KINETICS OF TOLUENE DEGRADATION BY DENITRIFYING AQUIFER MICROORGANISMS

By Pedro J. J. Alvarez,[1] Paul J. Anid,[2] and Timothy M. Vogel[3]

INTRODUCTION

Addition of nitrate to increase the electron acceptor pool for bioremediation of hydrocarbon-contaminated aquifers has been receiving attention (Anid et al. 1993). Field evidence of the beneficial addition of nitrate for enhancing in situ toluene biodegradation has been gathered in Karlsruhe, Germany (Werner 1985); Traverse City, Mich. (Hutchins et al. 1991a); San Diego, Calif. (Sheehan et al. 1988); and Borden, Ontario, Canada (Lemon et al. 1989). Assessing the kinetics of toluene degradation under denitrifying conditions is essential in determining the fate of toluene in nitrate-enhanced in situ bioremediation schemes and in estimating the duration of such cleanup operations.

Monod kinetics have been widely used to describe biodegradation rates of organic contaminants in aquifer systems (Alvarez et al. 1991; Borden and Bedient 1986; Chen et al. 1992; Widdowson et al. 1988). Much of the versatility of Monod's equation is due to its ability to describe biodegradation rates that follow zero- to first-order kinetics with respect to the target substrate concentration. In addition, Monod's equation describes the biodegradation-rate dependence on microbial concentration. However, Monod's equation is empirical (Monod 1949), and the two coefficients (k, Ks) that characterize the equation are system-specific. Therefore, caution should be exercised in extrapolating coefficients from one environment (e.g., wastewater treatment units) to another (e.g., aquifer systems).

Biokinetic data for toluene degradation under denitrifying conditions are scarce (Table 1), and no Monod coefficients have been reported for toluene degradation by indigenous denitrifying microorganisms from aquifers. The objectives of this study were to determine whether Monod's equation could adequately describe the kinetics of toluene degradation by such microorganisms and to measure the corresponding biokinetic coefficients.

THEORETICAL BACKGROUND

The hyperbolic equation proposed by Monod (1949) to describe the growth of bacterial cultures as a function of a limiting nutrient concentration was modified by Lawrence and McCarty (1970) to describe the effect of substrate

[1]Asst. Prof., Dept. of Civ. and Envir. Engrg., Univ. of Iowa, Iowa City, IA 52242-1527.

[2]Asst. Res. Sci., Dept. of Civ. and Envir. Engrg., University of Michigan, Ann Arbor, MI 48109-2125.

[3]Rhône-Poulenc Industralisiation, Dept. of Safety and Envir., 69153 Décines-Charpieu, France.

Note. Discussion open until March 1, 1995. To extend the closing date one month, a written request must be filed with the ASCE Manager of Journals. The manuscript for this technical note was submitted for review and possible publication on October 28, 1992. This technical note is part of the ***Journal of Environmental Engineering***, Vol. 120, No. 5, September/October, 1994. ©ASCE, ISSN 0733-9372/94/0005-1327/ $2.00 + $.25 per page. Technical Note No. 5013.

TABLE 1. Biokinetic Coefficients for Toluene Degradation under Denitrifying Conditions

k (g toluene per g cells per day) (1)	Ks (mg toluene/L) (2)	Y (mg cell per mg toluene) (3)	Specific first-order coefficient; k/Ks (L/mg/day) (4)	Overall first-order coefficient; $(k/Ks)^a X$ (day^{-1}) (5)	Method of measurement (6)	References (7)
1.12	—	0.62	—	—	Toluene disappearance in batch incubations with denitrifying pure culture of *Pseudomonas* sp. K-172 at 28°C, pH = 7.8	Altenschmidt and Fuchs (1991)
7.42[a]	—	—	—	—	Toluene disappearance in batch incubations with denitrifying pure culture of Strain T1 at 30°C, pH = 7.5	Evans et al (1991)
4.32	0.15	—	28.8	—	Toluene disappearance in batch incubations with denitrifying sewage sludge at 20°C, pH = 7.2	Jørgensen et al. (1990)
—	—	—	—	0.02–0.16	Toluene disappearance in denitrifying aquifer microcosms at 12°C	Hutchins et al. (1991b)
0.28	8.6	0.60	0.033	—	Toluene disappearance in denitrifying aquifer enrichment at 25°C, pH = 7	This work

[a]Reported as g toluene per g protein per day.

concentration (S) on the rate at which a given microbial concentration (X) removes a target substrate ($-\partial S/\partial t$)

$$-\frac{\partial S}{\partial t} = \frac{kSX}{Ks + S} \tag{1}$$

where k is the maximum specific substrate utilization rate, and Ks is the half-saturation coefficient. Alternatively, Monod's equation can be written in terms of microbial growth by incorporating the net yield coefficient (Y)

$$\frac{\partial X}{\partial t} = -Y\frac{\partial S}{\partial t} = \frac{YkSX}{Ks + S} \tag{2}$$

Representing biomass by the empirical formula $C_5H_7O_2N$ (McCarty 1972), and considering that a fraction (fs) of toluene is coupled to cell synthesis, the overall stoichiometric equation for toluene degradation under denitrifying conditions (on a one-electron basis) is

$$\frac{1}{36}C_7H_8 + \frac{(1 - fs)}{5}NO_3^- + \frac{(1 - fs)}{5}H^+ + \frac{fs}{20}HCO_3^- + \frac{fs}{20}NH_4^+ \rightarrow \frac{(35 - 36fs)}{180}CO_2 + \frac{(1 - fs)}{10}N_2 + \frac{fs}{20}C_5H_7O_2N + \frac{(38 - 27fs)}{180}H_2O \tag{3}$$

(for complete nitrate reduction to N_2 gas); or

$$\frac{1}{36}C_7H_8 + \frac{(1 - fs)}{2}NO_3^- + \frac{fs}{20}HCO_3^- + \frac{fs}{20}NH_4^+ \rightarrow \frac{(35 - 36fs)}{180}CO_2 + \frac{(1 - fs)}{2}NO_2^- + \frac{fs}{20}C_5H_7O_2N + \frac{(20 - 9fs)}{180}H_2O \tag{4}$$

(for partial nitrate reduction resulting in nitrite accumulation).

The relationship between the net yield coefficient (Y) and fs is given by the aforementioned stoichiometric relationships as

$$Y = \frac{fs(1/20)(113 \text{ g empirical cell/mole})}{(1/36)(92 \text{ g toluene/mole})} = 2.21fs \tag{5}$$

Therefore, the stoichiometric nitrate requirements for catabolic purposes (in grams of nitrate per gram of toluene) are

$$\frac{(1 - fs)(1/5)}{(1/36)}\frac{(62 \text{ g nitrate/mole})}{(92 \text{ g toluene/mole})} = 4.85(1 - fs) = 4.85 - 2.19Y \tag{6}$$

(for complete nitrate reduction to N_2 gas); or

$$\frac{(1 - fs(1/2)}{(1/36)}\frac{(62 \text{ g nitrate/mole})}{(92 \text{ g toluene/mole})} = 12.13(1 - fs) = 12.13 - 5.49Y \tag{7}$$

(for partial nitrate reduction to nitrite).

These stoichiometric requirements provide a compatibility constraint between cell yield and changes in nitrate and nitrite concentrations associated with toluene biodegradation.

METHODOLOGY

Experimental Design

A denitrifying enrichment was prepared by adding 500 g of sandy aquifer material to 2 L of mineral medium (buffered at pH 7.0). The mineral medium has been described elsewhere (Alvarez and Vogel 1991). Dissolved oxygen was removed from this slurry to less than 1 mg/L by purging oxygen-free nitrogen gas for 15 min. The deoxygenated slurry was incubated at 25°C inside an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, Mich.). Denitrification was induced by the addition of yeast extract (50 mg/L) and nitrate (200 mg/L as NO_3^-). The degradation of yeast extract reduced the dissolved oxygen concentration below the detection limit (0.1 mg/L) and increased the number of denitrifiers.

Two 250-mL serum bottles were filled with 200 mLs (each) of the anoxic supernatant from this slurry and capped with Mini-nert valves (Alltech Associates Inc., Deerfield, Ill.). These incubations were adapted to toluene (20 mg/L), which was degraded from 20 mg/L to less than 0.01 mg/L within one week. Kinetic measurements were started one week later. Toluene (80 mg/L) was added along with nitrate (400 mg/L as NO_3^-), and their decrease in concentration was monitored over time with the concomitant increase in denitrifiers concentration. The mass of toluene partitioning into the headspace at equilibrium was calculated to be less than 6% of the total mass added, and was neglected in the estimation of the yield coefficient. One of the incubations was autoclaved to distinguish biodegradation from potential volatization and sorption losses. Biokinetic coefficients were obtained by a nonlinear regression of these data using a commercially available statistical software (Program AR; BMDP, Statistical Software, Inc., Los Angeles, Calif.). Program AR was set to couple Monod's differential equation for substrate disappearance [(1)] with Monod's differential equation for biomass growth [(2)]. Since two equations were used to estimate three biokinetic parameters (Y, k, and Ks), the compatibility constraint given by (6) and (7) were used to evaluate the estimated cell yield against observed changes in nitrate and nitrite concentrations.

The estimated biokinetic parameters were tested with toluene degradation data from a separate aquifer slurry. This slurry was prepared by adding 12 g of the same aquifer material to a 250-mL serum bottle. The bottle was filled with nitrate-amended (100 mg/L as NO_3^-) medium. Unlike the denitrifying enrichment, no denitrification was induced by the addition of yeast extract prior to toluene addition (17 mg/L), and the initial concentration of denitrifiers in the slurry (0.00005 mg/L) was measured to be several orders of magnitude smaller than that of the enrichment (10 mg/L) described previously.

Analytical Procedures

Toluene was analyzed in a Hewlett-Packard 5890 gas chromatograph equipped with an HP 19395A headspace autosampler and a flame-ionization detector (Alvarez et al. 1991). The limit of detection for toluene was 0.01 mg/L.

Nitrate and nitrite were analyzed in a Dionex 4500i chromatograph using an AS4A ion exchange column (Dionex, Inc. Sunnyvale, Calif.) and a conductivity detector. The limit of detection was 1 mg/L for each compound.

A biological oxygen monitor YSI 530, equipped with a microchamber and an oxygen micro probe (YSI Inc., Yellow Springs, Ohio) was used to

verify that anoxic conditions prevailed in the incubations. The limit of detection for dissolved oxygen was 0.1 mg/L.

Microbial numbers in the denitrifying enrichment were measured by microscopy with the Acridine Orange Direct Count (AODC) procedure (Webster et al. 1985). A cell weight of 10^{-9} mg was assumed to convert cell numbers to biomass. This cell weight is based on a typically observed cell size of 1 μm^3 and a cell density of 1.04 g/cm^3 (Bratbak and Dundas 1984). Enumeration of denitrifiers in the aquifer material was performed with plate counts. Ten grams of aquifer material were diluted in 100 mL of 0.1% sodium pyrophosphate buffer as described by Webster et al. (1985). Appropriate dilutions were plated on agar amended with trypticase soy broth (200 g/L) and potassium nitrate (1 g/L). Plates were incubated inside the anaerobic chamber along with nitrate-free plates as controls.

RESULTS

The initial microbial concentration of the pregrown, toluene-adapted denitrifying enrichment was measured at 10 mg/L. A lag period of approximately two days was followed by the concomitant removal of toluene [Fig. 1(*a*)] and nitrate [Fig. 1(*b*)] with an increase in biomass [Fig. 1(*a*)] and appearance of nitrite [Fig. 1(*b*)]. No toluene was detected after 17 days of incubation. Approximately 397 mg/L of nitrate (as NO_3^-) were removed and 145 mg/L of nitrite (as NO_2^-) appeared during this period. The final biomass concentration was 57 mg/L. No removal of toluene or nitrate occurred in autoclaved controls.

A nonlinear regression of the biomass and toluene concentrations over time yielded the following biokinetic parameters ($\pm$ one standard deviation): $Y = 0.6 \pm 0.1$ g cells/g toluene; $k = 0.28 \pm 0.04$ g toluene/g cells/day; and $Ks = 8.6 \pm 9.3$ mg toluene/L. Best-fit simulations using these parameters [Fig. 1(*a*)] were performed by coupling Monod's equations for substrate disappearance [(1)] and biomass growth [(2)], expressed in finite difference form.

The estimated biokinetic parameters were tested with independent toluene degradation data from an aquifer slurry that had a much lower initial concentration of denitrifiers (5×10^{-5} mg/L). Approximately 16 mg/L of toluene were removed within 140 days following a lag of 110 days [Fig. 2(*a*)]. Toluene removal coincided with the disappearance of approximately 55 mg/L of nitrate (as NO_3^-) and the net accumulation of 3 mg/L of nitrite (as NO_2^-) [Fig. 2(*b*)]. Toluene degradation patterns were fit reasonably well using an initial denitrifiers concentration within one order of magnitude of the experimentally determined value [Fig. 2(*a*)].

ANALYSIS

Monod's equation adequately described the kinetics of toluene degradation and biomass production under denitrifying conditions [Fig. 1(*a*)]. The estimated yield coefficient (0.6 g cells/g toluene) is in excellent agreement to that reported for *Pseudomonas* sp. K-172 (0.62 g cells/g toluene), which was isolated from sewage sludge (Altenschmidt and Fuchs 1991). However, the estimated maximum specific substrate utilization rate (0.28 g toluene/g cells/day) is smaller than the value reported for this pseudomonad (1.12 g toluene/g cells/day) and that reported for denitrifying sewage sludge (4.32 g toluene/g cells/day) (Jørgensen et al. 1990). The discrepancy is prob-

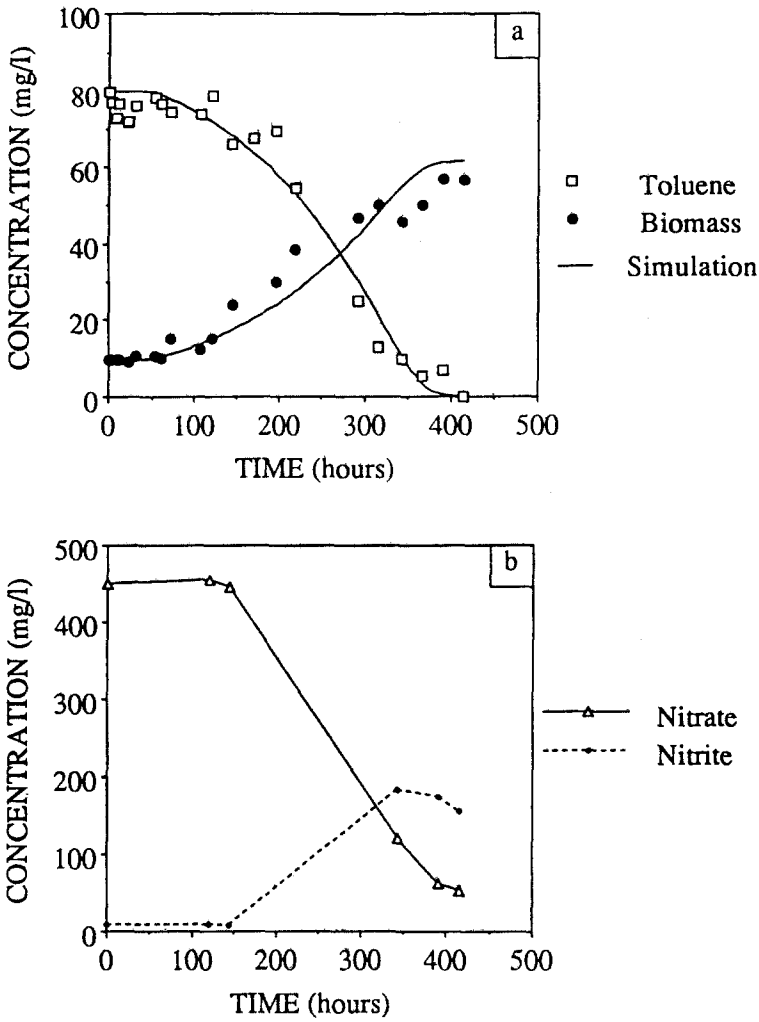

FIG. 1. Toluene Degradation by a Denitrifying Enrichment

ably due to intrinsic differences in the metabolic characteristics of microbes isolated from different ecosystems.

Approximately 80 mg/L of toluene were degraded within 17 days in the denitrifying enrichments grown on yeast extract (initial denitrifiers concentration of approximately 10 mg/L) [Fig. 1(*a*)], while it took nearly 140 days to degrade 16 mg/L of toluene in the aquifer slurry (initial denitrifiers concentration of 0.00005 mg/L) [Fig. 2(*a*)]. The longer lag period associated with the aquifer slurry (110 versus two days) probably reflects the time that active microorganisms require for growing to a sufficient concentration capable of exerting appreciable biodegradation rates. This explanation is consistent with model simulations and suggests the potential benefits of engineered manipulations to increase the active microbial concentration to enhance biodegradation kinetics.

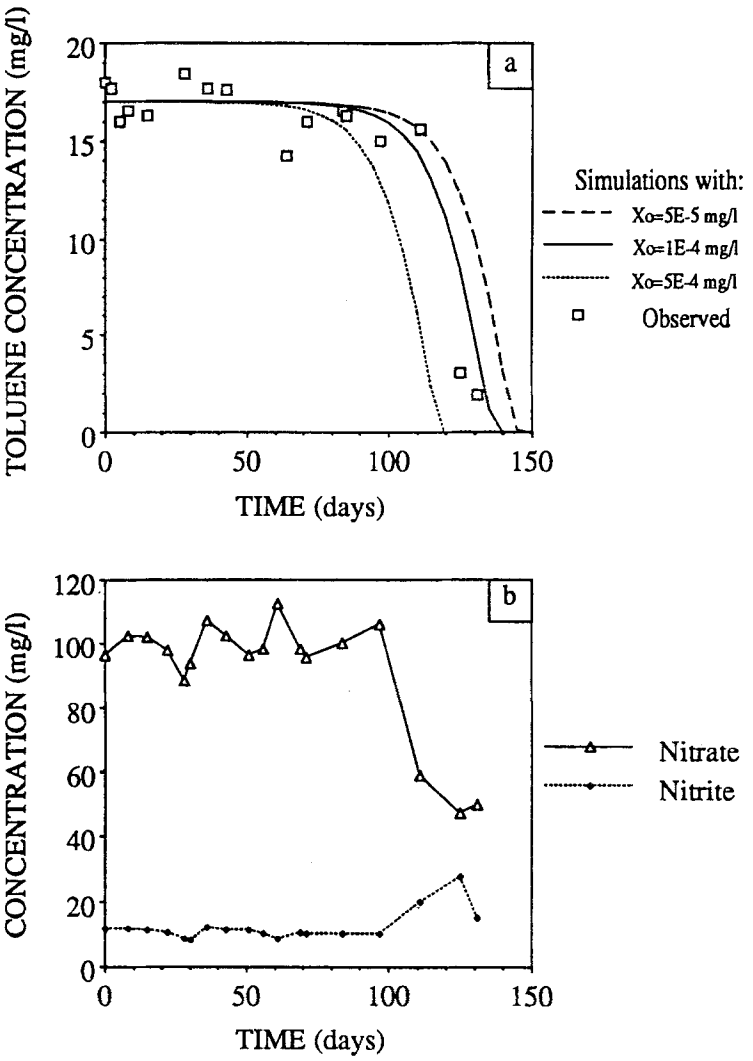

FIG. 2. Toluene Degradation in Denitrifying Aquifer Slurry

The concentration of toluene found in contaminated aquifers is often substantially lower than our estimated *Ks* values (8.6 mg/L). In this case, Monod's equation reduces to a first-order expression with respect to substrate concentration, with a specific first-order coefficient equal to k/Ks, which is numerically equal to the overall first-order coefficient (K') divided by the microbial concentration (X). The estimated specific first-order coefficient, $k/Ks = 0.033$ L/mg/day, indicates that toluene half-lives on the order of three weeks may be expected under denitrifying conditions, assuming a denitrifiers concentration on the order of 1 mg/L (i.e., 10^6 cells/g) in the biostimulated aquifer material.

Nitrate is a regulated compound because of its potential to cause methemoglobinemia. Therefore, in situ bioremediation projects utilizing nitrate

should prevent its accumulation above the permissible concentration of 45 mg/L (10 mg/L as N). Controlled nitrate addition requires an understanding of the stoichiometric nitrate requirements for catabolic purposes. However, the theoretical nitrate requirements to degrade toluene to CO_2, H_2O, and biomass may vary considerably, depending on the degree to which nitrate is reduced. The theoretical nitrate requirement to degrade one gram of toluene, assuming a cell yield of 0.6 g cells/g toluene, is 3.54 g (as NO_3^-) when denitrification to N_2 occurs [(6)]. However, 8.84 g would be required if nitrate is reduced only to nitrite [(7)]. Whether denitrification results in nitrite accumulation depends on the relative rates of reduction of nitrate to nitrite versus nitrite to nitric oxide. Faster nitrate than nitrite reduction rates are conducive to nitrite accumulation. Nitrite accumulation is more likely to occur when the available carbon sources are present at low concentrations relative to the amount of nitrate present, when incubation periods are short relative to the time required for complete denitrification, and when toxic intermediates that interfere with the completion of the denitrification reactions are produced (Tiedje 1988). Predicting the extent to which nitrite would acumulate in situ, if it does accumulate at all, is a difficult task. Therefore, estimates of nitrate requirements for field applications should be supported with laboratory experiments.

Approximately 397 mg/L of nitrate (89.6 mg/L as N) disappeared and 145 mg/L of nitrite (44 mg/L as N) appeared during the degradation of 80 mg/L of toluene in the denitrifying enrichment [Fig. 1(*b*)]. Assuming that 195 mg/L of nitrate (44 mg/L as N) was partially reduced to form 145 mg/L of nitrite (44 mg/L as N) and that the remaining 202 mg/L of nitrate was reduced to N_2 gas, the theoretical amount of toluene degraded would be

$$\frac{202 \text{ mg/L } NO_3^-}{3.54 \text{ g-}NO_3\text{-per g-T with } N_2 \text{ formed}} + \frac{195 \text{ mg/L } NO_3^-}{8.84 \text{ g-}NO_3\text{-per g-T with } NO_2^- \text{ formed}} = 79.1 \text{ mg/L of toluene} \quad (8)$$

In these experiments, 80 mg/L of toluene was degraded. Similarly, approximately 55 mg/L of nitrate (12.4 mg/L as N) disappeared and 3 mg/L of nitrite (0.9 mg/L as N) appeared during the degradation of 15 mg/L of toluene in the aquifer slurry [Fig. 2(*b*)]. Assuming that 4 mg/L of nitrate (0.9 mg/L as N) were partially reduced to form 3 mg/L of nitrite (0.9 mg/L as N) and that the remaining 51 mg/L of nitrate were reduced to N_2 gas, the theoretical amount of toluene degraded would be

$$\frac{51 \text{ mg/L } NO_3^-}{3.54 \text{ g-}NO_3\text{-per g-T with } N_2 \text{ formed}} + \frac{4 \text{ mg/L } NO_3^-}{8.84 \text{ g-}NO_3\text{-per g-T with } NO_2^- \text{ formed}} = 14.9 \text{ mg/L of toluene} \quad (9)$$

In these experiments, 16 mg/L of toluene was degraded. Therefore, the amount of toluene degraded in denitrifying incubations was in excellent agreement with stoichiometric calculations based on observed changes in nitrate and nitrite concentrations. These theoretical calculations assumed that toluene was completely degraded to CO_2, H_2O, and biomass, and that nitrate was not utilized for anabolism since ammonium chloride had been

provided as nitrogen source. Nitrate removed was assumed to be either partially reduced to nitrite or completely reduced to N_2 gas, and nitrite removed was assumed to be reduced to N_2 gas. These assumptions are consistent with the concept that other denitrification intermediates, such as nitric (NO) and nitrous (N_2O) oxides, do not usually accumulate (Knowles 1982; Tiedje 1988).

The excellent agreement between the observed and predicted amount of toluene degraded suggests that the assumptions undertaken in the stoichiometric calculations might be relatively accurate. These calculations reiterate that the net yield coefficient determined from biokinetics data (0.6 g cells/g toluene) is compatible with the observed changes in nitrate and nitrite concentrations.

SUMMARY AND CONCLUSIONS

The kinetics of toluene degradation by denitrifying aquifer microorganisms and the resulting biomass production were adequately characterized by Monod's equation. Toluene degradation was stoichiometrically coupled to nitrate reduction and biomass production. The estimated biokinetic parameters (Y = 0.6 g cells/g toluene; k = 0.28 g toluene/g cells/day; and Ks = 8.6 mg toluene/L) were tested in an independent kinetic experiment with a much lower initial denitrifier concentration. Simulations were in reasonable agreement with the observed toluene degradation pattern, and illustrated the strong dependence of the degradation time on the initial active biomass concentration.

Monod coefficients are system-specific, and caution should be exercised in extrapolating values measured in a given environment to another. Factors that contribute to the wide variability of reported coefficients include the metabolic diversity of microbes isolated from different systems, variations in environmental conditions, and the different experimental and analytical procedures used in their determination.

ACKNOWLEDGMENTS

This work was supported in part by the Michigan Oil and Gas Association (DRDA number 90-1112), the NIEHS Superfund Research Center (Grant 5 P42 ES04911-03), and the Office of Research and Development, U.S. Environmental Protection Agency to the Great Lakes and Mid-Atlantic Hazardous Substance Research Center (Grant R-815750-02).

APPENDIX I. REFERENCES

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