace and total abiotic losses were consistently less than 10%.

At timed intervals three biotic samples and one abiotic control were removed from the water bath and transferred to a chemical hood to determine rate of ^{14}C toluene degradation and rates of ^{14}C CO_2 and ^{14}C biomass production. For each biotic sample, three scintillation vials were labeled A, B, and C. One milliliter of NaOH (16*M*) was introduced into vials A and B and 1 mL of HCl (6*N*) acid was introduced into vial C. An aliquot of 0.5 mL of aqueous sample was withdrawn from a biotic vial, introduced into scintillation vial A, and combined with 6 mL of scintillation cocktail (Scintiverse II, Fisher Scientific Company, Fair Lawn, NJ). A 0.5-mL aliquot of the same sample was placed in vial B and air stripped for 5 min. The resulting solution was combined with 6 mL of scintillation cocktail. Similarly, 0.5 mL of the same sample was placed in vial C, air stripped for 5 min, and combined with scintillation cocktail. This procedure was repeated for the other two biotic samples, but only vials A and B were used for the abiotic controls. Radioactivity (^{14}C) in the resulting 11 (nine biotic and two abiotic) vials for each sample time were analyzed using a 2200CA Tri-Carb liquid scintillation analyzer (Packard Instrument Company, Laguna Hills, CA).

Quenched standards were run weekly to produce a standard curve used to correct for quenching. HCMM2 minimal medium in scintillation cocktail served as a blank to determine any background activity that was subtracted from the results of all other measurements. Radioactivity (^{14}C) obtained as disintegrations per minute (dpm, measured by the scintillation counter) was converted to milligrams per liter of carbon by determining the dpm for a known concentration of labeled toluene. The mass balance on ^{14}C activity was: total ^{14}C toluene introduced = ^{14}C toluene unreacted + ^{14}C CO_2 produced + ^{14}C biomass produced. The results obtained from scintillation vials were analyzed according to (all terms denote radioactivity due to ^{14}C): ^{14}C in vial A = ^{14}C-toluene + $^{14}CO_2$ + ^{14}C-biomass, ^{14}C in vial B = $^{14}CO_2$ + ^{14}C-biomass, and ^{14}C in vial C = ^{14}C-biomass.

The difference in radioactivity between vials A and B was the amount of toluene degraded, while the difference between vials B and C was the amount of CO_2 produced. Vial C was the amount of biomass produced. For abiotic controls, no CO_2 or biomass production was expected and the difference in vials A and B was calculated as the amount of unconverted toluene. The abiotic controls showed a decrease of less than 10% of the initial toluene concentration at the end of the experiment.

Biofilm Kinetics

Biofilm kinetics were investigated by supplying toluene to three packed-bed VPBRs in a countercurrent mode of operation (Fig. 1) at room temperature (18–23°C). House air was split into two streams with one stream sparged through a carboy containing water (to maintain humidity) and the other stream sparged through a liquid toluene reservoir. The two streams were mixed together forming a feed into the bottom of all three VPBRs. Liquid HCMM2 medium flowed downward through the VPBRs, providing a source of inorganic nutrients for biofilm growth. Physical parameters and packing characteristics involved in the biofilm kinetic study are shown in Table II. The influent toluene gas phase concentrations were 150 or 300 ppm. These experimental conditions are hereby designated as 150T and 300T, respectively. Exactly 250 ceramic Raschig® rings were in-

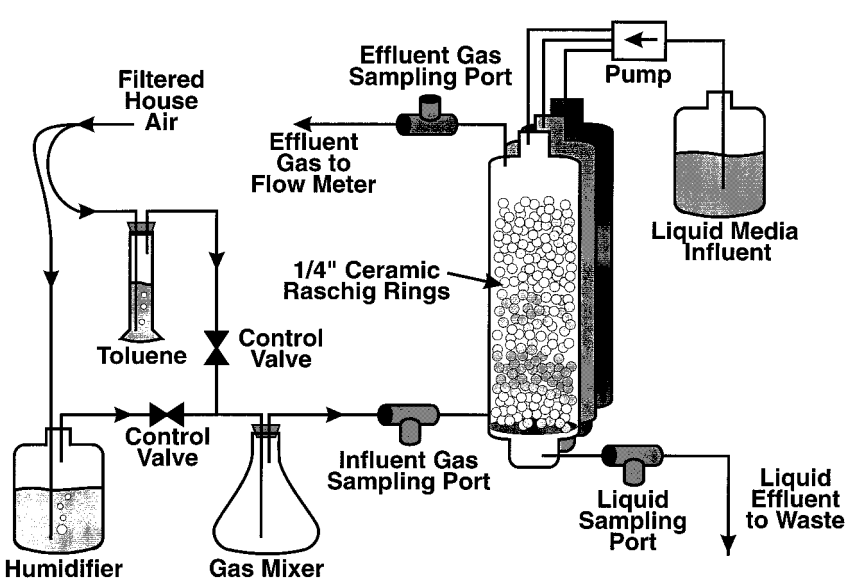

Figure 1. Schematic of reactor system used to develop biofilm for kinetic studies.

Table II. Physical parameters and packing characteristics for packed-bed VPBRs used in studying biofilm kinetics.

Physical parameters	
Vapor flow rate, 50 mL/min	
Liquid flow rate, 1 mL/min	
Toluene concentration, 150 and 300 ppm (vapor phase0	
Inoculation recycle time, 6 h	
Column description, 8 in. high, and 1 in. i.d.	
Packing characteristics	
Type, ceramic Raschig® rings	
Description, ¼ in. o.d. and ¼ in. long	

troduced into each reactor and the entire system was sterilized by autoclaving. After sterilizing, toluene vapor was fed into the column continuously until steady-state abiotic losses of approximately 10–15% were measured (approximately 2 days). A bacterial inoculum of *P. putida* 54G (5 mL) cells previously exposed to toluene vapors was introduced into the columns and the reactor liquid contents recycled for 6 h allowing the bacteria to attach to the ceramic rings. Following this, the VPBRs were switched back to a once-through mode and liquid flow was turned on. Vapor phase toluene concentration in the influent and effluent gas ports and liquid phase toluene concentration in the effluent were measured daily to determine reactor performance. Abiotic toluene losses were subtracted from observed reactor performance to obtain actual biological removal efficiencies.

After 1 week of operation, one VPBR was destructively sampled (week 1) by transferring the ceramic rings from the top, middle, and bottom of the column aseptically to three sterile petri dishes. For each column position, packing material was subjected to the following analysis: Six ceramic rings were combined with 6 mL of HCMM2 medium, then vortexed and sonicated for 3 min each, a procedure that was previously shown to yield a maximum number of viable cells. An aliquot (0.1 mL) of the resulting aqueous sample was used to enumerate cell numbers by diluting and spread plating on R2A and HT plates. Base (NaOH) was added to the remainder of the suspension containing the packing to a final concentration of 1*N* and the suspension was boiled in a 120°C incubator for 1.5 h to disrupt all the cells. The suspension was then vortexed and protein concentration of the suspension determined as discussed previously and then converted to biomass expressed as g biomass/m^2 packing.

Biofilm thickness was measured on three ceramic rings using confocal laser microscopy as described in the next section. For each ceramic ring, nine measurements of thickness were determined and averaged. Because three ceramic rings were sampled at each column position, a statistically significant value was obtained by evaluating a standard error.

Six ceramic rings were combined with liquid HCMM2 medium in 26-mL Supelco glass vials and vortexed to suspend the cells. Initial-rate ^{14}C toluene degradation studies (maximum time = 2.25 h) were conducted as discussed previously. Because the bacteria were suspended, mass transfer limitations were assumed to be negligible. Toluene concentrations used in the glass vials were similar to the concentration that the bacteria had been previously exposed to in the VPBRs. For example, 300 ppm vapor phase toluene concentration is equivalent to approximately 3 mg/L liquid phase toluene concentration. Packing material was left in the glass vials during the degradation studies. Comparable controls with the packing material removed from the vials did not show any appreciable differences in toluene degradation rates.

This procedure was repeated for another VPBR at 2 weeks from the start of the study (week 2). For the third VPBR, toluene concentrations were measured until constant removal efficiencies were obtained. This indicated that steady-state conditions, with respect to toluene degradation, prevailed in the VPBR. At this point (usually between 22 and 25 days from the start of the study) the third VPBR was destructively sampled (week 3).

Long-Term Toluene Exposure Study

This study was designed to investigate the effects of long-term toluene exposure on planktonic cells of *P. putida* 54G. Three 400-mL Erlenmeyer flasks were used in the VPBR system shown in Figure 1, replacing the packed bed columns, and no liquid flow was supplied. Toluene-contaminated air was bubbled through 250 mL of liquid HCMM2 medium in each flask at a vapor concentration of 150 ppm and a vapor flow rate of 50 mL/min. The gas flow provided sufficient mixing (visual observation), and all flasks were immersed in an insulated water bath at 24°C. At the start of the study, suspended cells of *P. putida* 54G, harvested in the early stationary phase after growth on toluene vapors, were inoculated into two flasks (biotic flasks). An abiotic control flask was operated in a similar fashion to the biotic flask except for the absence of cells. Toluene concentration was measured in the influent and effluent ports of the flasks. Liquid samples were withdrawn from the flasks daily for: cell enumeration on R2A and HT plates; measurement of nitrate, sulfate, and phosphate ion concentrations; measurement of toluene concentration; and measurement of cellular protein. Liquid and gas samples were removed at timed intervals until the end of the study (29 days). Liquid volume in the flasks was reduced by 20 mL (<10%) due to sampling at the end of this study.

Data Analysis

For planktonic cells, substrate degradation rates (dS/dt) were converted to specific growth rates (μ) using the following (Grady and Lim, 1980):

$$\mu = -\frac{Y_{x/s}}{X}\frac{dS}{dt}, \tag{2}$$

where X is biomass concentration and $Y_{x/s}$ is the yield. Spe-

cific growth rates were correlated to substrate concentration using the Andrews (1968) substrate inhibition model:

$$\mu = \frac{\mu_{max} S}{\left(K_S + S + \frac{S^2}{K_I} \right)}, \quad (3)$$

where μ_{max}(/day) is the maximum specific growth rate, K_S (mg/L) is a half-saturation constant, and K_I (mg/L) is a substrate inhibition constant. The kinetic coefficients μ_{max}, K_S, and K_I were determined by using a least-squares nonlinear regression fit (Microsoft Excel) of the Andrews inhibition model to the experimental results of μ vs. *S*.

Specific activity was used to compare the kinetics of cells with various histories and was calculated by dividing the absolute toluene degradation rate in a given assay (mg toluene/L/h) by the concentration of biomass (mg/L protein or cells as cfu/mL) present in that particular assay. Thus, specific activity was defined in two different units: milligrams of toluene degraded per milligram of protein per hour, designated as SAB (mg/mg/h) or milligrams of toluene degraded per number of cells grown on HT plates, designated as SAH (mg/cfu/h).

RESULTS AND DISCUSSION

Planktonic Cell Kinetics

Toluene degradation experiments were carried out for toluene concentrations ranging from 1 to 50 mg/L. Toluene degradation profiles are shown in Figure 2. All the profiles show a similar behavior: a lag phase, indicated by little or no change in toluene concentration, followed by a growth phase in which all the toluene is degraded. Cell numbers on R2A and HT plates were initially 5×10^4 cfu/mL and changed by a maximum of 4 orders of magnitude (data not shown) after complete mineralization of toluene. At toluene concentration of 20 mg/L and below, lag phases were shorter and suggested that higher toluene concentrations could inhibit growth of *P. putida* 54G cells. This observation is consistent with results shown in Figure 3, where growth rate decreased as toluene concentration increased above 20 mg/L. Growth rates for planktonic cells of *P. putida* 54G correlated well with the Andrews substrate inhibition model ($r^2 = 0.93$) of Equation (4). Values of yield ($Y_{x/s}$) were calculated to be 0.90 ± 0.13 mg biomass produced/mg toluene degraded for toluene concentrations ranging from 1 to 50 mg/L. Button (1985) presented kinetics of toluene degradation and induction of toluene degradation pathways in 1985. More recently Chang et al. (1993) and Oh et al. (1994) presented results on kinetic parameters estimated for aerobic growth of pseudomonads using toluene. Results of their studies in comparison to the present investigation are detailed in Table III. While Chang et al. (1993) used the Monod model to fit their data, Oh et al. (1994) determined that the Andrews inhibition model fit their data. We obtained K_S values lower than those obtained by Oh et al. (1994), while μ_{max} and K_I values were comparable. The different K_S values probably resulted from different nutritional conditions and differences in the strains of *P. putida* used. Bacterial injury levels during the planktonic cell studies, as calculated from Equation (1) were never higher than 10%.

Biofilm Kinetics

Reactors in the VPBR setup were destructively sampled every week as detailed previously. Representative data (week 2, 300T study) for attached cellular protein and biofilm thickness are shown in Figure 4. Biomass amounts and

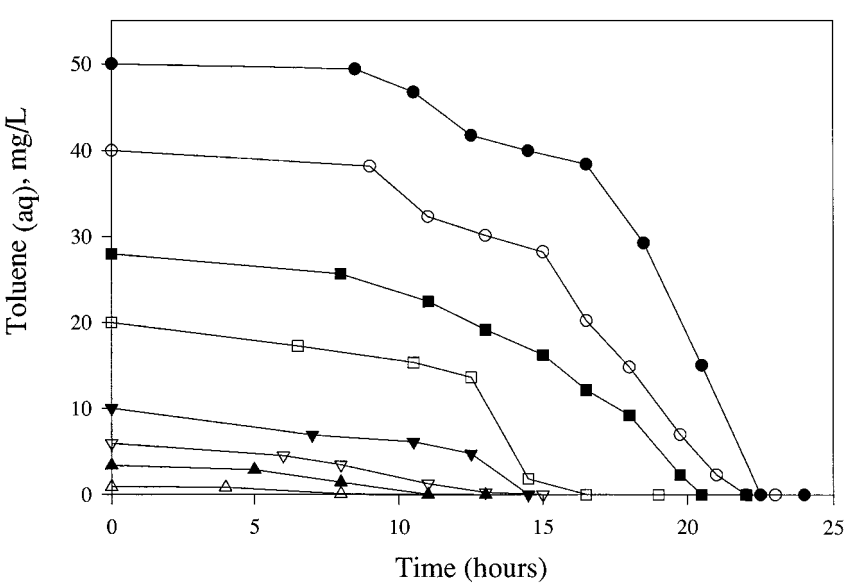

Figure 2. Degradation profiles (1–50 mg/L initial concentration) during ^{14}C toluene metabolism by planktonic cells of *P. putida* 54G in batch cultures.

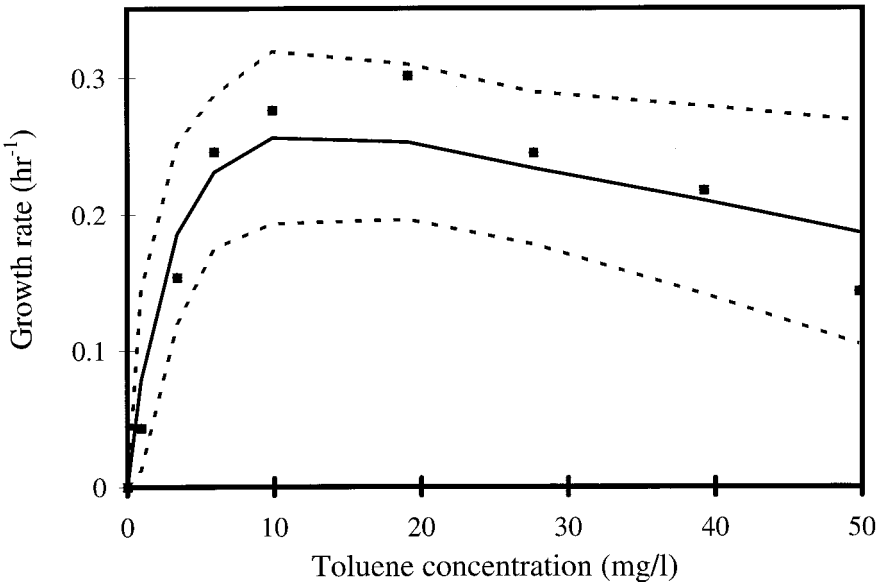

Figure 3. Specific growth rate for planktonic cells of *P. putida* 54G as a function of aqueous toluene concentration. Markers indicate experimental data, while the solid and dashed lines indicate model fit and 95% confidence intervals, respectively.

biofilm thicknesses were highest at the bottom (near the gas inlet) and decreased toward the top of the column, probably due to the gradient in toluene concentration. Similar results were obtained from each column for both experimental conditions. The difference in biofilm thicknesses between the top and bottom of the column decreased with time while toluene removal efficiency of the column increased (data not shown), which is consistent with unsteady-state operation of the VPBRs.

Toluene (^{14}C) degradation experiments were conducted on biofilm cells to evaluate specific activity. Representative results (same samples as Fig. 4) are shown in Figure 5. Consistent with the gradient in biomass and film thickness, the degradation rate in the specific activity assay was highest for the samples nearest the inlet. In comparison with the time elapsed in this assay, the doubling time for *P. putida* 54G at 3 mg/L aqueous toluene concentration was about 5.8 h, so that biomass growth in the specific activity assay could be neglected.

Similar toluene degradation experiments were conducted for all VPBRs during both the 150T and 300T studies, and toluene degradation rates were normalized to protein measurements to evaluate performance as specific activity (SAB). Figure 6 shows specific activity during both experi-

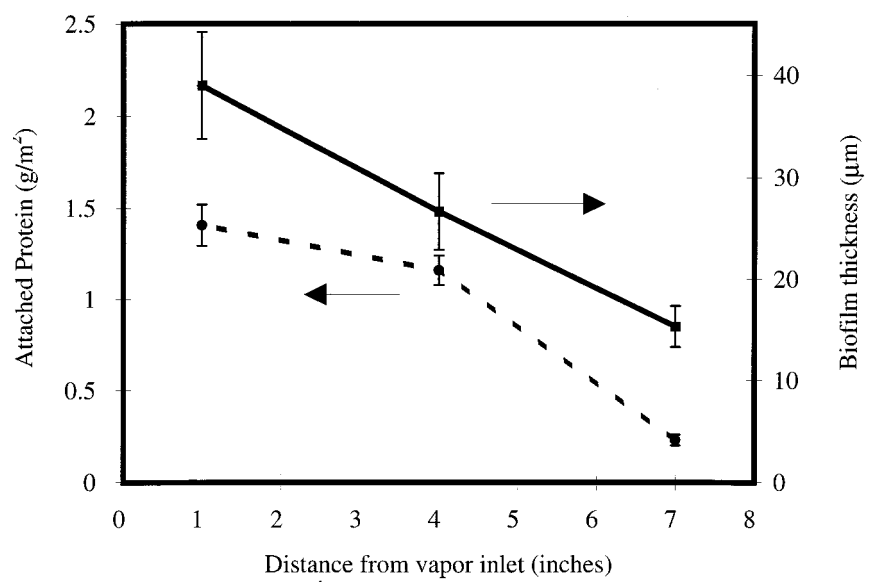

Figure 4. Protein (left ordinate) and biofilm thickness (right ordinate) for a week 2 VPBR sample during the 300T experiment at three different reactor positions.

Table III. Comparison of kinetic parameters for bacterial growth on toluene by *Pseudomonas* species.

Bacterial strain	μ_{max} (/day)	K_S (mg/L)	K_I (mg/L)	$Y_{S/s}$ (mg/mg)	Concn tested (mg/L)	References
Pseudomonas B1	13.03 ± 1.83	1.96 ± 0.91	—	1.22 ± 0.10	1–10	Chang et al. (1993)
P. X1	10.84 ± 2.77	1.88 ± 1.26	—	0.99 ± 1.26	1–10	
P. patida	17.28	15.07	44.43	0.64	5–70	Oh et al. (1994)
P. putida T2	3.1	0.43	—	0.28	0.01–4	Button (1985)
P. putida 54G	10.08 ± 1.2	3.98 ± 0.78	42.78 ± 3.87	0.90 ± 0.13	1–50	This study

mental conditions for biofilm cells at the top and bottom of the column. Also shown in Figure 6 is the specific activity for free cells (batch) evaluated as $\mu/Y_{x/S}$. The specific activity for free cells was computed at a substrate concentration of 3 mg/L, because this value was approximately equivalent to the gas phase toluene concentration to which the biofilm cells were exposed. Comparison of SABs for the VPBR experiments suggested higher activity for biofilm cells that had been exposed to the lower toluene concentration. When the toluene concentration was increased from 150 to 300 ppm, SAB decreased by a factor of 2 on the average. Biomass contents measured during the 300T study were consistently higher than those obtained during the 150T experiment (data not shown). This suggested that although an increase in toluene concentration produced an increase in biomass concentration, this increase was probably offset by a loss of biomass activity at the higher toluene concentration. Mass transport did not prevent toluene penetration throughout the film (maximum biofilm thickness = 55 μm, and Thiele modulus <3.4). Thus, we conclude that larger amounts of inactive biomass were being formed during the 300T experiment than during the 150T experiment. Specific activity (SAB) decreased from week 1 to week 3 samples for both the 150T and 300T studies, indicating that toluene degrading capability of the biofilm decreased with time.

Specific activity (SAB) for the biofilm experiments was between 3 and 5 times lower than that observed with planktonic cells. This implied that inactive biomass was formed during the 150T and 300T experiments and that the inactive biomass fraction increased with both toluene concentration and time. In the planktonic cell studies, which were only conducted for a day, insufficient time elapsed to allow accumulation of inactive cells. If cell counts on selective medium are a reasonable measure of active cells, then specific activity based on toluene degrading cells (SAH) should remain constant for both biofilm and planktonic cells, regardless of toluene concentration or time of exposure of the culture in the reactor.

Figure 7 shows that the specific activity (SAH) for biofilm and planktonic cells of *P. putida* 54G was approximately the same, 2.5×10^{-10} mg toluene degraded/cfu (HT) h. Further, this value was obtained for biofilm cells from all 3 weeks of exposure and for both gas-phase toluene concentrations tested.

Diks and Ottengraf (1991b) obtained similar results for a dichloromethane degrading biofilm in a biofilter. They observed that specific activity for a *Hyphomicrobium* spp. computed from batch dichloromethane degradation experiments was 0.64 g/g h, while that for biofilm-grown cells was only 0.08 g/g h under similar conditions, or about 12% of the batch experiment activity. In a study of *Desulfovibrio*

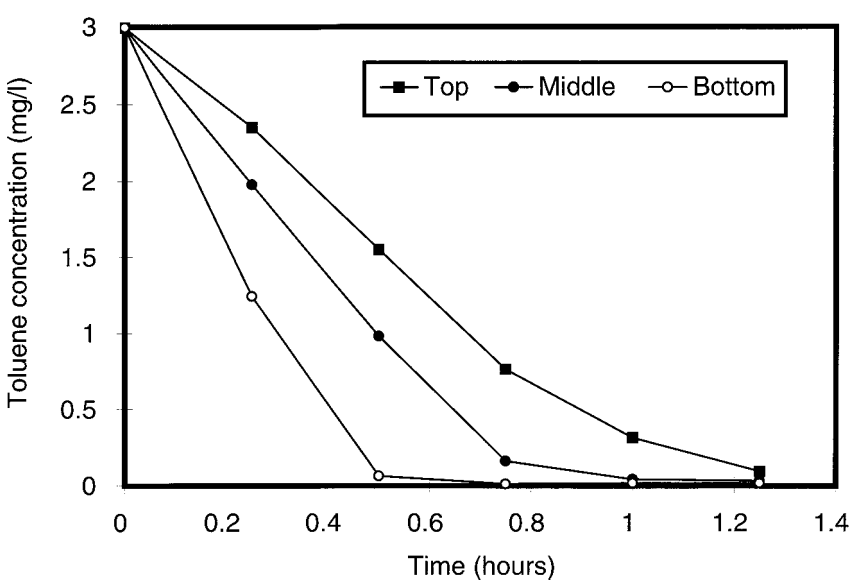

Figure 5. Aqueous toluene concentration profiles in the specific activity assay on the samples from Figure 4.

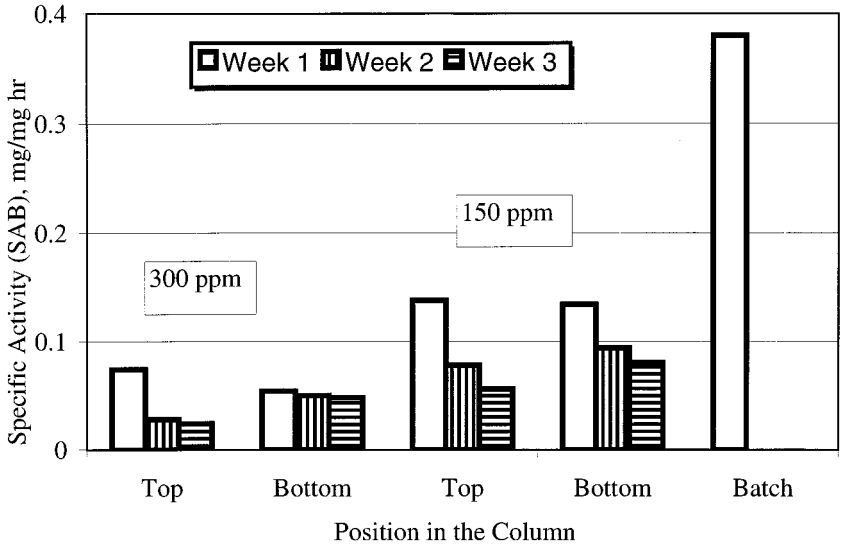

Figure 6. Specific activity (SAB) in units of mg toluene degraded/mg protein h for planktonic and biofilm cells. Planktonic cells are shown as batch while biofilm cells are shown for two different experimental conditions, influent vapor phase concentration of 150 and 300 ppm.

desulfuricans grown on lactate and sulfate, Okabe (1995) observed that maximal growth rate decreased from an initial value of 0.37 to 0.1/h at steady state within the biofilm, whereas the maximal growth rate for planktonic cells was stable at 0.37/h, all in the absence of nutrient limitation. While Okabe's study (1995) used a mass balance approach to calculate the specific growth rate in the biofilm, and Diks and Ottengraf's (1991b) used a similar approach to our study, both groups utilized total cell mass data rather than viable or active cell enumeration techniques.

Because mass transport limitations were similar (and minimal) in the specific activity assays used for both planktonic and biofilm-grown cells, other mechanisms are necessary to explain this difference. Reports of injury and/or toxicity due to toluene exposure have not been documented; to better understand these observations, we conducted long-term toluene exposure experiments. Our objective was to determine if the specific activities (SAB) for planktonic cells exhibited the same reduction as biofilm cells on exposure to toluene.

Long-Term Toluene Exposure Study

The results shown in Figure 8 are from an experiment where cells that were withdrawn from flasks after timed exposure to toluene were enumerated on R2A and HT plates. After the first day, cells that formed colonies on R2A plates remained constant at approximately 4×10^8 cfu/mL while cells on HT plates decreased continuously during the same

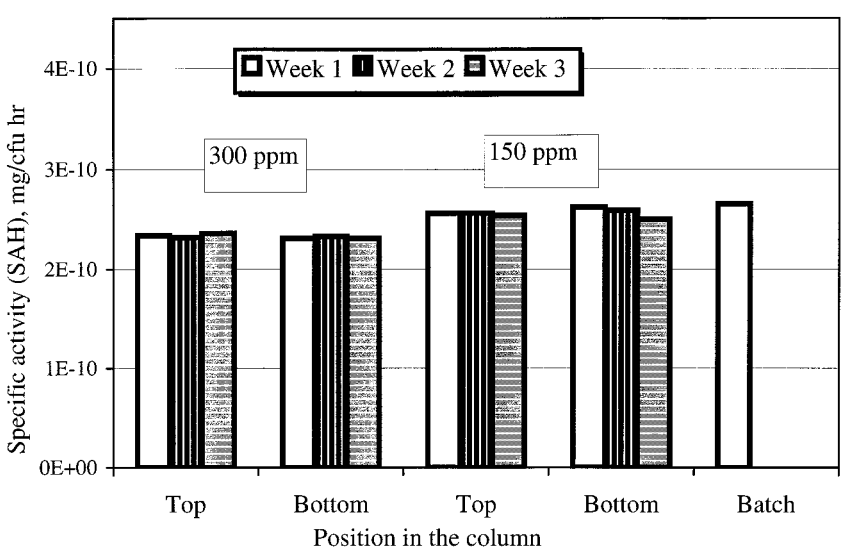

Figure 7. Specific activity (SAH) in units of mg toluene degraded/cfu on HT plates for planktonic and biofilm cells. Planktonic cells are shown as batch while biofilm cells are shown for two different experimental conditions, influent vapor phase concentration of 150 and 300 ppm.

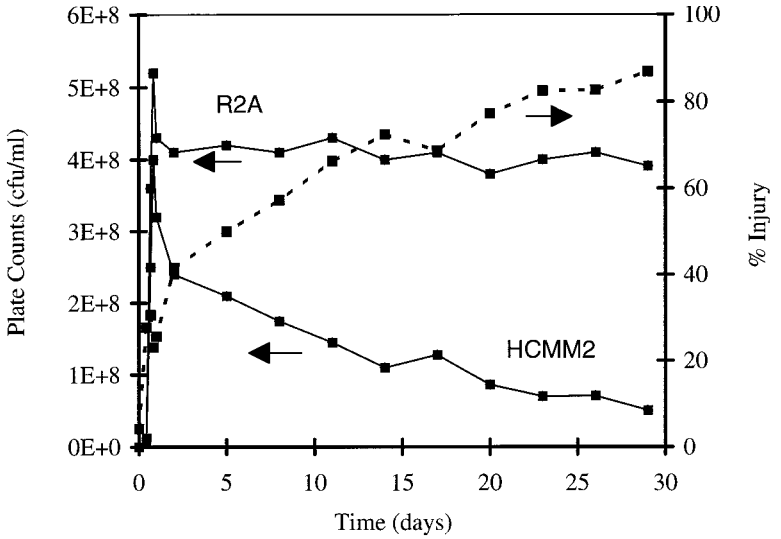

Figure 8. Cell numbers on R2A and HCMM2 + toluene plates (left ordinate) during the long-term exposure study for suspended cells. Dashed line with markers shows injury percent on the right ordinate.

time period from 3.2×10^8 to 5.1×10^7 cfu/mL. The difference in the cell numbers on R2A and HT plates is interpreted as a measure of toluene injury. Injury increased continuously for the duration of the study to 86% and suggests that long-term exposure to toluene caused significant levels of bacterial injury. Dissolved nitrate, phosphate, and sulfate ion concentrations decreased to approximately 50% of their initial concentration, indicating that these ions were not rate limiting (data not shown). Specific activity for the planktonic cells was determined by exposing the cells to a 1.5 mg/L liquid phase concentration of ^{14}C toluene, as explained previously. This procedure was repeated at timed intervals and, combined with biomass data, produced the specific activities shown in Figure 9. Values of specific activities (SAB) decreased from 0.25 to about 0.025 mg/mg h at the end of the experiment, while SAH decreased initially to 2.4×10^{-10} mg/cfu h in 1.5 days and then decreased only slightly to about 2.25×10^{-10} mg/cfu h at the end of the experiment at 26 days. The initial drop in SAH (day 1 to day 2) was characteristic of a stationary phase decline in activity (Robertson and Button, 1987; despite the continuing supply of toluene and oxygen), while cell numbers on HT plates remained nearly constant. The onset of injury in this experiment was at approximately 1 day, which correlated with transition of the cells from a log-phase culture to a stationary-phase culture. It is noteworthy that the trend and values for SAB and SAH were similar for biofilm and planktonic cultures.

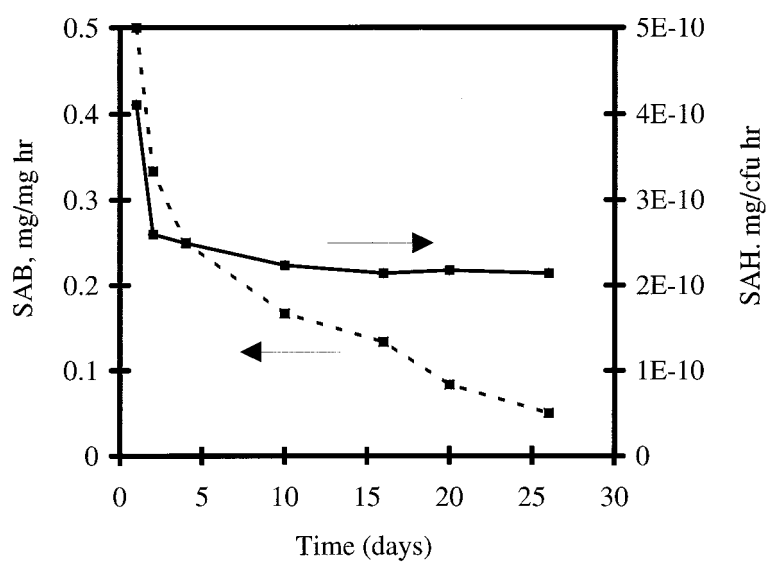

Figure 9. Specific activity in mg/mg h (SAB) and mg/cfu h (SAH) evaluated for planktonic cells from Figure 8. SAB is on the left and SAH is on the right ordinate.

Leddy et al. (1995) studied the growth of planktonic cells of *P. putida* 54G on toluene in batch cultures and observed that *tol*-mutants of the wild type cells were produced that increased in concentration with duration of toluene exposure. These *tol*-mutants possibly grew on organic compounds leaking from injured cells or intermediates formed due to incomplete toluene degradation. They also showed that benzyl alcohol, a degradation product, mediates formation of *tol*-mutants. We completed similar studies that support these findings.

Clearly, traditional bioreactor design strategies that have been used to model VOC degradation may be improved by incorporating effects of injury or toxicity caused by VOCs. Without coupling the processes of growth and injury or toxicity into a phenomenological model, bacterial activity could be grossly overestimated, compromising the predictive capabilities of such models.

CONCLUSIONS

In the present investigation, toluene degradation by biofilm and planktonic cells of *P. putida* was studied. We observed that specific activity (mg toluene degraded/mg biomass h and mg toluene degraded/cfu toluene degrading cells/h) for biofilm and planktonic cells were similar at toluene concentrations of 1–3 mg/L. We also noted that long-term toluene exposure and increase in toluene concentrations rather than surface attachment caused a difference in activity for free and attached cells of *P. putida* 54G. Levels of bacterial injury increased with duration and concentration of toluene exposure. During logarithmic growth, toluene degradation kinetics followed the Andrews inhibition model, with substrate inhibition being notable above 20 mg/L toluene. This value is much higher than the levels to which cells were exposed (1–3 mg/L) when injury was observed.

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