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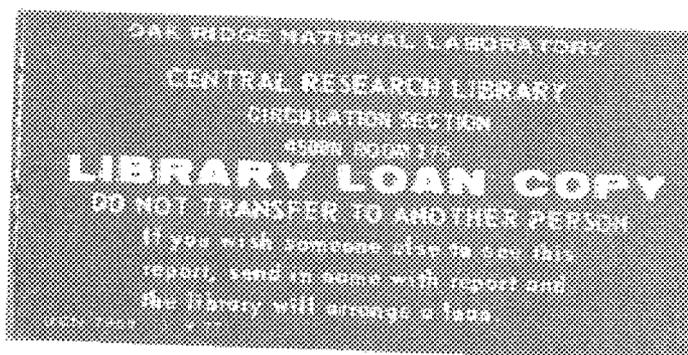
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**OAK RIDGE  
NATIONAL  
LABORATORY**

**MARTIN MARIETTA**

## Bioremediation of PCB-Contaminated Soil at the Y-12 Plant

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BIOREMEDIATION OF PCB-CONTAMINATED SOIL AT THE Y-12 PLANT

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## BIOREMEDIATION OF PCB-CONTAMINATED SOIL AT THE Y-12 PLANT

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### ABSTRACT

The technical feasibility of bioremediation of soils contaminated with polychlorinated biphenyls (PCBs) was investigated using six in-situ lysimeters and two slurry bioreactors during the summer and fall of 1987. Microbial degradation of PCBs was characterized, and microbial cultures were isolated and characterized.

Indigenous microorganisms present in contaminated soils from the floodplain of Bear Creek were shown to be capable of degrading monochlorinated biphenyl. Evidence included production of radiolabeled carbon dioxide from radiolabeled substrate and microbial characterization using gene probes and signature lipid analyses. Evidence was also obtained for dechlorination of highly chlorinated PCBs under laboratory conditions by microbial cultures isolated from other contaminated soils. These results are quite encouraging for further development of bioremediation technologies for PCBs.

In-situ treatment of the soils in lysimeters by aeration/mixing and water appeared to stimulate growth of microorganisms and increase the biodegradation of monochlorinated biphenyl in laboratory experiments using soil samples from the lysimeters. The effects of additional nutrients, carbon source, and inocula were unclear from these tests;

only one condition for each parameter was tested in the limited number of lysimeters.

Microbial inoculation and incubation of the soils appeared to affect the physico-chemical behavior of chlorinated biphenyls in the soil in a manner which inhibited their chemical extraction from the soil. The mechanism (or mechanisms) by which this effect occurred is not known.

Statistically significant degradation of the preexisting PCB contaminants in the soils was not detected in either the field lysimeters or the slurry bioreactors. This result should be interpreted in light of difficulties in obtaining appropriate PCB assays, the limited range of experimental conditions, and the short five-month duration of the study.

This work was conducted by a team of professional and technical staff from the Oak Ridge National Laboratory (ORNL) Chemical Technology and Environmental Sciences Divisions, the University of Tennessee, and the Oak Ridge Research Institute. Funding was obtained through the Hazardous Waste Technology Program from the U.S. Department of Energy/Oak Ridge Operations, the Hazardous Waste Remedial Action Program, and the environmental compliance program at the Y-12 plant. This work is an outgrowth from a recent ORNL Laboratory Director's R&D Fund project dealing with microbial treatment of hazardous wastes.

## BIOREMEDIATION OF PCB-CONTAMINATED SOIL AT THE Y-12 PLANT

### 1. INTRODUCTION

Polychlorinated biphenyls (PCB) contamination is a significant environmental problem on the Oak Ridge Reservation, as it is at many government and industrial installations. Oak Ridge sites include the oil land farm and other sites within the Y-12 burial grounds, portions of the old Z-oil system at Y-12, and at least one location along the floodplain of Bear Creek.

Feasible and practical remediation technologies for PCB contamination depend in part on the concentrations of PCBs. For relatively high concentrations, perhaps several hundred mg/kg or more, and a relatively small quantity of contaminated soil, removal of the soil from the site followed by storage or incineration may be a cost-effective approach. For low levels, perhaps less than 100 mg/kg, and relatively large quantities of soil, physical removal of the contaminated soil becomes extremely expensive. In-situ remediation methods would be much more attractive for these latter situations.

Until recently, it was widely believed that PCBs were not biodegradable. However, it is now known that many of these chlorinated compounds can be broken down biologically, and, in some cases, the carbon can be converted to  $\text{CO}_2$ . To date, no technology for PCB biodegradation has been successfully demonstrated in terms of cleaning up an actual contaminated site. However, research progress in biodegradation of PCBs and PCB-like compounds has been sufficiently positive to lead investigators to believe that effective technologies can be developed. The results of one such study, carried out on the Oak Ridge Reservation, are presented in this report.

### 2. OBJECTIVES

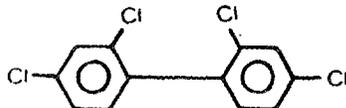
This project was designed to investigate the technical feasibility of bioremediation of PCB-contaminated soils at Y-12. The technical feasibility was addressed in the following ways:

- 1) Evaluation of in-situ bioremediation using lysimeters containing contaminated soil from the floodplain of Bear Creek.
- 2) Evaluation of a slurry bioreactor process using contaminated soil from the floodplain of Bear Creek.
- 3) Recovery and characterization of microbial populations from contaminated sites that could be further used for bioremediation processes.
- 4) Characterization of microbial degradation of PCBs under laboratory conditions.

### 3. BACKGROUND

#### 3.1 CHEMISTRY OF PCBs

Polychlorinated biphenyls are a class of compounds in which a biphenyl structure is augmented by chlorine (Cl) atoms at various positions. For example, 2,4,2',4'-tetrachlorobiphenyl has this structure:



There are other tetrachlors (4 chlorines) in which the chlorines are placed differently around the two rings. Since there are 10 possible positions for chlorine, 209 different compounds (called congeners) can be formed.

Several excellent reviews and news articles on PCBs have been published recently, and the following information is derived primarily from these sources.<sup>1-3</sup> Most pure-component chlorobiphenyls are solids at room temperature. However, commercial mixture preparations are generally resins or viscous liquids of density greater than water. Solubilities of PCBs in water generally decrease with increasing degree of chlorination and vary among different compounds with the same number of chlorines. The solubilities range from about 6 ppm for monochlorobiphenyls to 0.007 ppm for octachlorobiphenyls (8 Cl). Decachlorobiphenyl (10 Cl), despite its high chlorine content, is about twice as soluble as the octachlors.

PCBs have very low vapor pressures which also decrease with increasing chlorination. However, vaporization rates from aqueous solutions are anomalously high in view of the low vapor pressures and high molecular weights. Volatilization is greatly reduced in environments where PCBs are sorbed on soil or sediment surfaces.

PCBs are among the most stable organic compounds known. They have low dielectric constants and high heat capacities, which render them ideal for use in electrical capacitors and transformers. Complex mixtures of PCBs were commercially produced for a variety of uses, including dielectric fluids, printing inks, paints, dedusting agents, and pesticides. The major producer, Monsanto Corporation, marketed PCBs under the trade name Aroclor from 1930 to 1977. Common mixtures were Aroclors 1242, 1254, and 1260. The numbers indicate 42, 54, and 60% chlorine by weight, respectively. Aroclor 1242 is an average trichlor (3 Cl), Aroclor 1254 is an average pentachlor (5 Cl), and Aroclor 1260 is an average hexachlor (6 Cl). Aroclors 1254 and 1260 have been reported to contain over 60 different PCB congeners.

### 3.2 ENVIRONMENTAL CONSIDERATIONS

The chemical and physical stability which is the basis for the many industrial uses of PCBs is also the primary reason that they are a serious environmental problem. Because PCBs do not quickly degrade in the environment and are lipophilic (attracted to biological lipids), they persist in the environment and tend to bioaccumulate. Since the first report of PCBs in eagles, herring, and other aquatic fauna in 1966, these compounds have been shown to be nearly ubiquitous environmental pollutants. They have been found in many human and animal fatty tissues, milk, sediments, and numerous other matrices.

PCBs are apparent carcinogens; that is, increased rates of cancer have been observed in areas contaminated with high concentrations of PCBs. This observation, combined with an increased general environmental concern, prompted a public outcry which culminated in the United States with the regulation of PCBs under the Toxic Substances Control Act. In 1979 the U.S. Environmental Protection Agency (EPA) promulgated a total ban on the manufacture and sale of PCBs.

### 3.3 MICROBIAL TRANSFORMATIONS OF PCBs

Laboratory studies and evidence from contaminated soils and sediments indicate that rates of microbial transformations (roughly meaning biodegradation) are inversely proportional to the degree of chlorination. Monochlors (4-chlorobiphenyl, for example) are degraded relatively easily, and degradation has been observed in the laboratory for some tetrachlors and pentachlors. Analyses of contaminated soils and sediments which have weathered for years have shown that the PCBs still present are primarily pentachlors and higher chlorinated compounds. This suggests that the lower chlorinated compounds probably present in the initial mixtures have degraded over the years of weathering.

Metabolic pathways and intermediates are being actively investigated. Chlorobenzoic acids are commonly found intermediates, which suggests mechanisms involving hydroxylation and splitting of an aromatic ring. Radiolabeled tracer studies with  $^{14}\text{C}$ -PCBs have shown mineralization of some of the carbon to  $\text{CO}_2$  in some cases. Dechlorination is an important step, but is not generally understood. It appears to occur more readily under anaerobic conditions for the higher chlorinated congeners. Production of by-product chloride ions has been observed.

Some bacterial species are capable of growth on chlorinated biphenyls as the sole carbon source, according to published reports. The species include Acinetobacter sp. P6, Achromobacter sp., and Bacillus brevis sp. Selected strains of Alcaligenes sp., Pseudomonas sp., and Nocardia sp. have been shown to degrade PCBs in the presence of other carbon sources. Frequently, pure strains may be capable of certain transformations but unable to degrade the subsequent intermediates. Mixed populations of microorganisms therefore tend to exhibit broader degradative capabilities.

### 3.4 BIOREMEDIATION OF CONTAMINATED SOILS AND SEDIMENTS

A modest number of efforts have been made to remediate contaminated soils biologically. Results have been poor in general; little to no degradation is typically seen over periods of weeks to a year.

Nevertheless, samples of soils and sediments from contaminated sites have shown biodegradation of PCBs under laboratory conditions.

D. Focht (University of California, Riverside) has compared the rate of degradation of PCBs by indigenous soil bacteria with that achieved by inoculated bacteria.<sup>3</sup> A particular strain of Acinetobacter has been found that, although unable to grow on PCBs, can metabolize them when supplied as cometabolism. Focht has been able to demonstrate removal of PCBs from soil under laboratory conditions.

R. Untermann (General Electric) has found a bacterial strain which is capable of degrading a variety of PCB congeners in soil in the laboratory.<sup>3</sup> The U.S. EPA is supporting a test of this microorganism for actual bioremediation of a field site by seeding the site with a heavy inoculum of this bacterium.

#### 4. APPROACH

##### 4.1 STAFF AND FUNDING

The team of investigators was made up of scientists and engineers from:

- ORNL (Oak Ridge National Laboratory) Hazardous Waste Technology Program (C. P. McGinnis)
- ORNL Chemical Technology Division (T. L. Donaldson and G. W. Strandberg)
- ORNL Environmental Sciences Division (A. V. Palumbo and D. M. Genung)
- ORNL/University of Tennessee (UT) Distinguished Scientist Program (D. C. White and UT staff members T. J. Phelps and D. L. Hill)
- Oak Ridge Research Institute (N. W. Revis, C. T. Hadden, G. Holdsworth, and T. Osborne)
- Y-12 Health, Safety, Environment, and Accountability Division (C. W. Kimbrough and L. L. McCauley)

In addition, G. S. Sayler and J. W. Blackburn, both of the University of Tennessee, were consultants for the project. Both have extensive expertise in scientific and engineering aspects of

biodegradation of hazardous chemicals in general and of PCBs in particular.

Funding for the project was provided by Department of Energy-Oak Ridge Operations (DOE-ORO/)/Interim Ops and DOE-Hazardous Waste Remedial Action Program (HAZWRAP), and analytical services and materials were provided by the Y-12 Health, Safety, Environment, and Accountability Division. Arrangements for funding, reporting, and other business details were handled by the ORNL Hazardous Waste Technology Program.

#### 4.2 TECHNICAL OVERVIEW

An overview of the general technical approach is given below, and a chronological summary of the project is given in Table 1. Specific details will follow this overview.

##### 4.2.1 Site Selection for Field Tests

The oil land farm and the Z-oil system were initially considered for the in-situ tests. However, PCB concentrations at the oil land farm are relatively low for the most part,<sup>4</sup> which would make the analytical results more difficult to interpret in terms of real decreases in PCB concentrations. The oil land farm also contains a variety of other oils which might complicate the behavior of the microorganisms and make it more difficult to stimulate PCB degradation. Initial assays for PCBs in the Z-oil system indicated that the concentrations were quite low, so this site would not provide good conditions to measure decreases in PCB concentration, either.

Several years ago a site on the floodplain of Bear Creek, near the junction of Bear Creek Road and Highway 95, was found to have 50-100 mg/kg of PCBs at depths from 30 to 45 cm (12 to 18 in.).<sup>5</sup> This site was chosen for the field tests.

##### 4.2.2 In-situ Tests with Lysimeters

Six lysimeters were constructed, each from half of a 210-L (55-gal) stainless steel drum. These lysimeters were implanted at the contaminated site on the floodplain of Bear Creek and filled with soil from the site. Different conditions were established in each unit, as described later, including inoculation with Alcaligenes A5, a known PCB-degrading microorganism. Soil and leachate samples were taken

Table 1. Calendar of major events

Date (1987)	Site characterization and selection	Lysimeter tests <sup>a</sup>	Slurry bioreactor tests
May	11	Z-oil system sampled	
June	17	PCB analyses rec'd for Z-oil system	
	19	Bear Creek site chosen	
	26	Bear Creek soil sampled	Lysimeters emplaced
	30		Soils in lysimeters sampled
August	8	Oil landfarm sampled	
	17		Soils sampled (R. Turner)
October	12		Bioreactors started Slurries sampled
	14		Soils sampled
November	2		Slurries sampled
	5	Soils sampled	Inoculation with <u>Alcaligenes</u> A5
	19		Slurries sampled
December	3		Slurries sampled
	17		Slurries sampled Operation dis- continued

<sup>a</sup>Lysimeters were aerated and watered, and leachate was removed twice per week.

periodically and assayed for PCBs and bioactivity. From these data the disappearance (potential degradation) of PCBs and the microbiological characteristics of the soils can be determined.

#### 4.2.3 Slurry Bioreactor Tests

Two 120-L (30-gal) stainless steel drums were used to construct a control bioreactor and a test bioreactor. An aqueous slurry of 15% soil from the field site was recirculated, and air was sparged through the slurry. The off-gas was passed through a Tenax-GC trap to capture organic (PCB) vapors. After a start-up period, the test bioreactor was inoculated with Alcaligenes A5. Samples of the slurries were taken periodically and analyzed for PCBs.

#### 4.2.4 Isolation of Microbial Cultures

Soil samples were collected from various contaminated sites, and nutrient enrichment experiments were carried out in the laboratory to stimulate any indigenous PCB-degrading microorganisms that might be present. Sample sites included the old Z-oil system, the oil land farm, archived soil samples at K-25, and, of course, the contaminated area of the Bear Creek floodplain. In addition to tests for PCB-degrading activity, many of these samples were assayed by gene probe technology to look for PCB-degrading genotypes.

### 4.3 METHODS

#### 4.3.1 Analytical Methods for PCB Determination

Analyses of PCBs in field samples were done by the analytical laboratories at Y-12, K-25, and X-10 using packed column gas chromatography (GC) with an electron capture detector. Capillary GC combined with mass spectroscopy was also employed at X-10. Packed column GC provides a rough separation of PCB mixtures and an indication of total PCBs present, but is insufficient for separation and monitoring of individual species. Capillary columns have the high efficiencies required for closely spaced peaks, and are thus useful for tracking individual PCB congeners. Details on the procedures may be obtained from the analytical laboratories.

In certain laboratory samples (as opposed to field samples), PCB concentrations were measured by a high-pressure liquid chromatography

(HPLC) method developed for this project. The method employed a column of Phenomenex C18 (250 X 4.6 mm) containing a stationary phase of Spherex 5, with a C18 precolumn. The mobile phase was 75% (v/v) acetonitrile and 25% water. PCB congeners were detected using a Waters model 440 UV detector set at 254 nm. Capillary GC was also used for some laboratory studies.

#### 4.3.2 Lysimeters

Six lysimeters were constructed. Each consisted of one-half of a 208-L (55-gal) stainless steel drum (Fig. 1). A leachate sampling tube (3/8-in. stainless steel tubing) was welded to a hole at the side and bottom of the drum and was extended upward along the outside of the drum. A piece of 1/4-in. plastic tubing was placed within the stainless steel tubing and extended through the hole in the drum to about the center. The leachate was removed periodically from the bottom via the sampling tube by a battery-driven peristaltic pump.

A perforated stainless steel plate which fit snugly within the drum was supported 5 cm off the bottom by stainless steel blocks. A layer of fiberglass hardware cloth covered the stainless steel perforated plate. The purpose of the plate and cloth was to keep the dirt from the leachate collection zone.

Figure 2 shows the area where the lysimeters were placed. The original sampling data of Ralph Turner, Environmental Sciences Division, are shown in Fig. 3.<sup>5</sup> The lysimeters were placed in the general area of highest PCB concentration. Approximately 30 cm of dirt was removed, and then the next 15 cm was used for the lysimeters and slurry reactors. Approximately 60 kg of soil was placed in each lysimeter.

Two of the lysimeters were used as controls, one of which was covered with plastic film (clear, 6-mil, construction grade) held in place by plastic tape. The headspace over the dirt was sparged continuously at 10 mL/min with air from a tank. A Tenax-GC trap (~5 g, Supelco Co.) was used to collect any volatile PCBs that might be in the off-gas. The remaining four lysimeters were set up with four different treatment regimes, as shown in Table 2. The lysimeters were serviced twice a week. Stirring involved tilling the soil to facilitate aeration. Leachate from the bottom reservoir of each lysimeter was

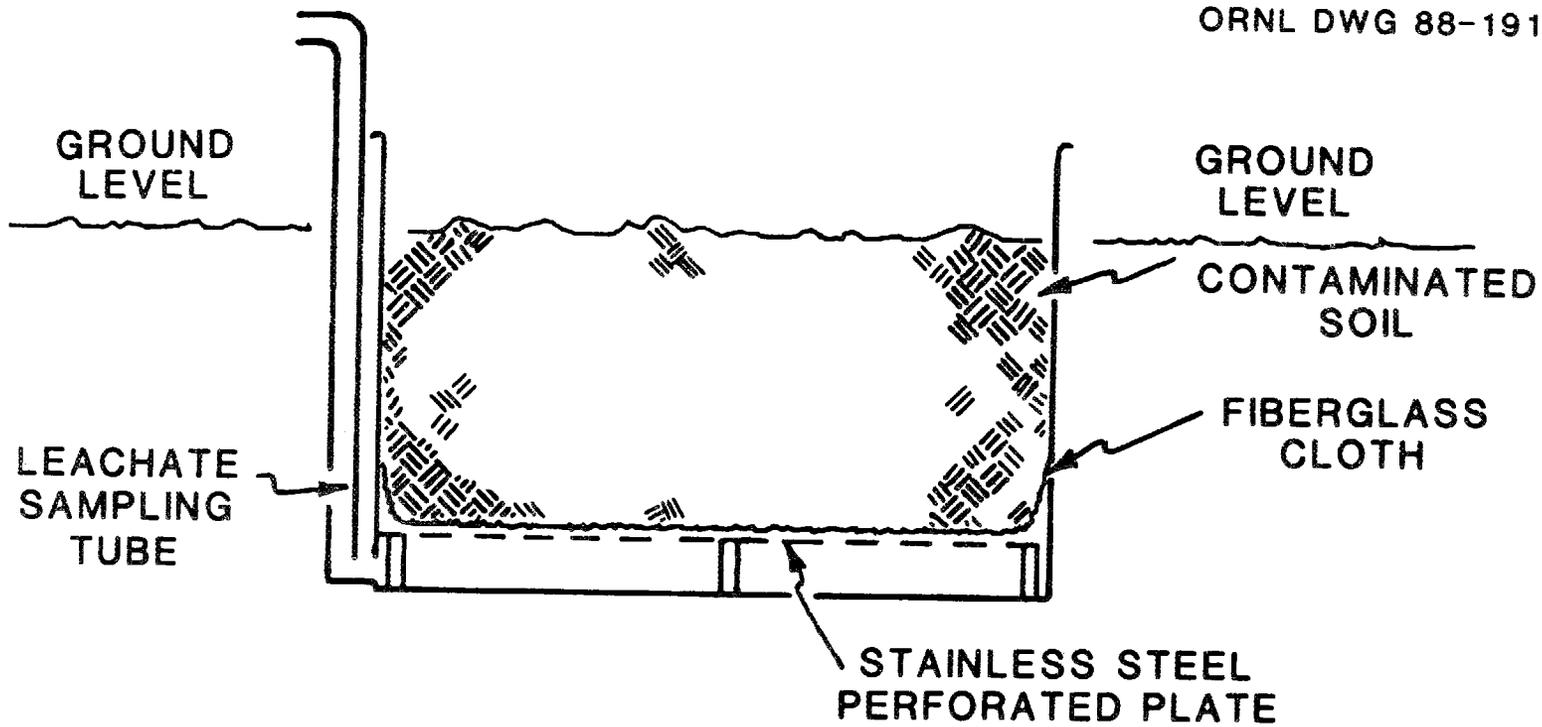


Figure 1. Cross-sectional schematic of lysimeter.

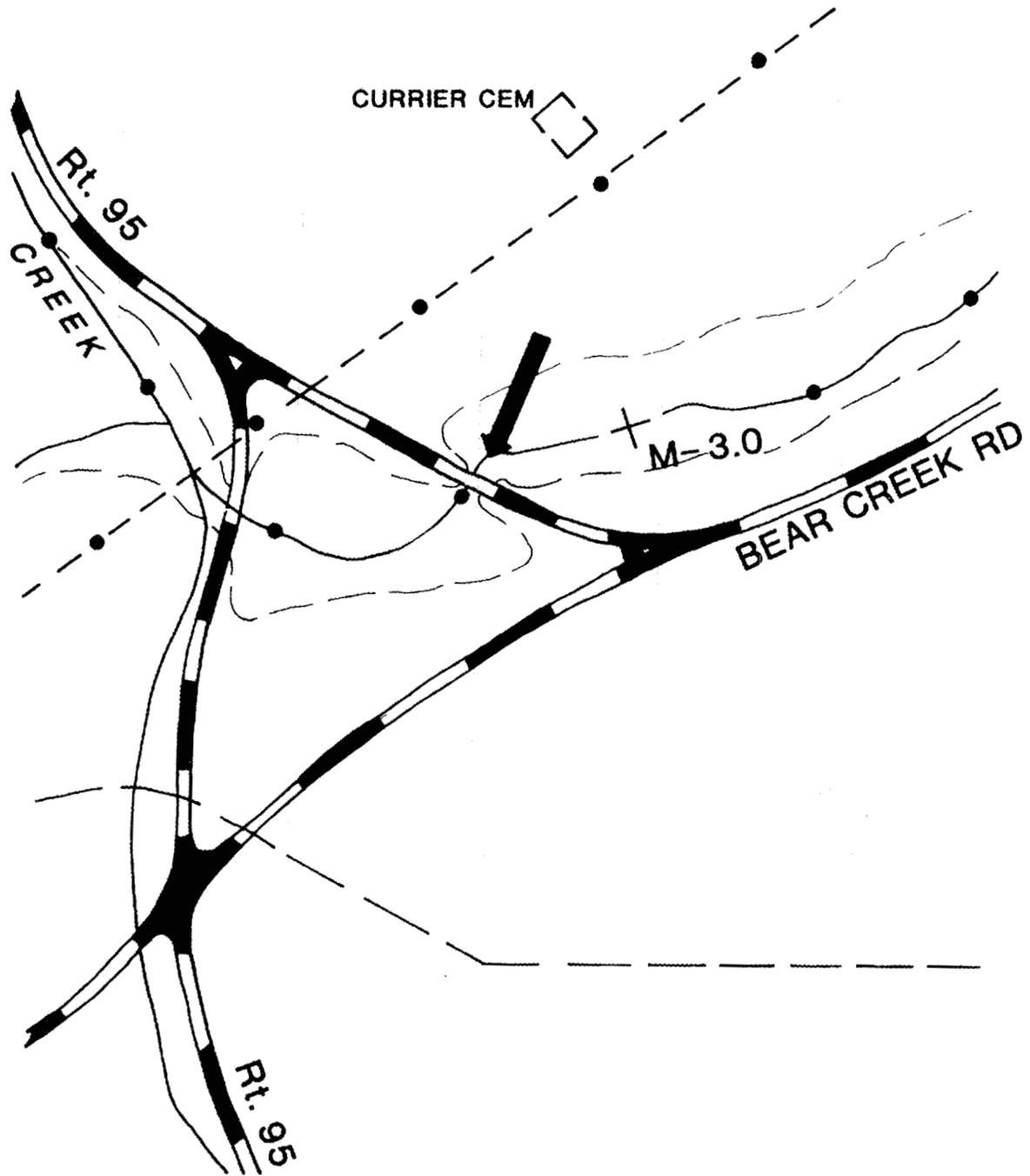


Figure 2. Location of lysimeters test site on Bear Breek floodplain.

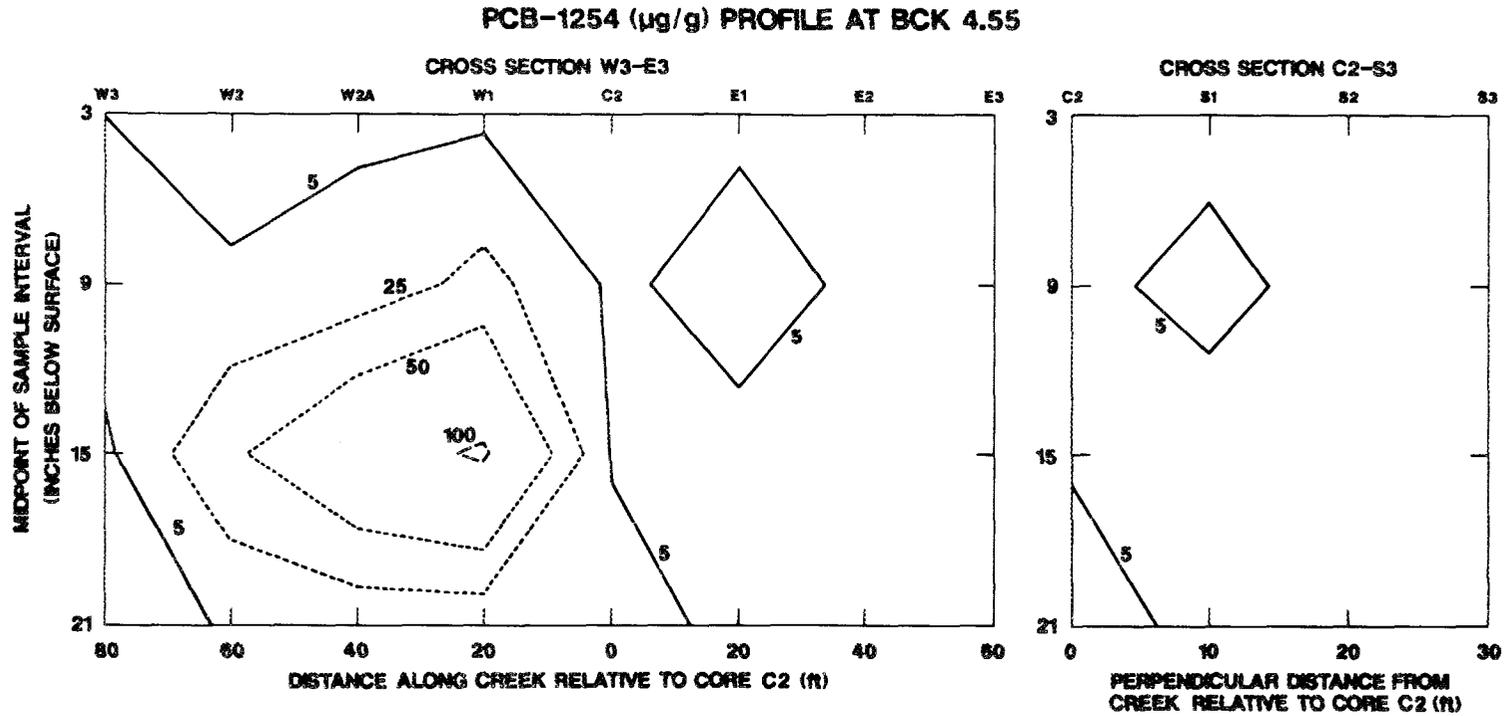


Figure 3. PCB concentrations at lysimeter site from previous characterization study. (Source: R. R. Turner, Distribution of Uranium and Polychlorinated Biphenyls in the Sediment and Floodplain of Bear Creek, Y/TS-mn, Martin Marietta Energy System, Inc., Oak Ridge, Tenn., 1988, in preparation.)

Table 2. Lysimeter treatment schemes

Lysimeter	Treatment <sup>a</sup>
1	(control) watered
2	(control) watered, stirred, sealed, 10 mL/min air blown over top of soil, Tenax trap outlet
3	watered, stirred, minerals, carbon, no inoculum
4	watered, stirred, minerals, inoculum
5	watered, stirred, minerals, carbon, inoculum
6	watered, stirred, minerals, carbon, biphenyl, inoculum

<sup>a</sup>Watered: 1 cm twice a week (3 L). Minerals: 2 mM phosphate, nitrate, ammonium chloride, calcium, plus low levels of trace minerals and vitamins. Carbon: 5 ppm yeast extract. Biphenyl: 2 ppm biphenyl.

pumped out before each addition of water, and a 25-mL subsample was placed in scintillation vials and stored at 2°C.

#### 4.3.3 Slurry Bioreactors

Two slurry reactors were constructed from 114-L (30-gal) stainless steel drums (Fig. 4). Cone-shaped bottoms were welded onto the drums. The dirt and water slurry was recirculated within the reactors using Wilden Model M1, air-driven, positive displacement pumps. The slurry was withdrawn from the bottom and reinjected into the top of the reactors. The recirculation rate was 11-19 L/min (3-5 gpm). Air was introduced near the bottom of the reactors through a porous metal frit at ~0.5 L/min. The off-gas was passed through Tenax-GC traps (5 g) to collect volatile PCBs and degradation products.

On October 12, 1987, two 10-kg lots of dirt from the lysimeter site were slurried, each in ~15 L of process water, and then sieved through a

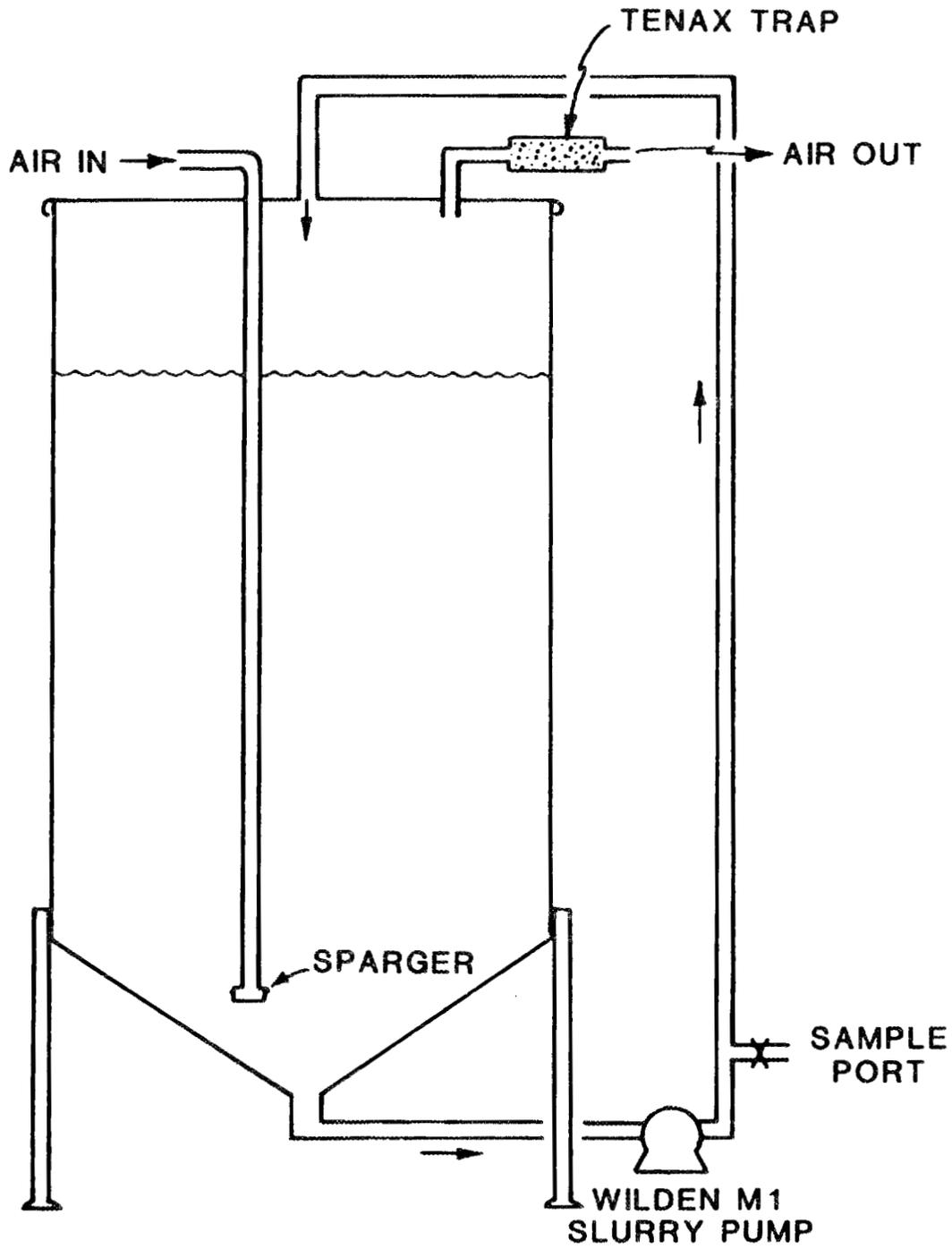


Figure 4. Cross-sectional schematic of slurry bioreactor.

1.2-mm screen to remove large particles. One lot was then added to the control reactor and the other to the test reactor. A mineral salts mixture consisting of 168 mg/L dibasic potassium phosphate, 170 mg/L sodium nitrate, 106 mg/L ammonium chloride, 5 mg/L Difco yeast extract, 0.5 mL/L trace elements, and 0.05 mL/L vitamin solution was added to the test reactor. The volume of the control and test reactors was brought to 75 L with process water.

On the days noted in Table 3, nutrient additions were made to the test reactor to stimulate the growth of PCB-degrading organisms. Also on November 5, 1987, 190 mL of a concentrated cell suspension of Alcaligenes sp. A5 was added to the test reactor. Samples were removed for PCB analysis on October 12 (initial), November 2, November 19, December 3, and December 17. The reactors were shut down on December 17, 1987.

#### 4.3.4 Enrichment Cultures

Enrichment cultures were established using soil from each of the lysimeters along with a mixture of biphenyls. The congeners used were biphenyl, a monochlorobiphenyl, and a dichlorobiphenyl, each at 50 ppm; and a trichlorobiphenyl and two tetrachlorobiphenyls, each at 10 ppm. Lysimeter subsamples and 20 mL of medium were incubated on a gyrotary shaker at 25°C in the dark in the presence of oxygen, oxygen and methane, and oxygen and propane mixtures. The enrichment cultures were later examined using the HPLC system to quantitate decreases in congener concentrations.

PCB-contaminated soil samples previously collected from Y-12 and stored at K-25 were enriched with a medium containing yeast extract, mineral salts, and biphenyl or 3-chlorobenzoic acid under aerobic and anaerobic conditions. After incubation for three to six weeks, samples were subcultured into medium without soil and allowed to grow for 7 d. At 7 d the cells were harvested, washed, and further incubated at room temperature for 4 d in a medium containing Tris buffer (10 mM, pH 7.0), 1% methanol, and PCB Aroclor 1254, a relatively highly chlorinated mixture. After incubation, an aliquot of Triton X-100 was added to the incubation medium and PCBs were extracted using hexane. An aliquot of

Table 3. Slurry bioreactor maintenance (test reactor only)

Date (1987)	Action
10/19	75 mg biphenyl in 8 mL ethanol
10/30	75 mg biphenyl in 8 mL ethanol
11/05	150 mL salt and vitamin solution plus 75 mg biphenyl and <u>Alcaligenes</u> A5
11/09	15 mL salt and vitamin solution and 37.5 mg biphenyl in 5 mL ethanol
11/12	Same as on 11/09
11/16	Same as on 11/09
11/19	Same as on 11/09
11/23	Same as on 11/09
11/30	Same as on 11/09
12/07	Same as on 11/09

the hexane extract was injected into a gas chromatograph and PCB determined by an electron capture detector.

#### 4.3.5 Gene Probe Assays

The presence of PCB-degrading microorganisms was tested using gene probe technology. Single-strand DNA isolated from microorganisms in the soils is mixed with a specially prepared, radiolabeled single-strand DNA that is known to code for PCB degradation. If the single-strand DNAs from these two sources are similar (complementary), they will combine (hybridize) to form double-stranded DNA. This double-stranded DNA is then detected via the radiolabel after the residual single-strand DNA has been washed away. The extent of formation of double-stranded DNA is an indication of the genetic capability of the microorganisms in the soil for degradation of PCBs.

Soil samples were probed with the pSS50 plasmid obtained from Dr. G. Sayler at UT.<sup>6</sup> The plasmid was isolated from a bacterial strain capable of degrading chlorinated biphenyls.<sup>7</sup> The pSS50 plasmid contains a pathway for degradation of 4-chlorobiphenyl (4CB), a monochlorinated biphenyl, and thus colonies that hybridize with this plasmid are likely to contain significant portions of the 4CB degradation pathway.

#### 4.3.6 Viable Cell Counts

The number of viable bacteria (colony-forming units, or CFU) in these soil samples was assayed using plate counts. In this method very dilute suspensions of cells are spread onto a rich growth medium plate of agar gel. After the cells grow up into visible colonies from single cell "seeds," they are counted. Soil samples (1-10 g) were blended with 100 mL of sterile 0.1% sodium pyrophosphate in a Waring blender to produce a well-distributed suspension.<sup>8</sup> Sterile dilutions of the resulting suspensions were prepared in the sterile sodium phosphate, and 0.1-mL fractions of the resulting dilutions were spread-plated in triplicate on the plating media. Both 1/10 tryptic soy agar (Difco) and YEPG agar were used for the initial samples (floodplain and oil land farm samples), and only the soy agar was used for the lysimeter samples. Plates were incubated aerobically at 20 to 25°C. Plates were evaluated by first counting the total number of colonies and then were used in the gene probe assays.

Culture tests for most probable numbers (MPN) of microorganisms were also carried out. Dilution experiments to determine the concentrations of microorganisms (both total numbers and numbers of biphenyl degraders) were done on the soils in each lysimeter. The concentration of the added inoculum (Alcaligenes A5) was determined by an MPN experiment using a minimal medium containing biphenyl as the carbon source. Serial dilutions, in triplicate, were carried out with fresh lysimeter soil subsamples starting with  $10^{-1}$  dilutions and continuing through  $10^{-8}$  dilutions.

#### 4.3.7 Acetate Incorporation into Lipids

The rate of acetate incorporation into cellular lipids is an indication of the amount of biological activity. Higher uptake rates are indicative of greater overall biological activity, which would be expected to correlate positively with increased degradation of PCBs. Acetate incorporation experiments were conducted on lysimeter sediments extracted by a modification<sup>9</sup> of the single phase chloroform-methanol method of Bligh and Dyer.<sup>10</sup> One-gram subsamples from each lysimeter were tested in triplicate, using 1  $\mu$ Ci of  $1-^{14}$ C acetate. Time points of

zero, 15 min, 1 h, 3 h, and 24 h were utilized. The lipid fraction was evaporated to dryness, and portions were counted by liquid to determine the amount of radioactivity incorporated into microbial lipids.

#### 4.3.8 Lipid Signatures

Individual microbial species tend to have unique combinations of lipids in their cell walls. Identification of these lipids helps to identify the particular microorganisms that are present (e.g., PCB degraders). Lipid determinations were conducted on sediments extracted by a modification of the single phase chloroform-methanol method of Bligh and Dyer.<sup>9,10</sup> The neutral lipids, glycolipids, and phospholipids were separated on silicic acid columns, converted to methyl esters of the fatty acids, and analyzed directly by glass capillary GC.

#### 4.3.9 Pure Culture Inocula

Alcaligenes A5 was grown for inoculation into the bioreactors and three of the lysimeters. A5 was obtained on agar plates and transferred to broth cultures using a mineral salts media containing 100 ppm biphenyl and 10 ppm yeast extract (media adjusted to pH 7.0). A 100-mL culture was used to inoculate 3 L of media, which was used in turn to inoculate 60 L of media contained in three fermentors (all grown at 25°C in the dark). The 60 L of cell suspension was concentrated using a Millipore Pellicon filtration system and used as inoculum for the lysimeters. Lysimeters 4, 5, and 6 each received 0.66 g (dry weight equivalent), which is approximately equal to  $10^7$  cells per gram of soil. For the slurry bioreactors, an inoculum composed of about 3 g (dry weight) of cells in 200 mL of buffer was prepared.

#### 4.3.10 PCB Degradation Kinetics

Laboratory experiments were carried out using  $^{14}\text{C}$ -radiolabeled model PCB compounds to evaluate the capability of indigenous microorganisms in the soils for biodegradation. Both radiolabeled 4-chlorobiphenyl (4CB) and a radiolabeled tetrachlorobiphenyl (TCB) were used. These compounds are straightforward to analyze, and production of  $^{14}\text{CO}_2$  is a clear indication of complete degradation.

Soil samples from the Bear Creek site before treatment were distributed into a series of reaction vials and amended with either

mineral salts, 60 ppm glucose, or 10 ppm yeast extract. Either  $^{14}\text{C}$  radiolabeled 4CB or radiolabeled TCB was added to each vial at a concentration of  $1\ \mu\text{g/g}$  soil. Parallel cultures of Alcaligenes A5 were included for comparison, and an autoclaved control was included for both soil and bacteria. After two weeks of incubation at room temperature, the incubations were terminated by the addition of HCl, and the amounts of  $\text{CO}_2$ , volatile PCB, solvent-extractable PCB, and water-soluble PCB were measured. Methods for recovery of these species and counting efficiencies were investigated in depth.<sup>11</sup>

Soil samples from each lysimeter were also tested for radiolabeled 4CB degradation to  $\text{CO}_2$  in time course experiments. Six replicate subsamples per lysimeter were used, with incubation times ranging from five minutes to two months. Samples from the head space of the reaction tubes were injected on a GC-gas proportional counter. The GC gives the total amount of  $\text{CO}_2$  produced in the sample and the gas proportional counter gives the amount of  $^{14}\text{CO}_2$  produced from the radiolabeled 4CB.

#### 4.3.11 Dechlorination of PCBs

Soils or sediments (from New Hope Pond) were added to a medium containing biphenyl or 3-chlorobenzoic acid or thyroxine and incubated for several months at room temperature under aerobic and anaerobic conditions. The cultures were then plated onto agar containing mineral salts and biphenyl, 3-chlorobenzoic acid, or thyroxine. After 7 d, the plates were examined for cell growth. Isolates were recovered and further cultured. For dechlorination experiments, aliquots of these cultures were sonicated to disrupt the cells and then incubated with 10 ppm of EPA standard Aroclor 1254. Appropriate controls were established to assess recovery of PCBs and effects of other organic materials in the culture, including heat-sterilized cells. At time zero and 96 h, samples were taken and PCBs were extracted into hexane. The extract was analyzed for PCBs by capillary GC with an electron capture detector. Dechlorination was determined by comparing the PCB content at time zero and 96 h. Changes in the peak heights for individual congeners were also examined.

## 5. RESULTS

### 5.1 PCB CONCENTRATIONS IN FIELD SITES

Initial analyses of the soil samples taken from the Y-12 Z-oil pumphouse and the area around the storage tank were performed by the analytical laboratory at Y-12. The PCB concentrations in all the samples were 1 ppm or less; this low concentration range was considered unsuitable for a biodegradation study. Therefore, it was decided to focus on a site on the Bear Creek floodplain near the junction of Bear Creek Road and Highway 95 (Fig. 2). The soil in this area had previously been characterized as containing 50-100 ppm of PCBs (estimated to be Aroclors 1254 and 1260) at depths from 30 to 45 cm (12 to 18 in.).<sup>2</sup>

Soil samples were taken from the lysimeters after they had been installed on the Bear Creek floodplain. The analytical results are summarized in Table 4. Results from Y-12 indicated that the total PCB levels in five of the six lysimeters were less than 1 ppm; the sixth contained ~5 ppm. These concentrations are substantially below the 50-100 ppm found in the earlier studies of the site. Splits of these samples were analyzed by the analytical laboratory at K-25. These were found to contain 30-45 ppm of Aroclor 1254. A subsequent sample set, submitted to the X-10 analytical laboratory, showed 2-20 ppm total of Aroclors 1254 and 1260. Both these sets of results conflict with the values of <1 ppm in five lysimeters and 5 ppm in one lysimeter reported by the Y-12 laboratory.

Selected archived soil samples from the Bear Creek floodplain (obtained from archives of Ralph Turner, Environmental Sciences Division) were submitted to the X-10 laboratory for analysis, along with a set of samples spiked with PCB. From these results, it was concluded that the results from the X-10 analytical laboratory were probably the most reliable, and the values of 2-20 ppm of PCB for soils from the Bear Creek floodplain lysimeters were accepted. It appears that the lysimeters were not filled with the most contaminated dirt. The discrepancies among results from individual laboratories is probably due

Table 4. PCB analyses by three laboratories  
(packed column gas chromatography)

Lysimeter	PCB concentrations in soils, mg/kg (Aroclors 1254 and 1260)			
	Y-12 <sup>a</sup>	K-25 <sup>b</sup>	X-10 <sup>c</sup>	
	(total)	(1254)	(1254)	(1260)
1	0.2	27.5	1.0	1.0
2	0.7	31.3	-	-
3	0.3	43.3	5.8	6.0
4	5.6	45.8	3.8	4.2
5	0.4	28.1	11	12
6	0.5	30.7	8.3	6.5

<sup>a</sup>Samples taken June 26, 1987; assayed July 8-14.

<sup>b</sup>Splits of samples from the Y-12 data in this table; submitted by Y-12 to K-25; analyzed July 30-August 21.

<sup>c</sup>New samples taken August 17 by Ralph Turner, Environmental Sciences Division, X-10; analyzed immediately by J. H. Stewart, Analytical Chemistry Division, X-10.

to variations in the sample handling technique, instrumentation, and extraction procedures.

## 5.2 MICROBIAL CHARACTERIZATION STUDIES

Samples were obtained for total viable counts and gene probe studies from various areas in Y-12 and from the soil lysimeters. Eight soil and water samples were taken on May 11, 1987, from the Z-oil storage area (Fig. 5) and the Z-oil pump house. Four samples were taken from the oil land farm on August 8, and samples were taken from two locations on the floodplain (samples 2/1 H and 2/0) on June 26. Samples were taken from the lysimeters on both October 14 and November 5, 1987.

### 5.2.1 Z-Oil Storage Area and Pump House

The soil and water from the Z-oil storage tank area and the Z-oil pump house contained appreciable numbers of viable bacteria. Five soil

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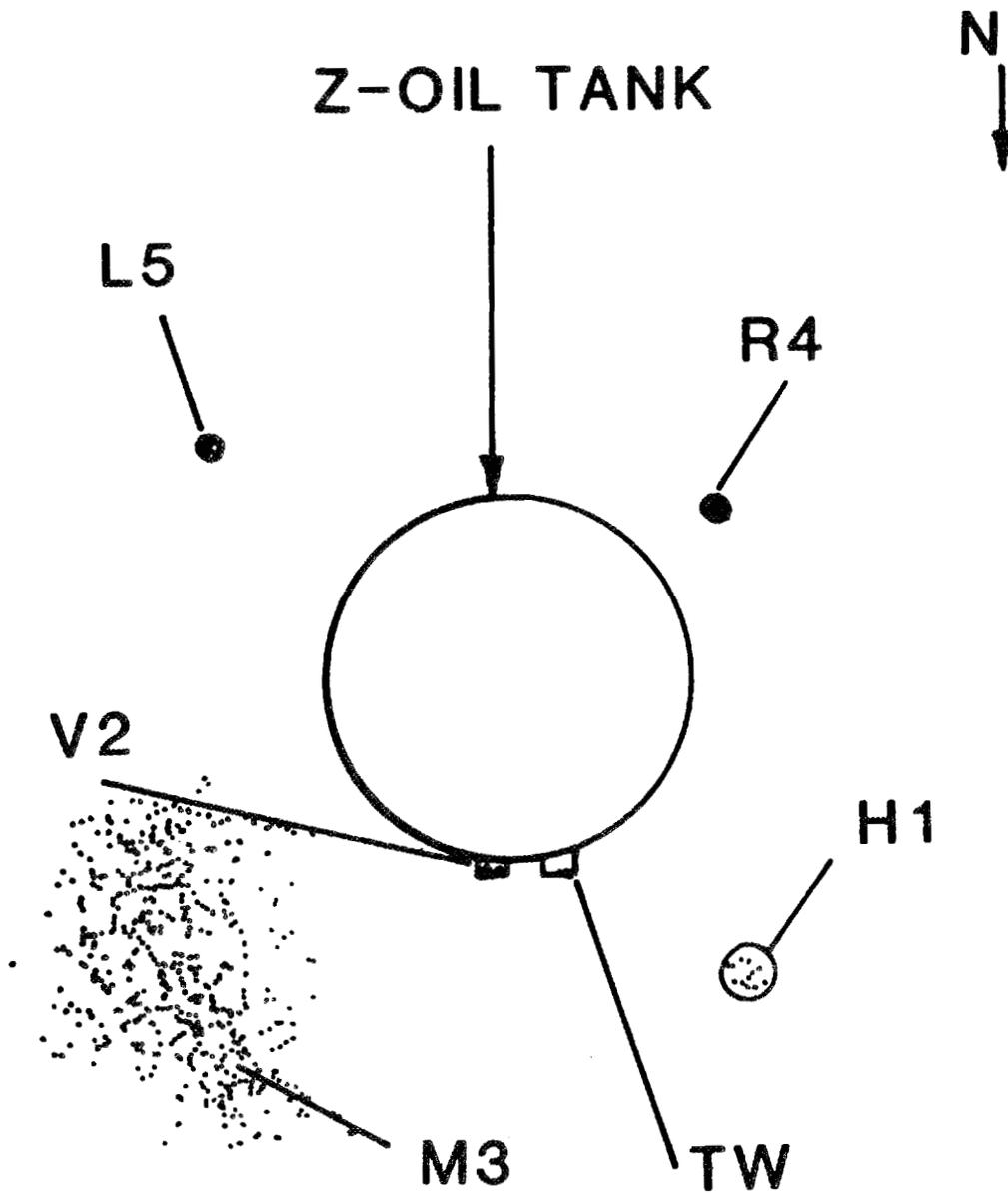


Figure 5. Location of soil samples sites around the Z-oil storage tank. Sample V2 was a water sample; all others were soil and mud samples.

samples were taken from the Z-oil storage tank area (Fig. 5). The viable counts in these samples covered a relatively narrow range—from  $1.5 \times 10^6$  to  $3.8 \times 10^6$  CFU/g. The concentration of bacteria in the one water sample from the valve area was  $5.2 \times 10^4$  CFU/mL. The total viable bacterial count from the mud in the pump house crawl space was  $1.1 \times 10^4$  CFU/mL and was  $1.0 \times 10^5$  CFU/mL in the water in the crawl space.

Colonies which had the 4CB degrading pathway appeared to be virtually absent in the samples from both the Z-oil storage tank area and the pump house. Colonies on 24 plates (three replicates from the same dilution for each of the eight samples) were probed using the pSS50 plasmid, and only two or three possibly positive results were obtained from over 2000 colonies on the 24 plates. This result is consistent with the absence of PCBs at this site, as reported above.

#### 5.2.2 Oil Land Farm

The number of viable bacteria detected in the soil from the oil land farm varied with the distance from the edge of the sample area and was generally low compared to the numbers observed in samples from less contaminated areas (Table 5). Total numbers of viable bacteria detected on the spread plates varied from  $4.8 \times 10^4$  CFU/g at the edge of the land farm to levels below detectability at 1.3 m from the edge.

The gene probe results indicate the existence of microorganisms with genetic 4CB-degrading potential, although the population appears to be small. Bacteria in three of the samples from the oil land farm hybridized with the plasmid containing the 4CB catabolic pathway, and the percentage in one sample was quite high, but the absolute number of colonies hybridizing with the probe was low because of the relatively low population of bacteria observed in these samples (Table 5). The highest percentage of bacteria that hybridized with the plasmid was in the sample taken 2 m from the edge of the land farm. This sample also contained the lowest concentration of viable bacteria (except for the adjacent sample, which had no detectable viable bacteria). In contrast, no colonies which hybridized to the plasmid were detected in the surface sample with the high oil concentration taken 2 m from the edge of the land farm.

Table 5. Gene probe and viable bacteria data for soil samples from the Bear Creek floodplain and the oil land farm

Sample <sup>a</sup>	Hybridization (% positive, mean ± standard error)	CFU (10 <sup>5</sup> /g, mean ± standard error)
FP - 2/1 H	2.94 ± 1.24	19.0 ± 0.60
FP - 2/0	4.82 ± 2.42	20.6 ± 1.99
OLF edge	1.67 ± 1.67	4.80 ± 0.81
OLF 2 ft	0.90 ± 0.90	0.54 ± 0.05
OLF 4 ft	(not done)	(none detected)
OLF 6 ft	16.8 ± 5.86	0.49 ± 0.03
OLF surface	(none detected)	1.96 ± 0.40

<sup>a</sup>FP = floodplain; OLF = oil land farm.

### 5.2.3 Lysimeters

The lysimeter experiments were started on June 26, 1987. Subsequent soil samples for microbial characterization were taken after 15 weeks (October 14) and 19 weeks (November 5). Total numbers of viable bacteria (Table 6) were more variable on the second sampling date (November 5) than on the first (October 14) and appeared to be related to the treatment of the lysimeters. On the first sampling date, very high levels of bacteria, in the range of 10<sup>7</sup> CFU/g, were in the samples taken from all of the lysimeters. This may be related to the disturbance of the soil required to set up the lysimeters. This hypothesis is consistent with the results of the analysis of the undisturbed floodplain samples, which had only 2 x 10<sup>6</sup> CFU/g (Table 5).

The variability among the lysimeters was greater on the second sampling date, and the number of viable organisms appeared to be related to the specific treatment of the lysimeter. The greatest reduction in the number of viable bacteria between the two sampling dates was observed in the lysimeters with the least amount of treatment (1 and 2), and the final concentration of bacteria was approximately equal to the

Table 6. Gene probe and viable bacteria data for soil samples from the lysimeters

Lysimeter	October 14, 1987		November 5, 1987	
	Hybridization (% positive $\pm$ standard error)	CFU ( $10^6$ /g)	Hybridization (% positive $\pm$ standard error)	CFU ( $10^6$ /g)
1	ND <sup>a</sup>	11.9	ND	1.47
2	ND	5.5	0.29 $\pm$ 0.29	1.18
3	2.33 $\pm$ 1.02	14.7	0.92 $\pm$ 0.50	19.0
4	3.90 $\pm$ 1.87	10.0	1.87 $\pm$ 0.38	8.6
5	0.84 $\pm$ 0.44	0.64	ND	5.6
6	0.63 $\pm$ 0.33	11.5	0.57 $\pm$ 0.57	6.1

<sup>a</sup>None detected.

number originally observed in the floodplain samples. The least reduction in total viable counts was observed in lysimeters 3 and 4.

The number of colonies isolated from the lysimeters which reacted with the plasmid varied according to the treatments. Low levels of colonies hybridizing with the pSS50 plasmid were generally found in the samples from the lysimeters, ranging from 0 to 3.9%. However, the patterns observed appeared to correlate with degradation capability (discussed later). On both sampling dates, lysimeters 3 and 4 had the highest number of colonies hybridizing with the pSS50 plasmid containing the 4CB degradation pathway (Table 6). The lysimeters with minimal treatment (1, which had no treatment, and 2, which was stirred) had the lowest number of positive results. No positive colonies were detected on either sample date for lysimeter 1, and only one positive colony was detected on one plate in the second lysimeter. Colonies hybridizing with the plasmid were detected in samples from the remaining lysimeters. Three of the final four lysimeters had positive results on both sample dates.

There was a general decrease in the number of colonies hybridizing with the probe from the first to the second sampling dates. In all four of the lysimeters that tested positive on the first sampling date, the percentage of the colonies which hybridized with the plasmid was lower on the second sampling date. Of the lysimeters in which colonies hybridizing with the plasmid were detected on the first sampling date, only in lysimeter 5 (which did receive additions of Alcaligenes A5 and yeast extract) were no colonies found hybridizing with the plasmid on the second sampling date.

### 5.3 PCB DEGRADATION, MINERALIZATION, AND DECHLORINATION

#### 5.3.1 Lysimeters

Samples of soil and leachate from each lysimeter were collected at least monthly and preserved for further analysis. Because of the difficulties in obtaining meaningful PCB analyses for our purposes, all of these samples have not been analyzed. The results of those samples that have been analyzed by the standard EPA method are summarized in Table 7. Based on the gross PCB concentrations obtained by packed column GC (J. H. Stewart, Analytical Chemistry Division, X-10), it appears that little (if any) PCB degradation occurred in either lysimeter 5 or 6. Samples from these two lysimeters were chosen for assay because other evidence from laboratory experiments suggested that the bioactivity level was highest in these two lysimeters. It herefore seems probable (but not demonstrated) that little if any degradation occurred in the other lysimeters as well. Additional perspectives on these results will be discussed later in this report.

Capillary GC was also used to analyze these same samples (J. E. Caton, Analytical Chemistry Division, X-10). A rough comparison of the individual peaks corresponding to individual congeners also indicated that little if any degradation occurred throughout July, August, and September. A selected group of samples was analyzed (M. V. Buchanan, Analytical Chemistry Division, X-10) using capillary GC and negative ion mass spectroscopy to see if loss of any particular congener could be seen. The results indicated that the PCBs in these soils are relatively highly chlorinated. This may reflect either the particular PCB mixtures

Table 7. PCB concentrations in lysimeter soils

Lysimeter	June 26 <sup>a</sup>		July <sup>b</sup>		August <sup>b</sup>		August 17 <sup>c</sup>		September <sup>b</sup>	
	1254	1260	1254	1260	1254	1260	1254	1260	1254	1260
5	6.2	4.3	6.2	6.1	3.7	3.2	11	12	8.1	6.6
6	and 4.9	and 4.2	7.0	6.5	5.3	4.3	8.3	8.0	7.8	6.5

<sup>a</sup>Two samples from original floodplain soil saved for slurry bioreactor experiments (not from lysimeters), analyzed by J. H. Stewart.

<sup>b</sup>J. H. Stewart, Analytical Chemistry Division, X-10, September 29, 1987.

<sup>c</sup>J. H. Stewart, August 17-24, 1987 (from Table 4).

that were deposited on the floodplain or biodegradation of the less chlorinated congeners over the years since the PCBs were deposited. Evidence for changes in the relative concentrations of individual congeners was inconclusive.

### 5.3.2 Slurry Bioreactors

Results from assays of selected slurry samples from the bioreactors are shown in Table 8. The peak areas from the capillary GC trace have been normalized by the peak with a retention time of 38.5 min (arbitrary choice of a relatively large peak). The peaks featured in Table 8 are those with retention times which correspond to known PCB peaks. Decreases in the normalized peak areas can be seen for several of the early peaks (presumably less chlorinated species) in both the control and test bioreactors. Increases in some normalized peak areas are also seen. The significance of these results is unclear to date. There is no proof that these peaks are PCBs other than the retention times. Again, a selected group of samples was assayed using capillary GC and negative ion mass spectroscopy to see if loss of any particular congener could be seen. The results were inconclusive. Samples of the initial soil placed in the slurry bioreactors have not yet been analyzed satisfactorily by capillary GC, so no comparison with initial conditions is yet possible.

### 5.3.3 Mineralization of 4-Chlorobiphenyl

Laboratory experiments were done at the Oak Ridge Research Institute with composited soil samples and with soil samples from individual lysimeters, each amended with 1 ppm of radiolabeled 4CB. Only about 1% mineralization (conversion to CO<sub>2</sub>) of substrate was observed in experiments with composited soil samples. Amendment of the soil with mineral salts or yeast extract had no significant effect on mineralization over a 14-d period. Addition of glucose increased mineralization of 4CB by about 30%. Cultures of Alcaligenes A5 in mineral salts showed less than 0.03% mineralization of 4CB when this compound was the only carbon source. Addition of yeast extract increased mineralization of 4CB slightly, whereas addition of glucose increased the mineralization of 4CB by about tenfold. This result may

Table 8. PCB assays of slurry samples from bioreactors  
(capillary gas chromatography)<sup>a</sup>

Retention time (min)	Aroclor <sup>b</sup>		Normalized peak area			
			Control bioreactor		Test bioreactor	
	1254	1260	Nov. 2	Nov. 19	Nov. 2	Nov. 19
18.95		+	102	5	8	3
22.29	+	+	18	9	12	9
22.5	+	+	40	22	30	22
23.67-.77	+		0	12	16	10
24.17-.2	+	+	45	29	39	28
24.55	+		16	11	15	10
25.7	+		0	8	11	7
26.07-.1	+		5	20	26	19
26.39-.41	+		11	7	9	7
26.82	+	+	55	39	51	38
27.75	+	+	28	29	36	28
28.1	+	+	13	15	17	15
28.67	+	+	44	32	38	30
28.85	+		64	49	60	46
30.16	+	+	23	19	21	17
30.64	+	+	144	125	150	123
30.9	+		0	14	16	13
31.6	+	+	58	46	54	44
32.8	+	+	78	98	105	97
33.5		+	15	14	15	13
34.2	++	+	70	61	63	58
34.64	+	+	18	16	12	20
35.0	+	+	12	9	11	10
36.1	+	+	50	43	44	41
36.55	+	+	5	25	27	24
38.5	+	++	100	100	100	100
40.9	+	+	42	45	44	46
41.66		+	24	22	21	20
42.12	+	+	27	25	24	25
46.46		+	13	2	14	16

<sup>a</sup>J. E. Caton, Analytical Chemistry Division, X-10, analyzed December 7, 1987; peak normalization by G. W. Strandberg.

<sup>b</sup>+ means peak present in standard for this Aroclor; ++ means peak is a major identifier for this Aroclor.

reflect an increase in biomass rather than a glucose-stimulated increase in the degradation rate.

Results of mineralization experiments with lysimeter soil samples showed that little activity was observed in lysimeters 1-5 (less than 2% mineralization of 4CB over 14 d), whereas 5-6% of the 4CB added to samples from lysimeter 6 was mineralized. Repeated assessments indicated that mineralization was greater with soil samples from lysimeter 6 than from lysimeters 1, 3, 4, and 5. However, the extent of mineralization was highly variable in replicates, ranging from 2 to 13% of the radiolabeled 4CB.

Soil samples were taken from the lysimeters in late September, amended with radiolabeled 4CB, and incubated in submerged culture form (slurry). The extent of the radiolabel converted to CO<sub>2</sub> is shown in Table 9. It can be readily seen that mineralization (conversion of carbon to CO<sub>2</sub>) was significantly more extensive in the samples from the manipulated lysimeters (2-6) than from the control. Moreover, the extent of mineralization appears to have reached a maximum after about two weeks. The reason may be simply a depletion of radiolabeled substrate. During biodegradation, carbon will have other fates in addition to CO<sub>2</sub>. The addition of Alcaligenes inoculum did not seem to have significantly improved the mineralization in samples from lysimeters 4-6 over lysimeters 2 and 3, which contained only indigenous microorganisms. The somewhat lower mineralization seen in the samples from lysimeter 6 is believed to be due to competition from added biphenyl substrate. This interpretation is consistent with acetate uptake results reported below.

An additional consideration is the fact that lysimeter 2 was covered with transparent plastic, which probably contributed to higher temperature and humidity than in the uncovered lysimeters. These conditions may have contributed to the relatively good mineralization of 4-chlorobiphenyl, shown in Table 9, by way of stimulating microbial activity in general.

Soil samples from several locations on the oil land farm were also amended with radiolabeled 4CB. The mineralization to CO<sub>2</sub> is summarized in Table 10. These soil samples appear to have comparable indigenous

Table 9. Biodegradation of  $^{14}\text{C}$ -radiolabeled 4CB (a monochlorinated biphenyl) by soil samples from lysimeters

Percentage of radiolabel converted to $^{14}\text{CO}_2$ under various lysimeter conditions						
Elapsed time, d	Control	Aerated	Aerated; nutrients	Inoculated with <u>Alcaligenes</u> ; aerated; nutrients		
	1	2	3	4	5	6
1,2			<1% in all cases			
3	1	1	2	3	2	2
5	1	4	6	6	4	4
9	3	11	13	16	15	6
12	2	15	16	15	14	9
17	2	19	25	23	16	10
20	2	21	23	21	16	10
27	2	24	25	22	18	10

bioactivity to the soils from the Bear Creek floodplain. These results suggest that bioremediation using indigenous microorganisms may be feasible at the land farm. (However, degradation of actual PCBs at the site has not yet been demonstrated.)

It can be seen that mineralization of radiolabeled 4CB differed substantially in experiments with humid soils compared to experiments with soils in submerged culture. The submerged (slurry) experiments consistently gave greater mineralization for all soils. The probable explanation is that in submerged culture the availability of 4CB to the microorganisms was greater than in humid soil samples, where adequate distribution of a small amount of radiolabeled 4CB is difficult to achieve.

#### 5.3.4 Mineralization of a Tetrachlorobiphenyl

Similar experiments were done with lysimeter soil samples amended with a radiolabeled tetrachlorobiphenyl (TCB). Although a small amount of radiolabeled  $\text{CO}_2$  was recovered, the amount was insufficient to claim

Table 10. Mineralization of radiolabeled 4CB by soil samples from the Y-12 oil land farm

Elapsed time, d	Percentage of radiolabel converted to $^{14}\text{CO}_2$
5	15
17	14

that biomineralization was occurring. These studies need more attention in the future to develop and verify biomineralization of TCB.

#### 5.3.5 Uptake of Radiolabeled Acetate

Soil from lysimeter 6 showed the highest rate of uptake of radiolabeled acetate, as seen in Table 11. This result is believed to be due to the extra carbon source added to lysimeter 6 in the form of biphenyl. This additional substrate probably stimulated the growth of more microorganisms in this lysimeter, which showed up as greater overall microbial metabolic activity in terms of uptake of acetate substrate. These results generally are in agreement with the levels of 4CB mineralization reported in Table 9 and with the MPN results also shown in Table 11. The combination of very high acetate uptake and modest 4CB mineralization in the soil from lysimeter 6 suggests that the biphenyl substrate may have been outcompeting the 4CB substrate. More work is needed to determine the right amount of biphenyl for stimulation of bioactivity while still allowing biodegradation of 4CB and PCBs. Alternatively, it may be argued that addition of biphenyl is not needed in these soils.

#### 5.3.6 Lipid Analysis of Microbial Communities

Table 12 shows the lipid analysis results. The phospholipid fatty acids (PLFAs) showed a small increase in lysimeter 1 while all others showed a larger increase. All of the treated lysimeters showed an increase in i15:0 and a15:0 (PLFAs common to microbial lipids). This increase was stronger with added yeast extract or inoculum (the inoculum

Table 11. Characterization of microbial populations in soil samples from lysimeters and oil land farm: Uptake of  $^{14}\text{C}$ -acetate and most probable numbers of biphenyl degraders

Test	Lysimeter						Oil land farm
	1	2	3	4	5	6	
$^{14}\text{C}$ uptake after 1 d, 1000 dpm/h							
September samples	159	104	160	83	127	775	450
October samples	215	145	217	226	236	770	
Most probable number of biphenyl degraders, cells/g soil	$<10^2$	$<10^2$	$1.5 \times 10^5$	$2.8 \times 10^5$	$2.8 \times 10^6$	$2.1 \times 10^7$	

Table 12. Specific phospholipid fatty acids in lysimeter subsamples

Fatty acid <sup>a</sup>	Lysimeter	Mol %		
		July	Sept.	Nov.
i15:0 and a15:0	1	2.1	3.08	1.46
	3	5.07	3.39	4.25
	4	1.59	5.83	3.33
	5	0.68	1.14	4.64
	6	<1.5	1.67	2.71
	16:1w7c	1	5.34	6.20
3		7.54	5.38	6.32
4		5.04	6.00	5.44
5		3.60	3.94	6.99
6		2.51	3.65	6.05
16:0		1	9.01	10.42
	3	10.44	11.52	9.46
	4	9.35	12.14	9.90
	5	6.60	8.91	9.84
	6	6.62	8.75	9.47

<sup>a</sup>Configurations: i = iso; a = anteiso; w = omega; c = cis.

in lysimeter 4 could serve a function similar to yeast extract in stimulating other populations). There is a shift in the community towards PLFAs which resemble Alcaligenes A5 (16:1w7c and 16:0) in those lysimeters with yeast extract and inoculum (4-6). The treated lysimeters showed significant increases in the patterns of two of the four common PFLAs of Alcaligenes A5, whereas lysimeter 1 decreased. This suggests shifts in the microbial community structure whereby lipids common to the degrader increased with inoculum and yeast extract and decreased in the control.

The pure culture of Alcaligenes A5 had four PLFAs yielding approximately 80% of total phospholipids. All four are common to microbial lipids. One unique, unidentified PLFA was observed in pure culture but was below detectable limits in all lysimeters. This is

understandable in that the inoculum provided less than 1% of the community, and the unique marker constituted only 0.3% of the Alcaligenes A5 lipids.

Trends in the total biomass populations in the lysimeters over the course of the study, as evidenced by PLFA analyses, are shown in Fig. 6. As would be expected, the biomass levels increased in response to favorable treatment to the soils. The addition of water alone to the lysimeter 1 (the control) was probably sufficient to stimulate increased biomass.

#### 5.3.7 Extractive Recovery of 4CB and PCBs from Soils

Since extraction of the PCBs from the soil sample is necessary before analysis by GC and mass spectroscopy, it is desirable that this extraction be as complete as possible. Several tests in which radiolabeled 4CB was added to soil samples provided an opportunity to evaluate extraction procedures.<sup>11</sup>

Figure 7 shows the recovery of radiolabeled 4CB in an HCl extract, vapor phase, and adsorbed to surfaces. While the total recovery is short of 100%, it is noticeably lower in the samples from the lysimeters that were inoculated with Alcaligenes (lysimeters 4, 5, and 6). This behavior suggests that the presence of the inoculated microorganisms has done something to the soil that inhibits chemical extraction of radiolabeled species.

Similar experiments with a radiolabeled tetrachlorobiphenyl (TCB) produced similar results. In this case, a hydrochloric acid (HCl) and a formic acid extraction were compared (Fig. 8). Recovery of radiolabel was higher for soils from lysimeters 1 and 3 using HCl than for soils from lysimeters 4, 5, and 6. When a formic acid extraction was tested, total recovery was higher in all cases and approximately uniform for samples from all lysimeters. These results indicate that the radiolabel not recovered by the HCl extraction is still present in the samples (as evidenced by increased recovery by the formic acid extraction). The variation in extractability between the inoculated and noninoculated lysimeters was shown to be reproducible.

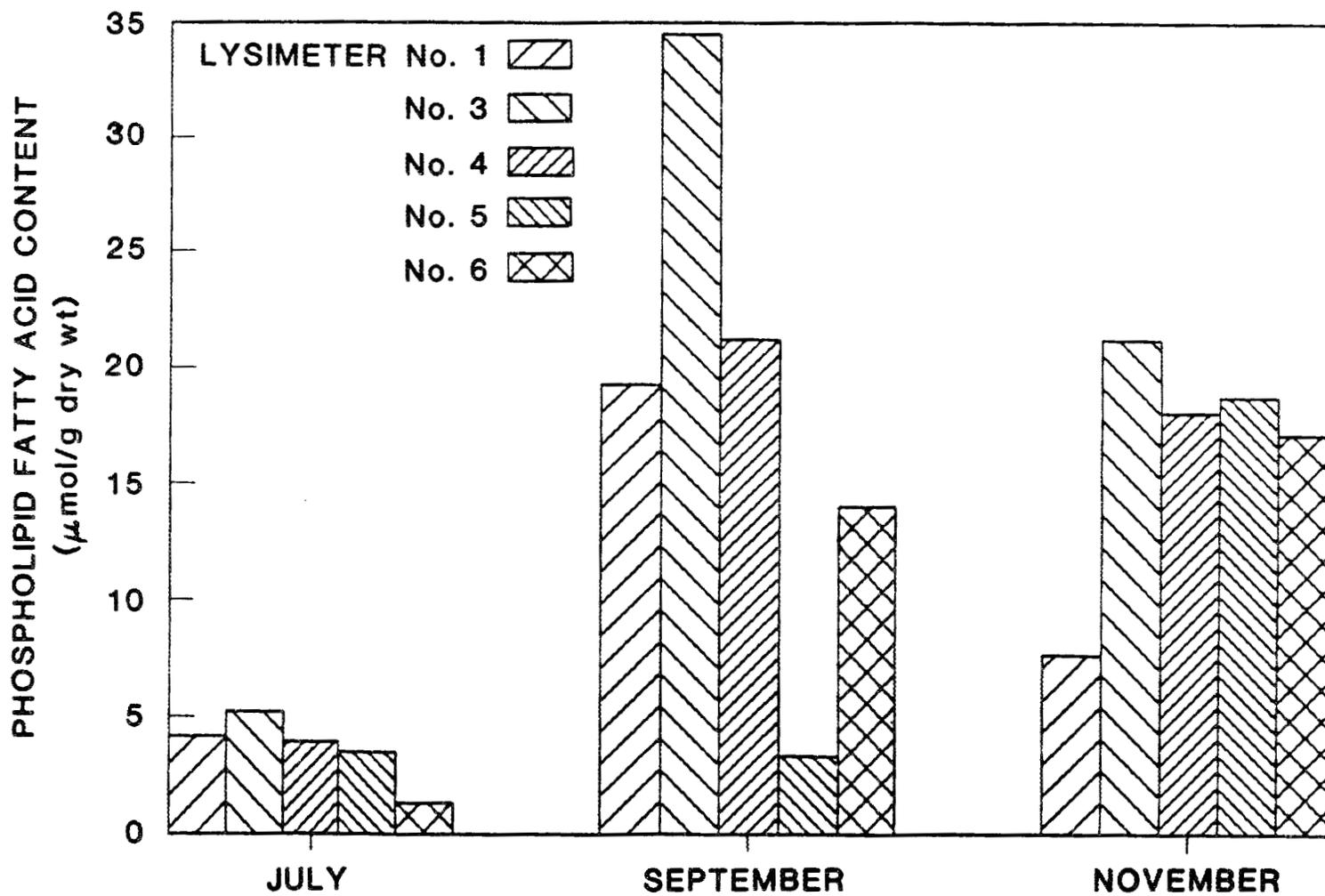


Figure 6. Microbial biomass in soil samples from lysimeters.

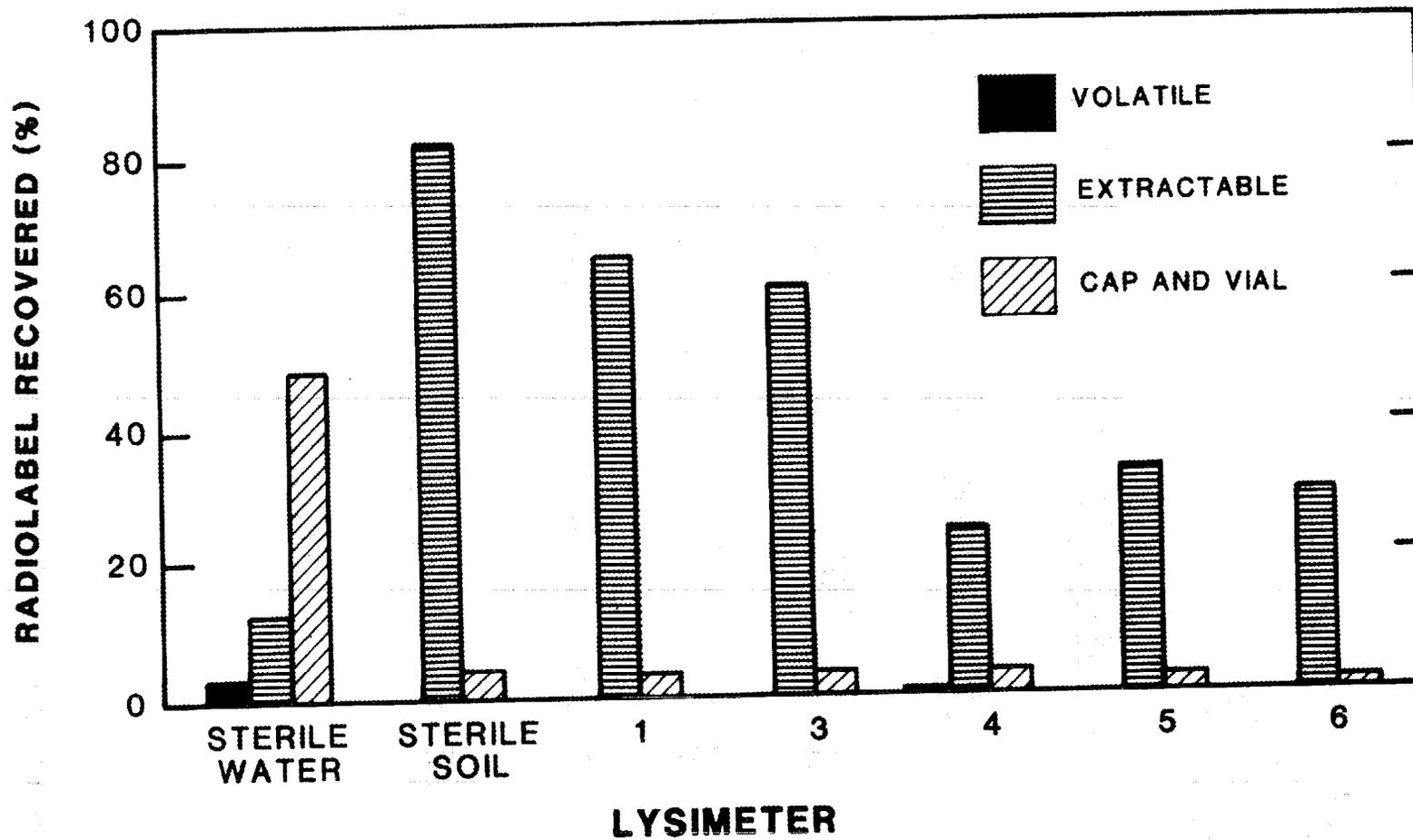


Figure 7. Recovery of radiolabel following addition of radiolabeled <sup>4</sup>CB to soil samples from lysimeters (14-d incubation).

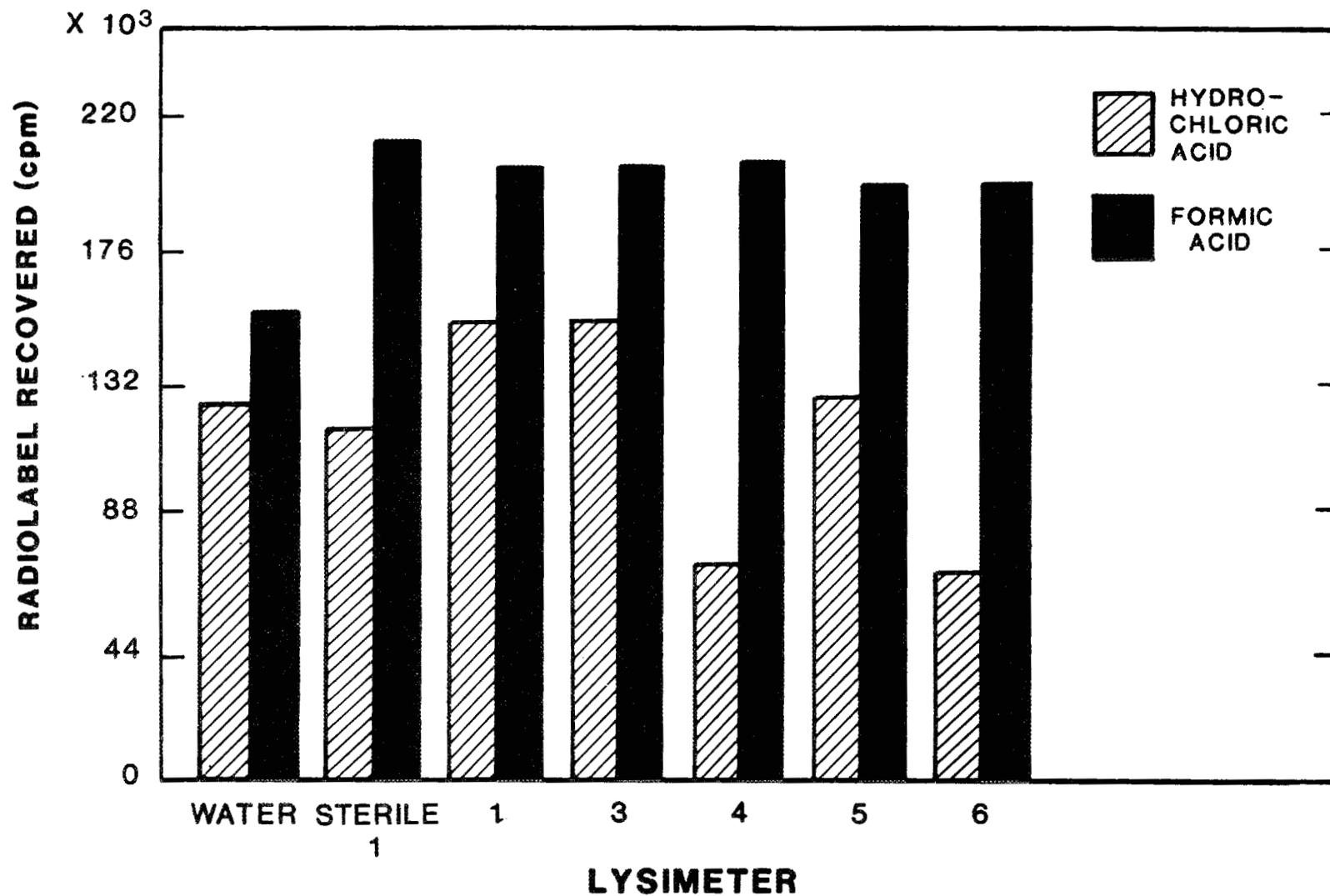


Figure 8. Recovery of radiolabel following addition of radiolabeled TCB to soil samples from lysimeters: Comparison of HCl and formic acid extractions.

An experiment was done to determine whether the decrease in extractability of PCBs is a slow process allowing easy extraction for a few days but not after two weeks. Multiple samples of soil and radiolabeled 4CB were incubated and then extracted at 2-3 d intervals with 1 N HCl and hexane/ether. Little to no change in extractability was observed up to 9 d, but the extent of extraction decreased at 11 and 14 d (Fig. 9). This result suggests that there may be a slow immobilization process in the soil. Alternatively, the radiolabeled compounds may be biotransformed in a manner that decreases the extractability.

Tests were done in which varying quantities of microbial cells were added to soil amended with TCB and incubated for several weeks. No difference in extractability was seen compared to samples without added cells. This suggests that a longer-term incubation is needed to decrease the extractability of TCB, or that biotransformation is a mechanism contributing to the decrease in extractability.

#### 5.3.8 Dechlorination of PCBs

Samples of soils and sediments were enriched with biphenyl, 3-chlorobenzoic acid, and thyroxine as described earlier. One to two isolates were obtained from each plate after 7 d of growth (Table 13). The larger number of isolates from the thyroxine enrichments suggests the presence of organisms with deiodinating activity but not dechlorinating activity.

The dechlorinating isolates were tested for dechlorination of Aroclor 1254 as described in Sect. 4.3.11. The percentages of PCB depletion over 96 h by cells grown under aerobic and anaerobic conditions in the presence of biphenyl or 3-chlorobenzoic acid are shown in Table 14. For the aerobic cultures, the PCB depletion ranged from 0 to 52% for those grown on biphenyl and 0 to 9% for those grown on 3-chlorobenzoic acid. In contrast, the anaerobic cultures showed 0 to 49% with biphenyl and 11 to 42% on 3-chlorobenzoic acid. When PCB depletion was observed with both aerobic and anaerobic cultures grown on 3-chlorobenzoic acid, the anaerobic cultures were two to ten times more effective in depleting PCBs than the aerobic cultures. This observation

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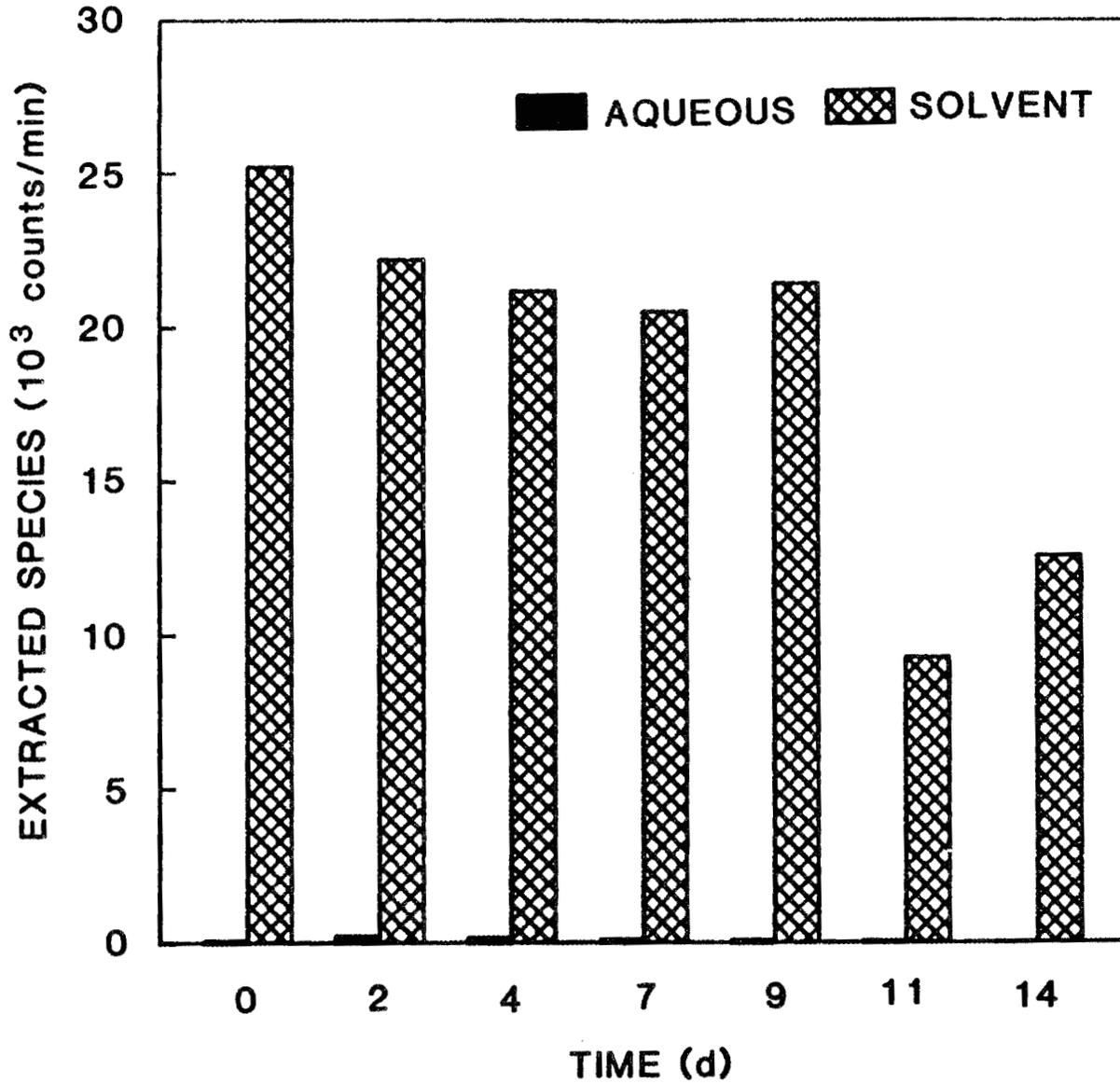


Figure 9. Extraction of radiolabeled TCB following incubation with soil.

Table 13. Numbers of different organisms isolated from soils and sediment

"-" means no isolates were obtained

Sample no.	Growth substrate					
	Biphenyl		3-Chlorobenzoic acid		Thyroxine	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
1	1	-	1	-	7	8
2	2	2	1	2	5	6
3	2	1	-	-	4	8
4	2	-	-	-	4	6
5	1	2	2	1	7	9
6	2	-	-	-	5	5
7	-	1	-	-	5	7
8	2	-	-	-	7	5
9	1	2	-	-	3	8
10	2	-	-	-	5	9
12	-	1	-	-	6	8
13	-	-	2	2	4	7
14	-	1	-	-	5	4
15	2	2	-	-	7	9
16	-	1	-	-	5	5
17	-	-	1	1	4	6
18	2	1	-	-	6	8
19	2	1	-	-	10	6
20	-	1	-	-	5	8
New Hope Pond	2	2	-	1	8	10

suggests that the anaerobic reductive pathways may be more effective for degradation of PCBs than the aerobic oxidative pathways.

Dechlorination was also evaluated by comparing capillary GC peak heights of the isomers of Aroclor 1254 in both control and experimental samples. The number of chlorines in the 13 to 15 isomers of Aroclor 1254 ranges from four to seven. Thus, if dechlorination occurs, we would anticipate changes in the relative amounts of the various isomers. Furthermore, isomers with three or less chlorines (which are

Table 14. Percent depletion of PCB (Aroclor 1254) by sonicated cell suspensions from aerobic and anaerobic cultures

"-" means no isolates were obtained

Sample no.	Growth substrate			
	Biphenyl		3-Chlorobenzoic acid	
	Aerobic	Anaerobic	Aerobic	Anaerobic
1	36	-	-	-
2	0	49	0	23
3	24	13	-	-
		-	-	-
5	0	33	0	11
6	26	-	-	-
7	-	16	-	-
8	2	-	9	27
9	16	27	-	-
10	43	-	-	-
12	-	14	-	-
13	-	-	1	42
14	-	12	-	-
15	34	35	-	-
16	-	4	-	-
17	-	-	-	-
18	52	10	-	-
19	33	0	-	-
20	-	4	-	-

not observed in chromatograms of Aroclor 1254) might be found in the experimental samples.

Figure 10 shows chromatograms of the PCB isomers from a control sample and an experimental sample. It appears that the peak heights of several isomers in the experimental sample are lower than in the control. It is also evident that the peak height of isomer 98 has been reduced and isomers 21 and 37 are present in the experimental chromatogram. Isomers 21 and 37 are not normally observed on chromatograms of Aroclor 1254. The number of chlorines in isomers 98, 21, and 37 are 5, 2, and 3, respectively.

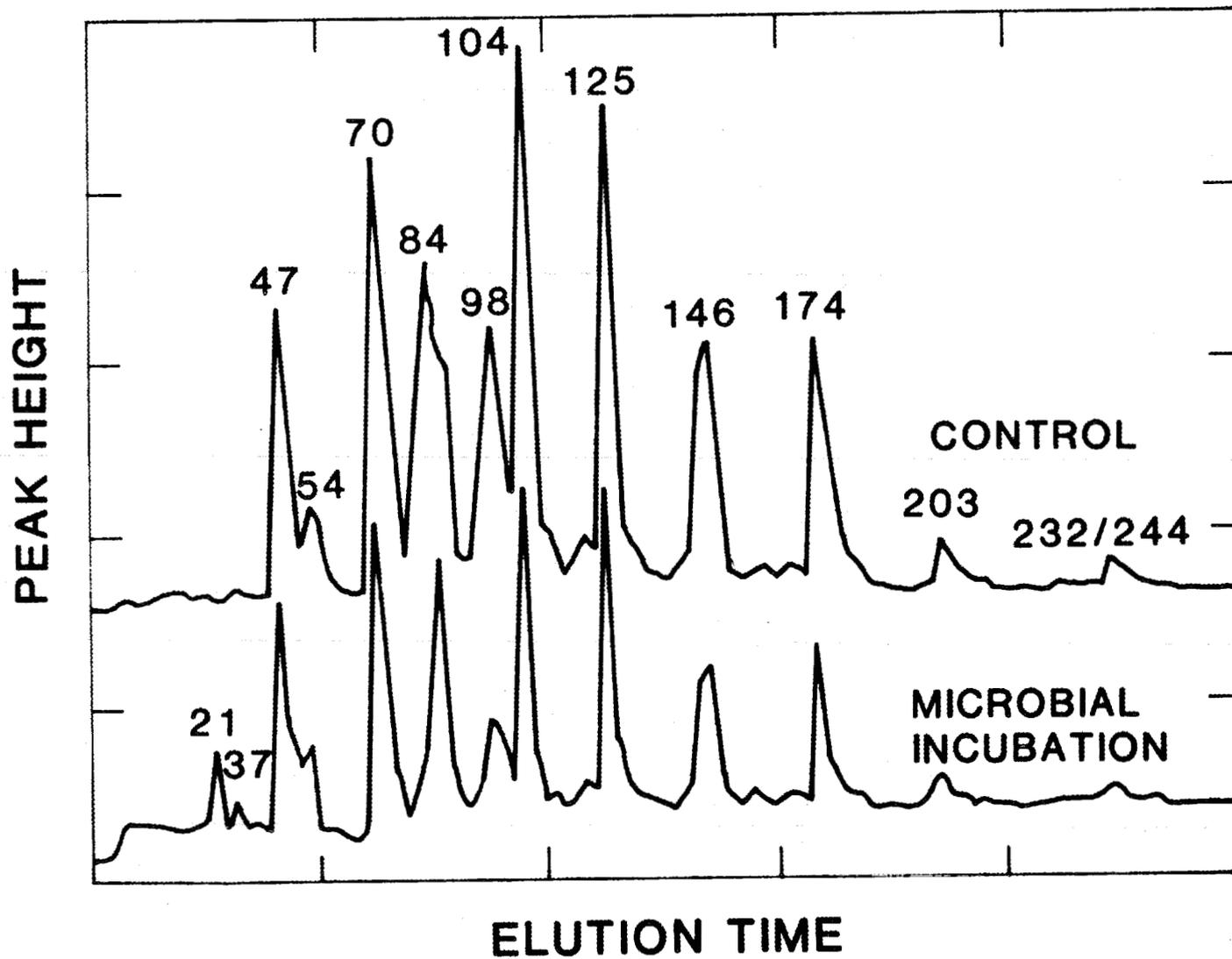


Figure 10. Characterization of PCB isomers in Aroclor 1254 before and after incubation with microbial cell fragments.

Shifts in the isomer content are summarized in Fig. 11 for this sample. It appears that the total decrease in particular isomers approximately equalled the total increase. The average increase in concentration was 60 ng of isomer per  $\mu\text{g}$  of total PCB, and the average decrease was 44 ng/ $\mu\text{g}$ . The isomers with two to four chlorines accounted for 85% of the increase. Similar results were observed in other experimental samples grown on biphenyl or 3-chlorobenzoic acid.<sup>11</sup>

## 6. CONCLUSIONS

The following conclusions and observations have been formulated based on the technical data presented in this report:

1. Indigenous microorganisms present in the contaminated soils used in this study were capable of degrading monochlorinated biphenyl. Evidence for this conclusion is the mineralization of radiolabeled substrate. Supporting evidence comes from gene probe assays and signature lipid analyses. This situation is quite encouraging for further development of bioremediation technology for PCBs.
2. In-situ treatment of the soils (in lysimeters) by aeration/mixing and water appears to stimulate growth of microorganisms and increase the biodegradation of monochlorinated biphenyl in laboratory experiments using soil samples from the lysimeters. The effects of additional nutrients, carbon source, and inocula are unclear from this study; only one condition for each parameter was tested in the limited number of lysimeters.
3. Evidence suggesting microbial dechlorination of PCBs was obtained under laboratory conditions. Decreases in the concentrations of higher chlorinated congeners were accompanied by increases in the concentrations of lower chlorinated congeners in the presence of selected aerobic and anaerobic cultures isolated from contaminated soils. This behavior warrants further investigation.
4. Microbial inoculation and incubation of the soils appear to affect the physico-chemical behavior of chlorinated biphenyls in the soil in a manner which inhibits their chemical extraction from the soil. The mechanism(s) by which this effect occurs is not known.

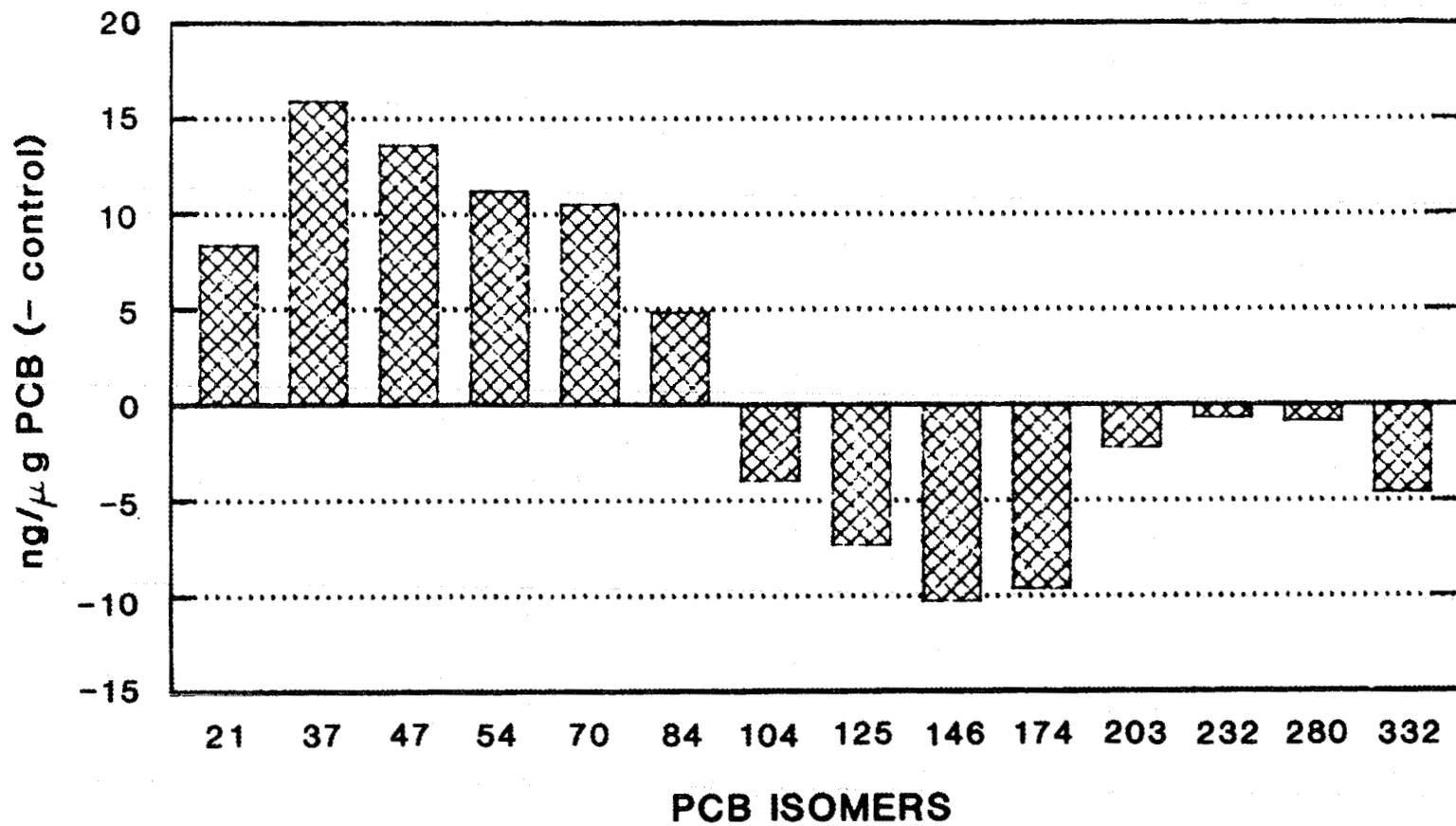


Figure 11. Changes in PCB isomers in Aroclor 1254 following 96-h incubation with microbial cell fragments from an anaerobic culture grown on biphenyl.

5. Statistically significant degradation of the PCB contaminants in the soils was not detected in either the field lysimeters or the slurry bioreactors. This result must be viewed in the following context:

- Analytical methods were less than desirable; further analyses are underway using better methods.
- Preliminary GC/mass spectrometry analyses have suggested that the PCBs in the soils are relatively highly chlorinated; these will be the most difficult congeners to degrade.
- No attempts were made in this limited test to look for more favorable conditions in the lysimeters and bioreactors.
- Relatively little time was available for acclimatization of the microbial populations under the favorable treatment conditions; longer exposure time could favor improvements in bioactivity.

6. Analysis of PCB concentrations for the purpose of evaluating biodegradation proved to be difficult. This is not an original conclusion or observation, of course; however, it has been a prevailing consideration in this project and has significantly affected the technical accomplishments.

Packed column gas chromatography, which gives a gross indication of the total amount of PCB material in the sample, is not sufficient. Furthermore, sample preparation and particular procedures are critical; analysis of the same or similar samples by different laboratories produced significantly different results. Capillary gas chromatography combined with mass spectroscopy is required for identification of individual PCB congeners in a relatively unambiguous manner.

#### 7. NEEDS FOR FURTHER PROCESS DEVELOPMENT

Current understanding of PCB biodegradation is insufficient to allow a prior design of successful in-situ and bioreactor processes for bioremediation of contaminated soil. This was certainly the case at the beginning of this feasibility test using lysimeters and slurry bioreactors, and it remains true. However, results from these tests indicate that bioremediation of PCB contamination is conceptually feasible.

Focused research and development are needed in three areas to improve our ability to bioremediate PCB-contaminated soils.

#### 7.1 STIMULATION AND MAINTENANCE OF DESIRED PCB-DEGRADING BIOACTIVITY

Mere presence of the appropriate strain(s) of microorganisms is not sufficient to ensure PCB-degrading bioactivity. The biochemical reactions are quite dynamic and can be turned on and off quite rapidly in response to the local microenvironment surrounding the microbial cells.<sup>12</sup> Factors that very probably affect the microbial transformations include the nature of the PCB substrates (including concentrations), presence of other carbon sources, trace nutrients and minerals, pH, moisture content, temperature, interactions among the various microbial species, build up of reaction intermediates, and so forth.

Fortunately, it is unlikely that all of these factors are equally important. The kinetic rates of PCB degradation could probably be increased by at least tenfold if we can identify the major controlling parameters and design process conditions to maintain these parameters at the optimal values.

#### 7.2 ANALYTICAL TOOLS TO MONITOR MICROBIAL POPULATIONS

In the same manner that it is advantageous to identify and monitor specific chemical species in chemical reaction systems to achieve and maintain performance, it is also advantageous to be able to identify and monitor specific microbial species to enhance biodegradation of PCBs. Several relatively new analytical methods are available to do this; gene probes and fatty acid signatures have been used in this study and have provided useful information that correlates positively with PCB degradation kinetics. Use of these technologies in this manner is new, and their practical utility largely remains to be proven. We and other investigators believe these techniques will lead to major advances in our abilities to monitor and subsequently control microbial populations. These abilities are necessary to harness microbial degradation capabilities for effective bioremediation technologies.

### 7.3 FORMULATION OF USEFUL NEW MICROBIAL MIXED CULTURES

This need can also be expressed by analogy to development of effective formulations for concrete, paints, plastics, and so forth, each of which meets a performance standard. Bioremediation technologies for PCBs will utilize mixed cultures for two reasons: natural biosystems are mixed cultures in the first place, and mixed cultures generally have broader capabilities to degrade a spectrum of substrates, which is the situation with PCB Aroclor mixtures.

Formulation of useful mixed cultures (and stimulation of indigenous mixed cultures) will require adequate characterization of the component microbial species and an understanding of how these component cultures interact to form stable populations. Development of these capabilities will draw on the capability needs described in Sects. 7.1 and 7.2. The starting points for this development work are the existing identified microbial species that biotransform PCBs and isolation of new cultures from contaminated sites. Genetic engineering techniques offer another route to new microorganisms with enhanced capabilities.

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