APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1989, p. 3221–3225
0099-2240/89/123221-05$02.00/0

Vol. 55, No. 12

Substrate Interactions during Aerobic Biodegradation of Benzene

ERIK ARVIN,* BJØRN K. JENSEN, AND ANDERS TORP GUNDERSEN

Department of Environmental Engineering, Building 115, Technical University of Denmark, DK-2800 Lyngby, Denmark

Received 30 May 1989/Accepted 5 September 1989

This study dealt with the interactions with benzene degradation of the following aromatic compounds in a mixed substrate: toluene, *o*-xylene, naphthalene, 1,4-dimethylnaphthalene, phenanthrene, and pyrrole. The experiment was performed as a factorial experiment with simple batch cultures. The effect of two different types of inocula was tested. One type of inoculum was grown on a mixture of aromatic hydrocarbons; the other was grown on a mixture of aromatic hydrocarbons and nitrogen-, sulfur-, and oxygen-containing aromatic compounds (NSO compounds), similar to some of the compounds identified in creosote waste. The culture grown on the aromatic hydrocarbons and NSO compounds was much less efficient in degrading benzene than the culture grown on only aromatic hydrocarbons. The experiments indicated that toluene- and *o*-xylene-degrading bacteria are also able to degrade benzene, whereas naphthalene-, 1,4-dimethylnaphthalene-, and phenanthrene-degrading bacteria have no or very little benzene-degrading ability. Surprisingly, the stimulating effect of toluene and *o*-xylene was true only if the two compounds were present alone. In combination an antagonistic effect was observed, i.e., the combined effect was smaller than the sum of the effect from each of the compounds. The reason for this behavior has not been identified. Pyrrole strongly inhibited benzene degradation even at concentrations of about 100 to 200 μg/liter. Future studies will investigate the generality of these findings.

The microbial degradation of organic pollutants in aquatic environments is influenced by a range of factors such as pollutant concentration, biomass concentration, temperature, pH, the availability of nutrients, inhibitors, primary substrates, adaptation, etc. This study focused on the interactions among organic pollutants and their ability both to stimulate and to inhibit biodegradation. Such interactions are important since most pollution sources discharge a whole spectrum of substances.

It is well established that the degradation of trace organics that do not support microbial growth (secondary substrates) depends on the biomass created by one or more easily degradable organic substances in relatively high concentrations (primary substrates) (9). However, little is known of the interactions among biodegradable organics which are present at concentrations supporting microbial growth. It has been observed that prior acclimation history significantly affects the degradation pattern (10, 11, 13). Bauer and Capone (1) observed that the degradation of specific polycyclic aromatic hydrocarbon compounds in marine sediment slurries may be stimulated by preexposure of other polycyclic aromatic hydrocarbon compounds and benzene (Ben). For example, anthracene, phenanthrene (Phe), and Ben stimulated the mineralization of naphthalene (Nap) significantly. Although Nap stimulated Phe degradation, it did not have the same effect toward anthracene.

The purpose of this study was to obtain more qualitative and quantitative information on interactions among aromatics during biodegradation. In the present phase of experiments, Ben, toluene (Tol), *o*-xylene (Xyl), Nap, 1,4-dimethylnaphthalene (Dmn), Phe, and pyrrole (Pyr) were selected as model compounds, with Ben as the dependent variable. The degradation of Ben in relation to the other aromatic substances is an important issue, since Ben is a compound of considerable environmental interest owing to its toxicity. Degradation of aromatics has become important in relation to wastes containing creosote and oil compounds.

* Corresponding author.

MATERIALS AND METHODS

Experimental system. The interactions were studied in simple batch systems consisting of 250-ml glass bottles with Teflon-lined screw caps. The aromatics were added from a stock solution according to the system described below. Initial concentrations of each of the compounds were 0.1 to 0.2 mg/liter.

The basal mineral medium contained the following (per liter of distilled water): 147 mg of $CaCl_2$, 0.4 mg of $FeCl_3 \cdot 6H_2O$, 9.4 mg of KCl, 50 mg of $MgSO_4 \cdot 7H_2O$, 12.2 mg of $NaNO_3$, 5.8 mg of Na_2HPO_4, and 100 mg of $NaHCO_3$. The trace elements were (per liter) 14 μg of Mn(II), 14 μg of Zn(II), 10 μg of Mo(VI), 10 μg of B, 8 μg of Cu(II), and 8 μg of Co(II).

The bottles were totally filled with air-saturated water at the beginning of the experiment to keep the system aerobic during the tests. Owing to the low initial concentrations of aromatic compounds, the water was aerobic throughout the experiment. This was confirmed by measurements at the end of the experiment.

After 5 days, a 15-ml sample was withdrawn from each bottle for analysis. Final samples were taken after 11 days.

The temperature was about 22°C and the pH was about 7 during the whole experimental run. The bottles were rotated in a dark box at 2 rpm.

Inoculation materials. Two types of inocula were used. Both inocula came from fixed biofilm systems in which the bacteria had been growing on the walls of a rotating drum system for about 0.5 year. The physical design of the rotating drum system is described by Kristensen and Jansen (5). Inoculum 1 (Inoc 1) had been grown on a substrate consisting only of aromatic hydrocarbons: Ben, Tol, Xyl, Nap, Dmn, biphenyl, and Phe. Inoculum 2 (Inoc 2) had been grown on a mixture of hydrocarbons and nitrogen-, sulfur-, and oxygen-containing compounds (NSO compounds): Ben, Tol, Xyl, indene, Nap, Dmn, biphenyl, Phe, phenol, cresol,

fluorenone, quinoline, indole, furan, dibenzothiophene, and Pyr. In all cases the biomass had been grown at substrate concentrations of the specific compounds below 300 μg/liter, most often in the range of 1 to 50 μg/liter. An exception from the degradation to 1 to 50 μg/liter was Ben and Pyr in the hydrocarbon-NSO mixture. Both compounds were only degraded significantly when the other compounds were degraded to concentrations of about 1 μg/liter. Then Ben was degraded to concentrations in the range of 20 to 80 μg/liter and Pyr was degraded to 120 to 210 μg/liter. The basal mineral substrate was the same in the continuous flow system as in this batch study.

The inocula had been stored for 3 months in a freezer before use. The same amount of biomass was added to each bottle, in a concentration of about 0.17 mg/liter. This concentration was calculated based on a measured ATP concentration of about 500 ng/liter and an ATP content in the biomass of 3 ng of ATP per μg of biomass (see Results).

Analytical procedure. The aromatic compounds were analyzed in a Shimadzu GC-9A gas chromatograph equipped with a OCI-G9 on-column injector and a flame ionization detector connected with a Shimadzu C-R 3A computing integrator. The gas chromatograph column was a 20-m Chrompack WCOT fused silica (liquid phase, CP-Sil-5 CB) column with an inner diameter of 0.53 mm. Samples of 10 ml were extracted with 0.5 ml of pentane, and the peaks were quantified by internal standardization with *n*-undecane as the standard.

ATP was measured by the luciferin-luciferase method (6). The sample (100 μl) was extracted with 100 μl of NRB (Lumac) for 10 min in sterile glass cuvettes. After enzyme (Lumit PM; Lumac) addition, measurements were done on a Lumac M2010 biocounter, which recorded the light output in relative light units. Relative light units were integrated over 10 s after the addition of purified enzyme. To convert the light output of the biocounter, we prepared a standard curve for each run with ATP standards purchased from Lumac.

Experimental design. The experiment was done as a factorial experiment. The percent degradation of Ben was the dependent variable; the other six aromatics Tol, Xyl, Nap, Dmn, Phe, and Pyr and the types of inocula were the independent variables. The factorial experiment was designed as a 2^5 confounded experiment with two blocks, one for each type of inoculum. There was no replication. The definition equation was I = ABCDEF = Tol Xyl Nap Dmn Phe Pyr; the alias relation for the sixth factor (Pyr) was Pyr = Tol Xyl Nap Dmn Phe, and for the blocks it was Blocks = Nap Dmn Phe (2, 4). The experimental design assumed that only the main factors and two-factor interactions may be significant. This design required 32 bottles. In addition to the 32 bottles, 2 additional bottles were used as controls to correct for evaporation losses. The two controls, containing the same amount of biomass as the other bottles, were treated with $HgCl_2$ and H_2SO_4, respectively. The reason for not using $HgCl_2$ in both controls is that it has been observed that mercury ions catalyze the degradation of Pyr (B. K. Jensen, unpublished data). In general, the evaporation loss was very small during the incubation. Samples were taken from each bottle at time zero and after 5 and 11 days.

Statistical analysis was performed by the statistical package SAS (12), version 6, for personal computers, procedure GLM. In all cases the significance of a parameter was evaluated based on the observed *P* value. The criterion for accepting a parameter was set to $P \leq 5\%$.

RESULTS

The percent degradation of the aromatics is shown in Table 1. From a qualitative inspection of Table 1, it appears that the degree of Ben degradation is lower with Inoc 2, i.e., the biomass grown on a mixture of aromatic hydrocarbons and NSO compounds. It also appears that within 11 days all the aromatic hydrocarbons except Ben (Tol, Xyl, Nap, Dmn, and Phe) were degraded. Most of the degradation occurred within the first 5 days. In contrast, Pyr, like Ben, was only partially degraded. Table 1 also indicates that degradation of Ben was relatively high when Tol and Xyl were present, while Pyr inhibited degradation.

The statistical analysis was performed to investigate the interactions among components in greater detail. Note that the effect of Tol, Xyl, Nap, Dmn, and Phe on Ben degradation was evaluated based on their initial concentrations. The reasoning was that most of the hydrocarbons were degraded after 5 days and that the effect of the hydrocarbons on Ben degradation most likely is related to the biomass created by their degradation.

Repeated-measures analysis. Since the samples after 5 and 11 days were taken from the same bottle they are not independent. The experiment can be treated according to repeated-measures analysis (3, 12). This allows the determination of significant factors after 5 and 11 days, in addition to overall significant factors in the experiment. The significant factors for benzene degradation were as follows: 5 days, Inoc, Tol, Pyr, Tol*Xyl, Xyl*Pyr, Nap*Phe; 11 days, Inoc, Tol, Pyr, Nap*Dmn, Dmn*Pyr; 5 plus 11 days, Inoc, Tol, Xyl, Pyr, Tol*Xyl. The asterisk (*) shows interaction, e.g., Tol*Xyl denotes interaction between Tol and Xyl. Not surprisingly, incubation time had a significant effect, but there were also significant interactions between incubation time and several of the independent factors. A close analysis of the most significant factors at 5 and 11 days showed that Inoc, Tol, Xyl, and Pyr were the most important independent variables, except for incubation time. The significance level (*P*) of the variables and the multiple correlation coefficient squared, R^2, are shown in Table 2.

Nonlinear model in time. The repeated-measures analysis assumed linear effects of all factors including incubation time. It is well known that degradation of organic compounds is seldom linear with time. The analysis may therefore be improved by assuming a nonlinear time dependency for Ben degradation and a linear dependency of the initial concentrations of the compounds Tol, Xyl, etc. Because sampling only was performed on days 5 and 11, it is necessary to have a simple time model in which only one constant needs to be determined. Let the percent degradation of Ben be called *D* and the incubation time be called *t*. The models tested were the following:

$$\text{Exponential model: } D = e^{k^*t} - 1 \rightarrow \ln(D + 1) = k^*t$$

$$\text{Quadratic model: } D = k^*t^2$$

The constant *k* was determined by linear regression in each of the 32 experiments, forcing the curve through the zero point. At time zero the percent degradation must be zero. Some other assumptions were made. (i) If a degradation of 0% was measured after 5 days, it was set to 1%. A degradation of 0% after 5 days is incompatible with the models. (ii) In several cases, degradation was 100% after 5 and 11 days. This means that the degradation may actually have reached 100% before these sampling times. If 100% degradation was measured on day 5, the degradation was set to 100% on that day. However, the measured degradation of 100% after 11

TABLE 1. Percent degradation of aromatics after 5 and 11 days, ATP concentration, and calculated biomass production[a]

Time (days)	Inoc	% Degradation of:							ATP (ng/liter)	Biomass produced (μg/liter)
		Tol	Xyl	Nap	Dmn	Phe	Pyr	Ben		
5	1	—[b]	—	—	—	40	14	8	671	0
5	1	—	—	—	0	—	12	8	539	0
5	1	—	—	100	—	—	0	0	1,016	73
5	1	—	—	100	80	100	4	18	1,918	374
5	1	—	100	—	—	100	—	39	1,940	381
5	1	—	56	—	74	—	—	86	1,599	268
5	1	—	100	100	—	—	—	48	1,490	231
5	1	—	100	100	100	100	—	73	2,074	426
5	1	100	—	—	—	80	—	100	1,289	164
5	1	100	—	—	10	—	—	100	1,116	107
5	1	100	—	100	—	—	—	88	1,993	399
5	1	100	—	100	66	100	—	100	2,034	413
5	1	100	100	—	—	100	5	52	2,235	480
5	1	100	76	—	100	—	5	60	1,829	344
5	1	100	100	100	—	—	14	54	3,593	932
5	1	100	100	100	100	100	0	51	3,127	777
5	2	—	—	—	—	—	—	12	801	2
5	2	—	—	—	70	100	—	1	1,220	141
5	2	—	—	100	—	100	—	1	1,877	360
5	2	—	—	100	0	—	—	0	1,506	237
5	2	—	100	—	—	—	0	1	1,390	198
5	2	—	100	—	100	100	8	5	2,047	417
5	2	—	100	100	—	100	15	0	1,590	265
5	2	—	100	100	100	—	17	0	1,298	167
5	2	33	—	—	—	—	0	0	1,164	123
5	2	82	—	—	91	100	18	3	1,760	321
5	2	100	—	100	—	100	25	5	1,795	333
5	2	50	—	100	61	—	0	0	1,459	221
5	2	100	100	—	—	—	—	29	1,670	291
5	2	100	100	—	100	100	—	10	2,878	694
5	2	100	100	100	—	100	—	16	2,777	660
5	2	100	100	100	100	—	—	11	1,921	375
11	1	—	—	—	—	100	40	18	864	23
11	1	—	—	—	100	—	30	12	1,098	101
11	1	—	—	100	—	—	17	12	1,634	279
11	1	—	—	100	100	100	40	30	2,284	496
11	1	—	100	—	—	100	—	100	1,801	335
11	1	—	100	—	100	—	—	100	2,288	497
11	1	—	100	100	—	—	—	100	2,458	554
11	1	—	100	100	100	100	—	100	2,033	412
11	1	100	—	—	—	100	—	100	1,596	267
11	1	100	—	—	100	—	—	100	2,206	470
11	1	100	—	100	—	—	—	100	2,700	635
11	1	100	—	100	100	100	—	100	3,169	791
11	1	100	100	—	—	100	25	50	3,100	768
11	1	100	100	—	100	—	18	69	2,200	468
11	1	100	100	100	—	—	32	69	3,762	989
11	1	100	100	100	100	100	18	69	5,173	1,459
11	2	—	—	—	—	—	—	100	2,297	500
11	2	—	—	—	100	100	—	5	1,255	153
11	2	—	—	100	—	100	—	46	3,487	897
11	2	—	—	100	100	—	—	50	2,750	651
11	2	—	100	—	—	—	26	5	1,495	233
11	2	—	100	—	100	100	51	11	1,900	368
11	2	—	100	100	—	100	49	5	3,345	850
11	2	—	100	100	100	—	28	5	1,752	319
11	2	100	—	—	—	—	23	5	2,188	464
11	2	100	—	—	100	100	51	15	1,596	267
11	2	100	—	100	—	100	32	11	4,525	1,243
11	2	100	—	100	100	—	20	8	1,986	397
11	2	100	100	—	—	—	—	100	1,956	387
11	2	100	100	—	100	100	—	46	3,024	743
11	2	100	100	100	—	100	—	100	2,090	431
11	2	100	100	100	100	—	—	100	3,265	823

[a] The biomass produced is calculated based on the calibration curve (Fig. 1). Initial concentrations (milligrams per liter): Ben, 0.18; Tol, 0.19; Xyl, 0.19; Nap, 0.22; Dmn, 0.20; Phe, 0.12; and Pyr, 0.18.

[b] —, Substance not present.

TABLE 2. Significance levels of variables

Day	Significance level P (%)					R^2
	Inoc	Tol	Pyr	Tol*Xyl	Xyl	
5	0.01	0.01	0.01	0.02		0.88
11	0.01	0.11	0.22		0.01	0.85

TABLE 3. Relationship of k values to aromatics

Model	Significance level P (%)				R^2
	Inoc	Tol	Pyr	Tol*Xyl	
Exponential	0.01	0.01	0.01	0.01	0.91
Quadratic	0.01	0.05	0.01	0.01	0.91

days was not always used. This applies to the situations in which degradation based on day 5 leads to a calculated 100% degradation before day 11.

The empirical degradation constants, the k values, were assumed to depend linearly on the initial concentrations of the aromatics: $k = f(\text{Tol, Xyl, Nap, Dmn, Phe, Pyr})$. The k values based on both the exponential and the quadratic models were related to the aromatics in a very clear and simple way (Table 3). The statistical analysis allows determination of a quantitative relationship between percent Ben degradation and the independent variables: incubation time, Inoc, and the initial concentrations of Tol, Xyl, and Pyr. The equations are as follows:

Exponential model Inoc 1:

$$\ln(D + 1) = t^*(0.65 + 1.48^*S_{\text{Tol}} + 0.77^*S_{\text{Xyl}} - 8.09^*S_{\text{Tol}}^*S\text{Xyl} - 1.83^*S_{\text{Pyr}}) \quad (1)$$

Exponential model Inoc 2:

$$\ln(D + 1) = t^*(0.36 + 1.48^*S_{\text{Tol}} + 0.77^*S_{\text{Xyl}} - 8.09^*S_{\text{Tol}}^*S_{\text{Xyl}} - 1.83^*S_{\text{Pyr}}) \quad (2)$$

Quadratic model Inoc 1:

$$D = t^{2*}(1.69 + 9.42^*S_{\text{Tol}} + 6.18^*S_{\text{Xyl}} - 65.04^*S_{Tol}^*S_{\text{Xyl}} - 8.97^*S_{\text{Pyr}}) \quad (3)$$

Quadratic model Inoc 2:

$$D = t^{2*}(0.29 + 9.42^*S_{\text{Tol}} + 6.18^*S_{\text{Xyl}} - 65.04^*S_{\text{Tol}}^*S_{\text{Xyl}} - 8.97^*S_{\text{Pyr}}) \quad (4)$$

Effect of ATP. The degradation of Ben after 5 and 11 days was tested to learn whether it was related to the biomass concentration, measured as ATP, in addition to the initial concentrations of some of the aromatics. ATP was not a significant factor.

ATP versus calculated biomass concentration. In connection with the statistical evaluation of the effect of ATP concentration, the correlation between ATP concentration and biomass was tested. The latter was calculated from the amount of aromatics transformed, using an average yield factor of 0.8. A plot of ATP versus the calculated total biomass is shown in Fig. 1. The yield factor 0.8 was determined for Nap (E. Arvin and B. K. Jensen, unpublished data). It is assumed that this yield factor is of the same order of magnitude also for the other aromatic compounds. The biomass produced is given in Table 1. This is calculated based on the measured ATP concentration (Table 1) minus. Inoc (796 ng of ATP per liter) divided by the average ATP concentration in the biomass (3 ng of ATP/μg of biomass) (Fig. 1).

DISCUSSION

A big difference in Ben degradation ability was observed between cultures that had been grown on aromatic hydrocarbons alone and those grown on aromatic hydrocarbons and NSO compounds. The NSO compounds obviously decreased the Ben-degrading ability. Future studies are needed to show whether this is generally true.

Equations 3 and 4 above show that when Tol or Xyl is present alone, it stimulates Ben degradation. This indicates that Tol- and Xyl-degrading bacteria are also able to degrade Ben. Surprisingly, when the two compounds were present together they exerted an antagonistic effect, i.e., the stimulating effect was smaller than the sum of the effects from Tol

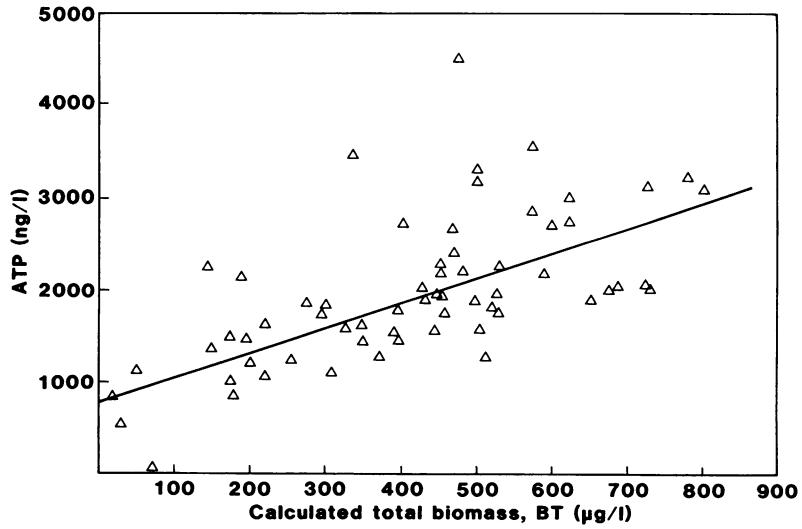

FIG. 1. ATP concentration versus the calculated total biomass (BT). The regression equation was ATP (nanograms per liter) = 796 + 3.00 BT (micrograms per liter). $R^2 = 0.45$. The intercept ± standard deviation was 796 ± 199 ng/liter. The slope ± standard deviation was 3.00 ± 0.42 ng of ATP per μg of biomass.

and Xyl alone. The reason for this has not yet been identified.

The dicyclic and tricyclic aromatic compounds Nap, Dmn, and Phe had no significant effect on Ben degradation, i.e., they do not seem to produce Ben degraders.

This study and the study by Bauer and Capone (1) clearly showed that the interaction pattern among the aromatic hydrocarbons is not simple despite the similarities in chemical structure. Much more research is needed to further clarify the interaction effects. In this regard factorial design has proved very useful. The information obtained can be further improved if more time steps are involved in the sampling from each bottle. This of course requires more analytical resources.

Equations 3 and 4 demonstrate the inhibiting effect of Pyr. It is conceivable that Pyr may act as an inhibitor, but the surprising aspect is that the inhibition occurs at such low Pyr concentrations, between 100 and 200 μg/liter. Accordingly, when dealing with the degradation of creosote waste, which contains Pyr, an inhibiting effect can be expected even at low substrate concentrations.

According to Fig. 1, there is some correlation between the ATP measured and the calculated biomass production (R^2 = 0.45). The ATP concentration in the biomass was 3 ± 0.42 ng of ATP per μg of biomass. This value is of the same order of magnitude as found in other investigations. Levin et al. (7) found values of 1.64 ± 0.30 for *Escherichia coli*, 7.71 ± 0.65 for *Zooglea ramigera*, and 2.9 ± 1.12 for a *Bacillus* sp.

ACKNOWLEDGMENTS

We thank the Technical Research Council of Denmark for the financial support for this project.

Valuable suggestions for improvements were obtained from Lewis Semprini and Dunja Grbič-Galič, Department of Civil Engineering, Environmental Engineering and Sciences, Stanford University. L. Brøndum and J. Monrad at the Engineering Academy of Denmark and H. Spliid, H. Holst, and B. Bilbo at the Institute of Mathematical Statistics and Operational Research at the Technical University of Denmark were very inspiring and helpful during discussions on the statistical treatment of data.

LITERATURE CITED

1. **Bauer, J. E., and D. G. Capone.** 1988. Effects of co-occurring aromatic hydrocarbons on degradation of individual polycyclic aromatic hydrocarbons in marine sediment slurries. Appl. Environ. Microbiol. **54**:1649–1655.
2. **Brøndum, L., and J. D. Monrad.** 1986. Statistical experimental planning II. (In Danish.) Den Private Ingeniørfond, Copenhagen.
3. **Cody, R. P., and J. K. Smith.** 1987. Applied statistics and the SAS programming language, 2nd ed. North-Holland Publishing Co., New York.
4. **Hicks, C. R.** 1982. Fundamental concepts in the design of experiments, 3rd ed. Holt, Rinehart & Winston, New York.
5. **Kristensen, G. H., and J. la Cour Jansen.** 1980. Fixed film kinetics—description of laboratory equipment. Department of Sanitary Engineering, Technical University of Denmark, Lyngby.
6. **Leach, F. R.** 1981. ATP determination with firefly luciferase. J. Appl. Biochem. **3**:473–517.
7. **Levin, G. V., J. R. Schrot, and W. C. Hess.** 1975. Methodology for application of adenosine triphosphate determination in wastewater treatment. Environ. Sci. Technol. **9**:961–965.
8. **Luong, J. H. T.** 1987. Generalization of Monod kinetics for analysis of growth data with substrate inhibition. Biotechnol. Bioeng. **19**:242–248.
9. **McCarty, P. L., B. E. Rittmann, and E. J. Bouwer.** 1984. Microbial processes affecting chemical transformations in groundwater, p. 89–115. *In* G. Bitton and C. P. Gerba (ed.), Groundwater pollution microbiology. John Wiley & Sons, Inc., New York.
10. **Papanastasiou, A. C., and W. J. Maier.** 1982. Kinetics of biodegradation of 2,4-dichlorophenoxyacetate in the presence of glucose. Biotechnol. Bioeng. **24**:2001–2011.
11. **Rozich, A. F., and R. J. Colvin.** 1986. Effects of glucose on phenol biodegradation by heterogeneous populations. Biotechnol. Bioeng. **18**:965–971.
12. **SAS.** 1985. Version 6, release 6.02. SAS Institute, Inc., Cary, N.C.
13. **Shimp, R. J., and F. K. Pfaender.** 1985. Influence of easily degradable naturally occurring carbon substrates on biodegradation of monosubstituted phenols by aquatic bacteria. Appl. Environ. Microbiol. **49**:394–401.