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Bioremediation of Polychlorinated Biphenyls

Degradation Capabilities in Field Lysimeters

Scientific Note

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ABSTRACT

The degradation of 4-chlorobiphenyl (4CB) was compared in field lysimeters containing 60 Kg of soil contaminated with 5–10 mg/Kg of polychlorinated biphenyls. *Alcaligenes* A5, a bacterium carrying a plasmid for 4CB degradation, was inoculated into three lysimeters. When compared to an untreated control, soil samples from water, mineral, and yeast extract treated lysimeters with and without a bacterial inoculum exhibited greater than 10-fold increases in the rate of [1-^{14}]C-acetate incorporation into lipids and $^{14}CO_2$ production from [U-^{14}C-] 4-chlorobiphenyl. Gene probe analyses for the 4CB plasmid and most-probable-number enumerations demonstrated the presence of biodegradative populations in lysimeters and the probable survival of the added *Alcaligenes* A5.

Index Entries: PCB; polychlorinated; biodegradation; bioremediation; chlorobiphenyl.

INTRODUCTION

Polychlorinated biphenyls (PCBs), biphenyl rings containing 1–10 chlorine atoms, are ubiquitous pollutants that are physically inert and poorly soluble in water. Until recently, it was thought that PCBs were not

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biodegradable. It is now understood that many PCBs containing less than four chlorines per molecule can be degraded biologically (*1–5*), and in some cases mineralized to carbon dioxide by microbial enrichments and pure cultures (*4,5*). Highly substituted PCBs can also be degraded biologically, although probably at slower rates (*6–9*).

Although biological degradation of PCBs could provide a practical and economically feasible technology for the remediation of contaminated soils, this technology has not been successfully demonstrated. Biological treatments may be most favorable for relatively low levels of contamination, perhaps less than 100 mg/Kg, and relatively large quantities of soil, where physical removal and burial become extremely expensive. Long-term field studies examining the degradative capabilities of natural microbial consortia have not been performed, nor have mechanisms for the maintenance of degradative capabilities been examined in field studies. The objectives of this study were to examine PCB degradation potentials and metabolic acivities of microorganisms in field lysimeters containing PCB-contaminated soil and receiving different treatment regimes.

METHODS

Description of the Lysimeters and Experimental Site

A site on the floodplain of Bear Creek, near the junction of Bear Creek Road and Highway 95, near Oak Ridge, TN, was identified as containing greater than 10 mg/Kg of PCBs, with a congener distribution typical of Aroclor 1254 (Monsanto, St. Louis, MO) at depths of 30–45 cm below the surface. This site was chosen as the source of soils and the location of the experimental lysimeters. Six lysimeters were constructed from halves of 210 L (55 gal) stainless steel drums. Each lysimeter contained approximately 60 Kg of soil that was placed on top of a stainless steel screen covered with fiberglass cloth (Fig. 1). Percolated waters were collected in the bottom 10 cm of the lysimeters and were removed through the leachate sampling tubes. Lysimeters were serviced twice weekly July–November 1987.

The lysimeters were treated twice weekly as follows. Lysimeter 1 was a watered control receiving 3 L of water twice each week. Lysimeter 2 was watered and then sealed with a plastic cover. Air was blown over the soil of lysimeter 2 at a rate of 10 mL/min, which then passed through a 5.0 gm Tenax (Alltech Associates, Deerfield, IL) trap to collect volatile PCBs. Lysimeter 3 was stirred and received 5 mg/L yeast extract and minerals. The mineral solution (3 L twice weekly) contained a 2.0 m*M* phosphate, nitrate, ammonium chloride, calcium, and a 0.1 strength trace mineral and vitamin solution (*12*). Lysimeter 4 received stirring, 3 L of mineral solution, and an inoculum of *Alcaligenes* strain A5. The inoculum (0.66 g

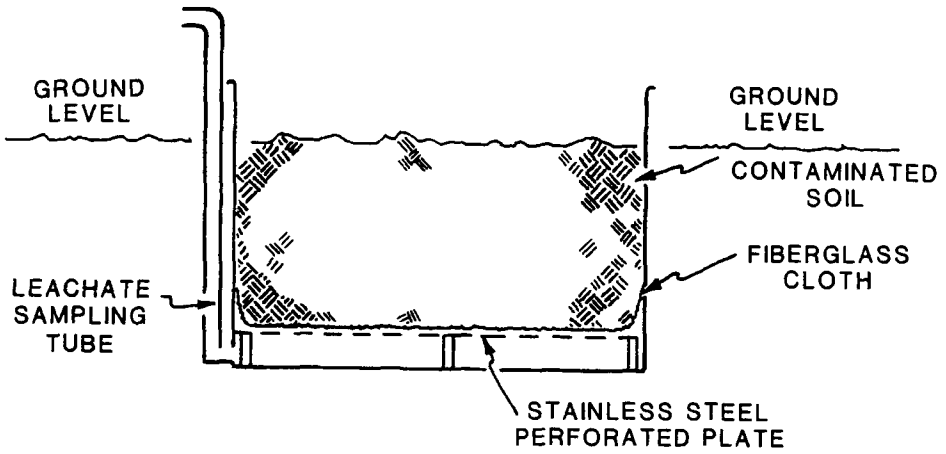

Fig. 1. Cross sectional schematic of a lysimeter. Each lysimeter contained 60 Kg of contaminated soil from the site location and depth.

dry weight equivalents approximately 10^7 cells/g of soil) was added one time to lysimeters 4, 5, and 6. Lysimeter 5 received water, stirring, 3 L of mineral solution, yeast extract and inoculum, whereas lysimeter 6 received the above plus 6.0 mg biphenyl with each treatment. Stirring was done manually using a multipronged hand gardening tool.

Microbial Activity Measures

The rate of [1-^{14}C]acetate incorporation into cellular lipids, a general measure of microbial activity, was determined using radiotracer time course experiments. Acetate incorporation experiments were performed in triplicate using 1.0 g lysimeter soil samples, 1.0 mL sterile deionized water, and 5.0 μCi of [1-^{14}C]acetate (56 mCi/mmol, New England Nuclear Corp., Boston, MA). Time points included 0.0, 0.25, 1, 3, and 24 hours. At each time point triplicate tubes were inhibited with 3.0 mL of a first phase of a Bligh and Dyer (*13*) lipid extraction solution. Microbial lipids were extracted by a modification (*14*) of the single phase chloroform–methanol method of Bligh and Dyer (*13*). The lipid fraction was evaporated to dryness and portions were counted by liquid scintillation counting to determine the amount of radioactivity incorporated into microbial lipids over time.

Soil samples from each lysimeter were monitored for 4-chlorobiphenyl (4CB) and 2,2′,4,4′tetra-chlorobiphenyl mineralization rates. Two grams of soil was placed into 27 mL crimp top tubes (Bellco, Vineland, NJ) containing 2 mL of water and 1.0 μCi of [U-^{14}C]tetra-chlorobiphenyl or [U-^{14}C]4-chlorobiphenyl (7.2 mCi/mmol, Sigma Chemical Co., St. Louis, MO) and sealed with teflon septa (Altech, Deerfield, IL). Time course experiments utilizing six replicate subsamples per lysimeter soil sample consisted of points from 5 min to two mo. Samples from the head space of these tubes

were injected onto a gas chromatograph-gas proportional counter as described previously (*11,15*). The gas chromatograph separated and quantified the total amount of carbon dioxide and the gas proportional counter measured the amount of $^{14}CO_2$ produced from the microbial mineralization of [U-^{14}C] substrate.

Culturing of Microorganisms

Alcaligenes strain A5, which was provided by G. Sayler, University of Tennessee, was cultured in the following medium: in g/L; $NaNO_3$, 4.0; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.01; $FeCl_3 \cdot 6H_2O$, 0.5; biphenyl, 0.1; yeast extract, 0.01; plus minerals and vitamins (*12*). Medium pH was adjusted to 7.0 and incubations were in the dark at 25°C. Concentrations of cell mass for lysimeter inoculation was accomplished using a Pellicon tangential flow filtration system (Millipore, Bedford, MA).

Three-tube most-probable-number (MPN) enumeration series for 4CB degraders were performed using the above medium in screw cap tubes. Incubations were one month at 22°C. Total numbers of colony forming units were determined using 0.1 strength Tryptic Soy Agar or the following medium: in g/L; dextrose, 1.0; peptone, 2.0; yeast extract, 0.2; NH_4NO_3, 0.2; agar, 18.0. Spread plates were incubated aerobically at 20°C for 10 d.

DNA–DNA Colony Hybridization Experiments

Colony forming units from plate count experiments were probed with the pSS50 plasmid (*4*) containing a pathway for the degradation of 4CB. The probe was prepared by nick translation of the purified plasmid using the protocol supplied with the kit (Bethesda Research Laboratories, Gaithersburg, MD). The probe was prepared with [^{32}P]dCTP and after nick translation the labeled probe was separated from the labeled nucleotide using a 3 cm^3 Sephadex G-50 column (*4*). Biotrans (ICN Biomedicals) Nylon 66 membranes were used as hybridization supports and colony lifts were made using the supplied protocol and as described by Shields et al. (*4*).

Kodak X-Omat X-ray film and a single intensifier screen (Fotodyne, Dupont Cronex) were used in the autoradiography of the membranes with the [^{32}P]-labeled plasmid probe. The cassettes were exposed at −70°C for 8–24 h, as described previously (*4*).

Analytical Procedures

Microbial phospholipids were extracted from lysimeter soil samples by a modification (*14*) of the single phase chloroform–methanol method (*13*). The phospholipids were separated on silicic acid columns, subjected to methanolysis (*14*), evaporated to dryness under a stream of N_2 gas, and the methyl esters were stored at −20°C. The esters were analyzed directly by glass capillary-gas liquid chromatography as previously de-

Table 1
Specific Phospholipid Fatty Acid Shifts in Lysimeter Subsamples

Fatty acid PLFAME[a,b]	Lysimeter	Mole % July	Mole % Sept	Mole % Nov	Mole % factor change	Δ pmol PLFAME gdw^{-1}
	1	2.1	3.08	1.46	0.7	26
	3	5.07	3.39	4.25	0.83	664
il5:0 and	4	1.59	5.83	3.33	2.09	544
al5:0	5	0.68	1.14	4.64	6.8	848
	6	<1.5	1.67	2.71	1.8	446
	1	5.34	6.20	3.53	0.66	47
	3	7.54	5.38	6.32	0.83	954
l6:lw7c	4	5.04	6.00	5.44	1.07	789
	5	3.60	3.94	6.99	1.94	1190
	6	2.51	3.65	6.05	2.41	1007
	1	9.01	10.42	8.43	0.93	272
	3	10.44	11.52	9.46	0.9	1473
l6:0	4	9.35	12.14	9.90	1.05	1427
	5	6.60	8.91	9.84	1.49	1611
	6	6.62	8.75	9.47	1.43	1548

[a]Methyl esters of phospholipid fatty acids.
[b]Configurations. i = iso; a = anteiso; w = omega; c = cis.

scribed (*10,14*). Tentative peak identification was based upon relative retention times on polar and nonpolar columns as compared to standards.

RESULTS

Lysimeters 4–6 received an inoculum of *Alcaligenes* strain A5 corresponding to approximately 10^7 bacteria/g of soil, and the effects of this addition were evident in the phospholipid data (Table 1). Phospholipid fatty acid analysis of the *Alcaligenes* species did not reveal unique lipid biomarkers, but three fatty acids each accounted for 25–30% of the total. The three major PLFA were 16:1w7c, 16:0, and 18:1w7c. Although all are common in microbial lipids, the 16-carbon PLFA exhibited substantial increases in lysimeters 5 and 6. Substantial increases in the 16-carbon acids in lysimeters 5 and 6 may have reflected survival of the inoculated strain. Assuming 100 μmol of PLFA/g of cells, survival of *Alcaligenes* A5 without growth would have contributed approximately 350 pmol of 16:0 and 16:1w7c PLFA/g of soil in lysimeters 4–6. These results demonstrated stimulation of microbial biomass in treated lysimeters and that the increases in lysimeters 4–6 were consistent with survival of *Alcaligenes* A5. *Iso* and *anteiso* 15:0, PLFA common in many microorganisms but not

Table 2
Microbial Biomass and Activities from Lysimeter Subsamples[a]

Soil sample data from October	Lysimeter 1	2	3	4	5	6
Colony forming units ($\times 10^6$/gdw)	12	5.5	15	10	0.6	11
MPN for biphenyl degrading microorganisms per gram	$<10^2$	$<10^2$	10^5	10^5	10^6	10^7
Acetate incorporation into cellular lipids (dpm/h×103)	215	145	217	226	236	770
Mineralization of [^{14}C]4-chlorobiphenyl to $^{14}CO_2$ (% at 20 d)	1.8	24	25	22	18	10
Mineralization of [^{14}C]2,2',4,4'tetra-chlorobiphenyl to $^{14}CO_2$ (% at 20 d)	ND	ND	<2	<2	<2	<2

[a]ND = not detected. Experimental design as described in text.

observed in *Alcaligenes* strain A5, also increased in these lysimeters (Table 1). Increased 15-carbon PLFA from these lysimeters were indicative of general microbial stimulation likely resulting from the 660 mg of bacterial biomass and other nutrients added to the lysimeters. The mole percent of each of the three fatty acids shown in Table 1 decreased in the lysimeter that received only water. Lysimeter 3, which received minerals plus yeast extract, exhibited decreased mole percents even though there were substantial increases in the total pmols of the three marker acids.

Other measures of microbial biomass and activity also illustrated differences among the lysimeters (Table 2). Bacterial plate counts in October revealed approximately 10^7 bacteria/g of soil for most lysimeters. The low value for lysimeter 5 agreed with low PLFA for that month and may have reflected differences between monthly subsamples. Microorganisms capable of growing in an MPN medium containing 100 mg/L biphenyl as the sole energy source differed dramatically among lysimeters. The lowest MPN was observed in lysimeters 1 and 2 and the highest were observed in lysimeter 6, which received biphenyl, yeast extract, and an inoculum containing greater than 10^7 biphenyl degraders/g of soil. The predominant organisms in lysimeter 6 were characteristic of *Alcaligenes*. Lysimeter 5 did not receive biphenyl supplements but did exhibit 10^6 biphenyl degraders typical/g of *Alcaligenes*. Lysimeter 3, which received yeast extract

but no inoculum, appeared to stimulate resident microorganisms that could utilize biphenyl as an energy source.

Incorporation of acetate into cellular lipids was lowest in lysimeter 2, a covered control that did not indicate significant volatilization of PCBs, while lysimeters 1, 3, 4, and 5 were similar (Table 2). Lysimeter 6, which received biphenyl additions, exhibited three times as much activity as the other lysimeters.

Lysimeter 6 also exhibited ten times more biphenyl degraders, yet mineralized less 4CB to $^{14}CO_2$. Lysimeter 1 degraded very little 4CB, whereas lysimeter 3 degraded as much 4CB at an equivalent rate as lysimeters receiving inocula (Table 2). High mineralization in lysimeter 2 was attributed to humid and warm conditions caused by the cover, whereas the low rates of lysimeter 6 were possibly due to substrate competition by the biphenyl additions. Regardless, lysimeter 3, which did not receive an inoculum, degraded as much 4CB as did those lysimeters inoculated with a known 4CB degrader. *Alcaligenes* A5 did not degrade 2,2′,4,4′-tetrachlorobiphenyl, nor did resident microorganisms in any of the lysimeters during the course of these studies.

The DNA probe results (Fig. 2), with the exception of data from lysimeter 2, generally corresponded well with the mineralization data and confirmed that PCB degradation activity was stimulated in the lysimeters. The highest number and percentage of colonies which hybridized with the probe were present in lysimeter 4.

Yeast extract which was added to lysimeter 3 appeared to stimulate the extant population of organisms that degraded 4CB (Table 2) and that hybridized with the probe containing the PCB degradation pathway (Fig. 2). There were no positive hybridizations in the sample from lysimeter 2, but samples from this lysimeter did show significant 4CB degradation (Table 2). Interestingly, the MPN for PCB-degrading microorganisms also showed minimal numbers in lysimeter 2 despite the relatively high degradation rate. In this case, the DNA probe data corresponded better to the MPN estimate of the presence of PCB-degrading organisms than with the 4CB degradation rate data. There were no colonies isolated from lysimeter 1 that hybridized with the probe, and lysimeter 1 also had the lowest mineralization of 4CB and the lowest MPN for biphenyl degrading microorganisms.

Lysimeters 5 and 6, which received an inoculum of the bacteria containing the 4CB degradative plasmid, had detectable levels of bacteria that hybridized with the plasmid. This may indicate survival of the inoculated bacteria or hybridization with other microorganisms containing the pathway. The MPN data tend to indicate that the positive hybridizations were probably the result of the survival of the *Alcaligenes* in the soil. The MPN data indicated that lysimeters 5 and 6 had the highest concentrations of biphenyl-degrading microorganisms, but both the DNA probe results and the 4CB mineralization data indicate that the potential for PCB degra-

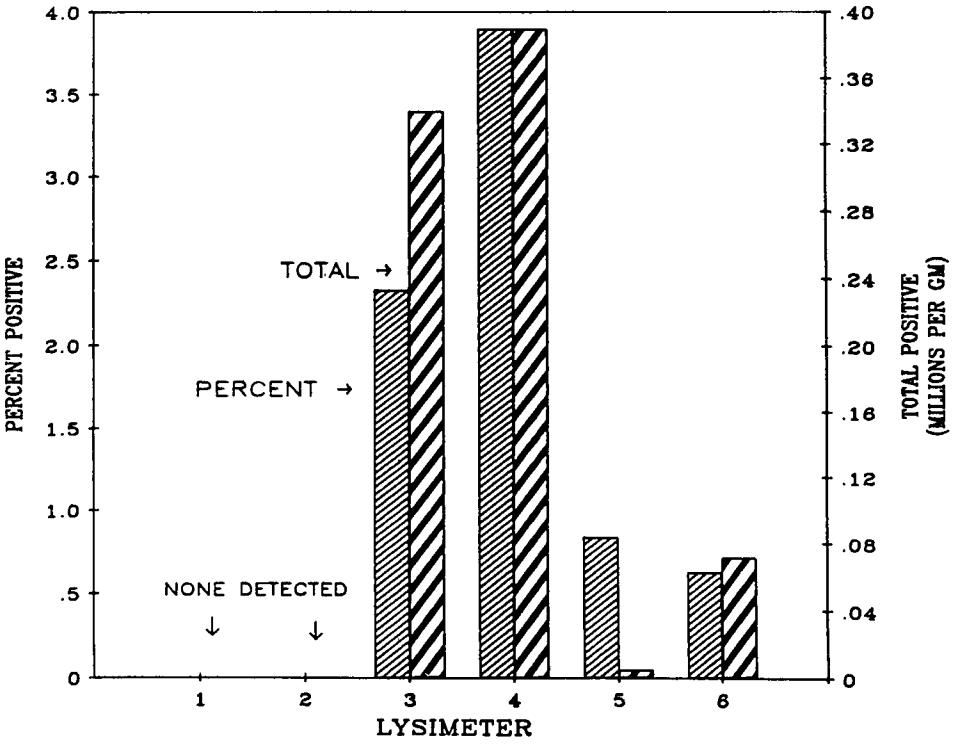

Fig. 2. Results of DNA probe analysis of samples taken from the lysimeters on October 14, 1987. Data is given as percent of the total population that hybridizes with the pSS50 plasmid (narrow hatching) and the total number hybridizing with the plasmid (wide hatching).

dation was lower than in lysimeter 3 that did not receive an inoculum of the *Alcaligenes*.

DISCUSSION

These results demonstrated that the ecology of microbial communities in field lysimeters could be altered through water and nutrient treatments. Inoculated microorganisms appeared to survive, as evidenced by MPNs, phospholipids, biomass, and DNA hybridization studies. General microbial activity and biomass were influenced by water and stirring, by inoculation of a bacterial culture, and biphenyl treatments. Even though the microbial ecology of the lysimeters was shifted, degradation of 4CB was little influenced by inoculation of a known 4CB-degrader. Treatments with water, minerals, and yeast extract stimulated resident microorganisms in noninoculated lysimeters that degraded 4CB as efficiently as inoculated lysimeters.

General microbial biomass and activity measures have often been used to assess shifts in microbial populations (*10,14*), and activities (*15*). Phospholipid analyses and bacterial plate counts both showed that biomass increased in all treated lysimeters during the five-month study. Rates of radiolabeled acetate incorporation into microbial lipids were similar in the control lysimeter to other surface soils (Phelps et al., manuscript submitted). Increased acetate incorporation by lysimeter 6 subsamples revealed stimulation of microbial activities. It is likely that yeast extract supplements plus the 150 mg of biphenyl added during the course of the treatments contributed to the increased anabolic activity. It was not surprising that lysimeter 6 also contained the highest population of microorganisms capable of growing in a medium containing biphenyl as the only energy source.

Survival of microorganisms used in laboratory studies that are inoculated into natural environments is a major concern for microbially mediated *in situ* remediation technologies. Other investigators (*8*) have also reported increased PCB degradative activity by adding bacterial inocula. MPN enumerations suggest the survival of substantial populations of biphenyl degraders in lysimeters receiving inocula. Furthermore, with yeast extract or biphenyl treatments, recovery of biphenyl degraders increased two orders of magnitude. The shift in PLFA was consistent with the survival of *Alcaligenes* A5 inoculated in lysimeters 4–6. Although 16-carbon fatty acids are ubiquitous in many microorganisms, their mole percent decreased in uninoculated lysimeters, increased slightly in the inoculated lysimeter that did not receive yeast extract, and increased substantially in the lysimeters receiving nutrients.

Degradation of 4CB was little influenced by inoculation of a known 4CB degrader. If substrate competition with biphenyl depressed the mineralization of radiolabeled 4CB from lysimeter 6, then it is possible that lysimeter 6 could have exhibited greater mineralization of the total chlorobiphenyl pool. Future experiments may clarify the role of biphenyl stimulation in these lysimeters. Inoculum alone or inoculum plus yeast extract did not result in increased mineralization of 4CB over the stimulation of resident microorganisms. In other experiments (*16*), soils near these lysimeters exhibited 2% mineralization as did lysimeter 1, but soils from an oil land farm exhibited 30% mineralization of 4CB within 20 d. These findings support the concept that resident microorganisms may be enriched for toxicant degradation in some contaminated habitats. Similar findings were observed for trichloroethylene degradation in contaminated subsurface soils (*17*).

Interestingly, addition of water, frequent mixing and nutrient additions appeared successful mechanisms for the stimulation of 4CB degradation in these studies. However, it should be noted that higher congeners and Aroclors did not show significant degradation during the course of this study highlighting the need for considerable research effort to effectively remediate PCB contaminated environments.

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REFERENCES

1. Kipli, S., Himberg, K., Yrjala, K., and Backstrom, V. (1988), *F.E.M.S. Microbiol. Ecol.* **53,** 19–26.
2. Focht, D. D. and Brunner, W. (1985), *Appl. Environ. Microbiol.* **50,** 1058–1063.
3. Rochkind-Dubinsky, M. L., Sayler, G. S., and Blackburn, J. W. (1987), *Microbiological Decomposition of Chlorinated Aromatic Compounds.* ch. 11, Dekker, New York.
4. Shields, M. S., Hooper, S. W., and Sayler, G. S. (1986), *J. Bacteriol.* **163,** 882–889.
5. Kong, H. L. and Sayler, G. S. (1983), *Appl. Environ. Microbiol.* **46,** 667–672.
6. Bedard, D. L., Wagner, R. E., Brenan, M. J., Haberl, M. L., and Brown, J. F. Jr. (1987), *Appl. Environ. Microbiol.* **53,** 1094–1102.
7. Bedard, D. L., Haberl, M. L., May, R. J., and Brenan, M. J. (1987), *Appl. Environ. Microbiol.* **53,** 1103–1112.
8. Brunner, W., Sutherland, F. H., and Focht, D. D. (1985), *J. Environ. Qual.* **14,** 324–328.
9. Waid, J. S. (1986), *PCBs and the Environment,* vols. I and II, CRC Press, Boca Raton, FL.
10. Smith, G. A., Nichols, P. D., and White, D. C. (1986), *F.E.M.S. Microbiol. Ecol.* **38,** 219–231.
11. Nelson, D. R. and Zeikus, J. G. (1974), *Appl. Microbiol.* **28,** 258–261.
12. Lynd, L., Kerby, R., Zeikus, J. G. (1982), *J. Bacteriol.* **149,** 255–263.
13. Bligh, E. G. and Dyer, W. J. (1959), *Can. J. Biochem. Physiol.* **35,** 911–917.
14. White, D. C., Bobbie, R. J., King, J. D., Nickels, J., and Amoe, P. (1979), *Methodology for Biomass Determinations and Microbial Activities in Sediments,* ASTM STP 673, Litchfield, C. D. and Seyfried, P. L., eds., American Society for Testing Materials, pp. 87–103.
15. Phelps, T. J. and Zeikus, J. G. (1985), *Appl. Microbiol.* **50,** 1285–1291.
16. Donaldson, T. L., Strandberg, G. W., McGinnis, C. P., Palumbo, A. V., White, D. C., Hill, D. L., Phelps, T. J., Hadden, C. T., Revis, N. W., Holdsworth, G., and Osborne, T. (1988), Bioremediation of PCB-Contaminated Soil at the Y-12 Plant, ORNL/TM-10750, Martin Marietta Energy Systems, Inc., Oak Ridge, TN.

17. Fliermans, C. B., Phelps, T. J., Ringelberg, D., Mikell, A. T., and White, D. C. (1988), *Appl. Environ. Microbiol.* **54,** 1709–1714.