1 of 1

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**AN ASSESSMENT OF PCB DEGRADATION BY MICROORGANISMS
INCLUDING METHODS FOR MEASURING MINERALIZATION**

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Objectives

The objectives of these studies were:

- (1) Isolate and identify organism(s) from PCB contaminated soil and sediment that degrade PCB;
- (2) Provide information on the potential of organisms in soil samples taken from a PCB-contaminated area to mineralize or dechlorinate PCB congeners;
- (3) Provide preliminary information on potential enhancement of PCB biodegradation as a result of nutritional amendment of the samples;
- (4) Carry out material balance studies to provide some indications as to the potential relative rates of biodegradation and loss of PCB congeners in the treatment system;
- (5) Carry out analyses of successive lysimeter samples to determine whether field treatments have had an effect on the capacity of soil microbes to mineralize PCBs.

In attempting to achieve these objectives, we have expended considerable effort to validate the fractionation procedure used to assess mineralization and conversion of PCB substrates. The assessment relies on the ability to measure (volatile) [^{14}C]-labeled CO_2 in the presence of potentially volatile [^{14}C]-labeled PCB and degradation products, to differentiate between volatile and non-volatile [^{14}C]-labeled compounds, between water-soluble products of metabolism and a mixture of unchanged substrate and other water-insoluble products, and between metabolism and loss or non-extractability of the substrate.

and minimal requirements for energy input. Therefore an intensive effort should be made to develop bioreclamation technologies as an economical way to degrade PCB wastes. Systems for assessing the biodegradability of hazardous organic wastes have been proposed and discussed by a number of authors (18, 19, 20, 21 and 22). A field assessment of groundwater transport and degradation of hazardous organics has also been reported (23).

In situ biodegradation can be an effective means of removing organic compounds from soil, by complete degradation to inorganic constituents or by transformation to non-hazardous compounds. Microbes in groundwater can migrate to localized concentrations of pollutants and attack them in situ, decontaminating soil particles that serve as a reservoir for hazardous organic compounds. A variety of technologies are currently being developed for the delivery of organisms and nutrients to contaminated zones in soil (24, 25, 26, 27, 28 and 29). These technologies should be pursued to find optimal methods for the treatment of oil-contaminated soil at Y-12. Once developed, these technologies could be applied at other DOE sites.

Pathways for PCB Degradation

The metabolism of chlorinated hydrocarbons including PCBs by microorganisms is considered to be one of the major pathways of environmental degradation for these widespread pollutants. The observations of Ahmed and Focht (3) that bacteria were capable of growing on biphenyl and 4-chlorobiphenyl, stimulated interest in determining whether microorganisms were capable of degrading PCBs (30). Environmental and laboratory studies have both demonstrated PCB degradation by microorganisms, with the less-chlorinated biphenyls being degraded more rapidly than the highly chlorine substituted biphenyls (31 and 32). Bacteria and fungi have been shown to degrade chlorinated hydrocarbons through, 1) hydroxylation, cleavage of the aromatic ring, dechlorination and mineralization and 2) reductive dechlorination and mineralization (33, 34 and 35). The oxidative dechlorination pathways are associated with aerobic bacteria and fungi and the reductive dechlorination pathways with anaerobic bacteria. Aerobic bacteria have been studied the most followed by anaerobic bacteria and fungi. The aerobic microorganisms associated with PCB degradation include, (1) the bacterial genera, Pseudomonas, Achromobacter, Acetobacter, Acinetobacter, Alcaligenes, Klebsiella and (2) the fungus, Phanerochaete chrysosporium. Studies of PCB degradation by anaerobic bacteria have not to date identified specific genera, although reductive dechlorination has been demonstrated for a variety of chlorinated hydrocarbons under anaerobic conditions.

Under aerobic conditions the initial biochemical reaction of

methods for dechlorinating PCB to the less toxic biphenyl. One such method is the microbial dechlorination of this toxic substance. This report describes preliminary results of studies designed to isolate and enrich bacteria capable of dechlorinating PCB. Most studies have enriched for dechlorinating bacteria from environmental samples incubated with biphenyl or monochlorinated compounds. The enriched cultures have then been characterized for their ability to dechlorinate substrates such as those described above. In the present studies, PCB-contaminated soil was used to isolate microbes capable of dechlorinating PCB. The soil was enriched with yeast extract, mineral salts and either biphenyl or 3-chlorobenzoic acid. After several transfers, cells were harvested and incubated with the Aroclor 1254. Dechlorination of the 1254 was determined by gas chromatography. A decrease in the total concentration and changes in peak heights of the various isomers of the 1254 was used to confirm dechlorination. Attempts were made to enhance dechlorination by the addition of various carbon substrates.

In similar studies iodinated hydrocarbons were used in place of the chlorinated substrate because previous investigators have shown that the iodinated substrates are dehalogenated at rates faster than the chlorinated hydrocarbons. If the iodinated substrate has to induce a non specific dehalogenase and if this enzyme were equally effective on both substrates, then the incubation time required for the induction of the dehalogenases would be reduced. Cultures containing soil, mineral salts and 3,5,3',5'-thyronine (thyroxine) were grown anaerobically at 32°C. After several transfers, cells were added to a medium containing mineral salts and Aroclor 1254 and incubated anaerobically at 32°C for 5 days. Dechlorination was determined as described above.

Methods

a. Isolation Procedures

Collection soil and sediment samples. Soil and sediment samples (20) were collected from a repository at K-25 and sediment samples from New Hope Pound. The soil and sediment samples were collected at various times from Y-12 and stored at K-25, because they contain PCB contamination. Sediment samples from New Hope Pound were collected because the PCB levels were greater than 50 ppm. The PCB concentration was determined on all samples and they ranged from 10 to 100 ppm. After collecting the samples they were thoroughly mixed and 0.5 grams of soil and sediment (wet weight) was added to the medium described below and incubated aerobically or anaerobically at 32°C for the times indicated below. Dry weight was determined on all samples after allowing the sample to air dry to a constant weight at room temperature.

The mixed soil and sediment samples were added to a medium containing mineral salts and biphenyl or 3-chlorobenzoic acid

thyroxine, 1mg/mL of medium) in 1% methanol (final PCB conc 10ug/mL).

2) Controls

- a) 9 mL Tris buffer (pH 7.5) containing 0.1% BSA and 1 mL PCB (1254) (or thyroxine 1mg/mL of medium) in 1% methanol (final PCB conc 10ug/mL).
- b) 4 mL of the sonicated suspension (previously heated for 30 minutes at 90°C), 5 mL Tris buffer (pH 7.5) containing 0.1% BSA and 1 mL PCB (1254) (or thyroxine 1 mg/mL of medium) in 1% methanol (final PCB conc 10 ug/mL).
- c) 4 mL of the sonicated suspension, 5 mL Tris buffer (pH 7.5) containing 0.1% BSA and 1 mL of 1% methanol.

These mixtures were incubated at room temperature in sealed vials and 3 mL aliquots were removed at the indicated times and added to Triton X-100 and the PCB 1254 was extracted into hexane.

c. Analyses of PCBs and dechlorination.

A 25 gram (wet weight) sample of soil and sediment was added to an extraction thimble in a Soxhlet extractor. After adding 300 mL of hexane (pesticide grade) to the thimble, the Soxhlet system was connected and the PCBs in the sample were extracted for 2.5 hours. The hexane extract, containing the PCBs, was taken to dryness using a rotary evaporator and the residue was dissolved in 50 mL of hexane. A 5 mL aliquot of this hexane mixture was added to a vial (and sealed) containing 0.5 grams of Florisil (100 to 200 mesh) and mixed for 1 minute before injecting 2 uL into a Hewlett-Packard 5880A gas chromatograph. The dry weight of each soil sample was determined by allowing the sample to air dry to a constant weight at room temperature.

In the dechlorination experiments a 0.15 mL aliquot of Triton X-100 was added to the mixture followed by 6 mL of hexane. After mixing this slurry for 20 minutes the sample was centrifuged to separate the layers and 3 mL of the hexane layer was removed and added to a vial (and sealed) containing 0.5 grams of Florisil. After mixing, a 2 uL aliquot of sample was injected into the Hewlett-Packard gas chromatograph. PCBs were analyzed using a gas chromatograph equipped with an electron capture detector and glass column packed with 2% OV-101 (43).

Dechlorination was determined by comparing the total amount of PCB extracted at 0-time with the experimental values at 96 hours. Results showing increases in the mono-, di- and trichlorobiphenyl (i.e. isomers 11, 14, 16, 21, 28, 32, 37 and 40) and corresponding decreases in the tetra (47, 54, 58 and 70), penta (70, 84, 98, 104, and 125) or hexachlorobiphenyl (125, 146, 160, 174 and 203) at 96 hours incubation were used

to provide further evidence of dechlorination. Results from control (a) were used to determine the recovery efficiency of Aroclor 1254 from the incubation media. Control (c) was used to determine the presence of bacterial organics that were extracted into hexane and co-chromatographed with the isomers of 1254. Control (b) was used to determine the presence of biological dechlorination.

RESULTS AND DISCUSSION

I. Mineralization of PCBs by soil organisms

A. Validation of methods for measuring mineralization of [14C]-4-chlorobiphenyl and [14C]-tetrachlorobiphenyl.

The protocol initially devised to assess mineralization and material balance with the soil samples was based on studies by Saylor's group, as prescribed in the statement of work. This protocol was subsequently revised on the basis of experimental results with model soil and water suspensions and solutions of labeled 4-CB, TCB, and CO_2 . The revised protocol is included as Appendix A. Studies aimed at validating the protocol are described below.

This section describes progress in establishing reliable procedures for carrying out materials balance studies in the assessment of mineralization of PCBs. The studies reported here are related to the analytical protocol itself, and are intended to validate the procedures set out in the protocol. The current protocol includes changes made in procedures as a result of other experiments which showed certain aspects of the protocol not to be feasible.

Assessments described here include the kinetics of removal of CO_2 from aqueous and soil samples, fractionation of 4-chlorobiphenyl (4-CB) from aqueous samples, efficiency of Florisil and phenethylamine for trapping CO_2 and 4-CB, and the effects of various additions on the counting efficiency of ^{14}C in the scintillant used.

1. Kinetics of removal of CO_2 from control samples

a. Aqueous samples

A series of experiments was carried out to characterize the rates of removal of $^{14}\text{CO}_2$ from solution in the test system. The test vial contained 0.01 $\mu\text{Ci Na}_2^{14}\text{CO}_3$ in 1 ml 0.01 N NaOH and was sealed with a Teflon-lined septum. A cannula was inserted through the septum and connected to a trap consisting of 1.5 ml phenethylamine in 6 ml Scintiverse E. A hypodermic needle connected to an air tube from a multichannel peristaltic pump was inserted through the septum to the bottom of the vial. 0.1 ml of 1 N HCl was injected through the septum, and the vial was

bubbled with air at 2 or 8 cc/min. At various times the phenethylamine trap was changed, and the radioactivity in each trap was determined. Figure 1 shows that essentially all of the CO_2 was removed from the solution by 60 minutes at 2 cc/min and by 30 min at 8 cc/min.

b. Soil samples

A similar study was made to determine how the soil matrix affected the rate of CO_2 removal. The test vial contained 0.01 $\mu\text{Ci Na}_2^{14}\text{CO}_3$ in 0.1 ml 0.01 N NaOH added to 1 g soil taken from the initial lysimeter sample. The vial was sealed with a Teflon-lined septum and attached to the air pump and phenethylamine trap as described above. 1 ml of HCl solution was injected and the rate of $^{14}\text{CO}_2$ evolution was measured. The results in Figure 2 showed that 0.1 N HCl was not sufficient to release the CO_2 quantitatively from the soil, but that 1 N HCl did so. A bubbling time of 40 min appeared to be sufficient to remove essentially all of the $^{14}\text{CO}_2$ from the soil after addition of 1 N HCl.

2. Fractionation of [^{14}C]-4CB from control samples

The procedure for fractionation of [^{14}C]-4CB is based on the expectation that a significant amount of the compound will be volatilized by the bubbling process, that it will be trapped in Florisil, and that the remainder of the compound in the reaction vial will be selectively soluble in hexane/ether. In this study, the test vial containing 1 μg 4CB + [^{14}C]-4CB in 0.5 ml H_2O was sealed with a Teflon-lined septum and connected to the air pump and Florisil and phenethylamine traps as described above. 0.5 ml of 0.2 N HCl was injected, and air was bubbled through the suspension at 8 cc/min. At 60 min the Florisil trap was removed and eluted with 2 ml 15% ether in hexane. The residual [^{14}C]-4CB in the Florisil was counted separately. To the sample vial were added 0.1 ml of 10% Triton X-100, 0.2 g anhydrous Na_2SO_4 , and 4 ml of hexane/ether (1:1). The suspension was shaken for 30 min and then centrifuged to separate the layers, and the aqueous and solvent layers were counted. The results shown in Table 1 indicated that about 87% of the [^{14}C]-4CB was removed from solution by 60 min of bubbling, and that essentially all of the remainder was extracted into the hexane/ether fraction. A separate experiment demonstrated that a sample of [^{14}C]-4CB held in 0.5 ml of 0.2 N HCl for 60 min before fractionation was quantitatively recovered, and therefore did not appear to be bound appreciably to the glass or to the Teflon septum.

TABLE 1
FRACTIONATION OF 4-CB

Fraction	cpm	% of Total	Cumulative %
Phenethylamine	319	0.14	0.14
Florisl eluate	203,094	86.8	86.9
Florisl residue	1,834	0.78	87.7
Aqueous	12	0.005	87.7
Hexane/ether	53,892	23.0	110.7

3. Trapping efficiencies

a. Phenethylamine traps

The efficiency of phenethylamine traps was evaluated by bubbling $^{14}\text{CO}_2$ into a phenethylamine trap consisting of 0.75 ml phenethylamine in 3 ml Scintiverse E in a vial sealed with a Teflon-lined septum. This vial was connected by a similar cannula to a second phenethylamine trap. Air was bubbled through the sample and traps at 8 cc/min for 60 min, and the total ^{14}C radioactivity in each trap was measured. In two experiments, the amount of $^{14}\text{CO}_2$ that escaped the primary trap and was trapped in the secondary trap was about 0.3%.

b. Florisl traps, [^{14}C]-4CB

The test vial containing 1 μg 4CB + [^{14}C]-4CB in 0.5 ml H_2O was sealed with a Teflon-lined septum and connected to the air pump as described above. A Florisl trap containing 0.5 g Florisl in a 3-cc plastic syringe was attached to the vial via a hypodermic needle inserted through the septum. The Florisl trap was capped with a rubber stopper through which a hypodermic needle had been inserted, and the needle was connected to a phenethylamine trap. 0.5 ml of 0.2 N HCl was injected through the septum. Air was pumped through the 4CB suspension and traps at 8 cc/min for 30 min, and the total ^{14}C radioactivity in each trap was determined. Table 2 shows that less than 0.01% of the [^{14}C]-4CB escaped the primary Florisl trap and was trapped in the phenethylamine secondary trap.

scintillation vial contains 0.5 g of Florisil (dry or wet with 0.1 N NaOH and drained). Therefore, it is possible to elute $^{14}\text{CO}_2$ selectively from the Florisil trap with NaOH solution and then transfer the Florisil to a scintillation vial to count the residual [^{14}C]-PCB.

TABLE 3
ELUTION OF CO_2 AND 4CB FROM FLORISIL
BY NaOH AND HEXANE/ETHER

FRACTION	CO_2		4CB	
	cpm	%	cpm	%
A.				
(Standard)	61,410	100.0	241,631	100.0
Phenethylamine	31,343	51.0	0	0
Florisil				
Hexane/ether	10,591	17.2	146,900	60.8
0.1 N NaOH	7,918	12.9	15,836	6.6
Solution res.	64	0.1	105,434	43.6
B.				
(Standard)	97,462	100.0	320,512	100.0
0.1 N NaOH	93,665	96.0	199	0.06
Hexane/ether	2,164	2.2	259,623	81.0
(Florisil res.)	4,208	4.3	30,282	9.45

TABLE 4
COUNTING EFFICIENCY OF [^{14}C]-4CB
IN WET OR DRY FLORISIL

Condition	cpm	avg.	%
Direct	385,572 370,156	377,864	100.0
Dry Florisil	366,705 379,860 380,830	375,778	99.5
Wet Florisil	370,691 373,449	372,070	98.5
Mean	375,323		
Std. Dev.	6,346		

4. Counting efficiencies

a. Effect of water and phenethylamine on ^{14}C counting efficiency

100 μl (0.01 μCi) of $\text{Na}_2^{14}\text{CO}_3$ was added to 6 ml of Scintiverse E with or without an additional 1.5 ml of phenethylamine. The ^{14}C cpm and the external standard channel ratio were determined, and 1 ml of water was added to each vial. The process was repeated until 8 ml of water had been added. Figure 4 shows that the scintillant containing phenethylamine was able to accommodate 3 ml of water and still maintain at least 95% of the initial counting efficiency, whereas without phenethylamine, the same amount of water had reduced the counting efficiency by more than 30%. Because the proposed fractionation procedure requires elution of $^{14}\text{CO}_2$ from Florisil by the addition of aqueous solution, it appears that addition of phenethylamine to the scintillant is essential to count these fractions efficiently. A plot of counting rate as a function of channel ratio showed that the counting efficiency decreased with channel ratio at different rates depending on whether the scintillant contained phenethylamine. Therefore channel ratios cannot be used reliably to correct for decreases in counting efficiency unless all samples contain phenethylamine.

B. Mineralization assessments on lysimeter soil samples.

Composited soil samples were distributed into a series of reaction vials and amended with either mineral salts, 60 ppm glucose, or 10 ppm yeast extract as indicated in the protocol. Either 4-chloro-[^{14}C]-biphenyl (4CB) or tetrachloro-[^{14}C]-biphenyl (TCB) was added to each vial at a concentration of 1 $\mu\text{g}/\text{gram}$ soil. Parallel cultures of *Alcaligenes* A5, a PCB-degrader, 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 CO_2 , volatile PCB, solvent-extractable PCB, and water-soluble PCB were measured.

Only a small amount of mineralization of either substrate was observed (Figure 5). The non-amended soil samples mineralized only approximately 1% of the available 4CB and about 0.2% of the available TCB. Amendment of the soil with mineral salts or yeast extract had no significant effect on mineralization during the 14-day period. Addition of glucose increased mineralization of 4CB by about 30%, but mineralization of TCB was not affected. Cultures of *Alcaligenes* A5 in mineral salts showed very little mineralization when the ^{14}C -labeled PCB was the only carbon source, less than 0.03% of either 4CB or TCB. Addition of yeast extract increased mineralization of 4CB, but not above 0.1%, whereas addition of glucose increased the mineralization of 4CB by about ten-fold. Supplementation with either glucose or yeast extract did not increase mineralization of TCB.

Results of mineralization experiments on lysimeter soil samples showed that little activity was observed in lysimeters 1-5 (less than 2% of the add 4-CB mineralized in 14 days), whereas 5-6% of the label was mineralized by soil from lysimeter 6 (Figure 6). Repeated assessments of mineralization confirmed the result that mineralization was greater with soil from lysimeter 6 than from lysimeters 1, 3, 4, and 5. The recovery of added substrate was also consistently lower from amended soils, implying that binding of PCB to soil was greater under conditions allowing better microbial growth.

These results indicate that although initial background PCB-degrading activity in the lysimeter soil is low, some PCB degradation does occur, and that nutritional supplementation may be effective in increasing the rate of mineralization by soil organisms. Increased mineralization in soil samples amended with glucose may reflect an increase in biomass of PCB-degrading organisms rather than a glucose-stimulated increase in the rate of degradation in the organisms initially present.

II. Binding of PCB to soil

A. Variability of recovery from lysimeter soils

Mineralization experiments showed that recovery of PCB from lysimeter soil was lower after prolonged incubation than in the initial validation experiments, which used very short incubation times. In addition, recovery was markedly less in soils which had been amended and inoculated (Figure 7), leading to the hypothesis that PCB might be binding to organic matter in the soil, especially soil microbes.

As a result of group discussion, the decision was made to compare different extraction conditions to see whether complete recovery of PCBs from soil could be achieved. It was hypothesized that the limited recovery of PCB from the lysimeter soils might be related to binding of PCB to bacterial cells in the soil. Therefore, an experiment was designed in which various numbers of cells were added to soil, a constant amount of PCB was added, and the efficiency of extraction was measured. The results indicated that there was no significant difference in the efficiency of extraction over a range of 10^5 to 10^7 cells per gram of soil when the test soils were allowed to incubate for three days. Because the extraction efficiency was near 100% in each case, it appears that a longer time may be necessary to see any matrix-related effects on PCB binding in soil. However, a series of duplicate soil samples which had been incubated with tetrachlorobiphenyl for 14 days and then frozen was available. This series was extracted with either 1N HCl at room temperature or 88% formic acid at 120° C for 60 min, then, after addition of 1% Triton X-100, with four volumes of hexane:ether (1:1). Extraction was essentially complete in all the samples autoclaved in formic acid, whereas the pattern previously observed, of less complete extraction in soils which had been amended with nutrients and bacteria, was seen in samples which were extracted with HCl at room temperature (Figure 8).

The results suggested that a longer-term incubation with a non-metabolized PCB label could be useful to address the question of binding of PCB to bacterial cells in a soil matrix. Therefore, various numbers of bacterial cells, ranging from 10^4 to 10^8 per gram, were added to soil from lysimeter 1, and each was made 1 ppm in [14 C]TCB. The soils were incubated for 14 days, acidified with 1 ml 1 N HCl, and then extracted. No significant difference was seen in the extractability of the label into hexane-ether as a function of bacterial cell concentration (Table 5).

TABLE 5
BINDING OF TCB TO BACTERIAL CELLS IN SOIL

	cpm	% of Total
Total TCB Added	24,165	100.0
Cells Added/g Soil		
None	13,800	
	12,725	55.0
1 x 10 ⁶	6,831	
	13,055	41.2
3.2 x 10 ⁶	7,870	
	14,589	46.6
1 x 10 ⁷	14,892	
	11,815	55.4
3.2 x 10 ⁷	8,012	
	12,208	42.0
1 x 10 ⁸	15,206	
	11,186	54.6
Mean	11,849	49.0
Std. Dev.	2,866	11.9

Time course of extractability: To determine whether the binding of PCB to soil was a slow process, leading to the ready extractability of the compound after a few days of incubation but not after two weeks, an experiment was done to assess the time course of extractability of TCB. Parallel samples of [¹⁴C]-TCB were incubated in 1 g soil in sealed vials, and were extracted with 1 N HCl and hexane/ether at intervals of 2-3 days. Little or no difference in extractability was observed up to 9 days, but the extent of extraction was decreased at 11 and 14 days (Figure 9). This result suggests that there is indeed some slow binding process in the soil, and that assessments made on freshly prepared soil samples or slurries may not reflect the kinetics of reaction or metabolism of PCBs which have been in the soil for extended periods of time.

TABLE 6
EXTRACTION OF TCB FROM SOIL UNDER VARIOUS
EXTRACTION CONDITIONS

	cpm	% of Total
Total TCB Added	24,165	100.0
Extraction Condition		
1 N HCl, rm. temp.	13,058	
	11,919	51.5
1 N HCl, autoclaved	2,125	
	1,695	7.9
1 N HCl, hexane/ether 60°	16,140	
	18,330	72.5
Hexane/ether only, 60°	9,022	
	15,599	50.9
2% Tween 80, HCl	6,625	
	5,726	25.6
0.1 % DMSO, HCl	6,894	
	6,433	27.6
Conc. Formic, rm. temp.	25,900	
	25,792	106.9
Conc. Formic, autoclaved	26,078	
	26,011	107.8

B. Variability of mineralization by lysimeter soil

Lysimeter 6 consistently showed higher activity than all other lysimeters in this assay, which was characterized by the addition of PCB to soil rather than to a soil slurry. Slurry experiments gave higher levels of mineralization for all soils. This contrast may indicate that in the slurry experiment, availability of substrate to the bacterial population was increased, and that in soil, mineralization by soil microbes may be limited by mobility of both microbes and PCB. Thus the

amount of mineralization seen in the soil experiments may depend in part on a fortuitous distribution of cells and added substrate. If so, it seems likely that there would be considerable variation in the mineralization results for soil samples, and less for slurries. It appears that the variation in mineralization among replicates and repeat experiments is high with lysimeter 6 soil. Table 7 shows the results of mineralization determinations for several samples of lysimeter 6 soil. In these experiments, soil containing [^{14}C]4-CB was incubated in sealed vials for 14 days before being fractionated according to the protocol. The high level of variability is reflected by the ratio of standard deviation to the mean. A comparison of this extent of variability with that observed with soil slurries might be informative in regard to the question of localization of added PCB.

TABLE 7
VARIABILITY OF MINERALIZATION RATES
IN SOIL FROM LYSIMETER 6

Expt. Date	Vial	% of [^{14}C] as CO_2
9/30	1	2.67
	2	13.5
10/16	1	7.74
	2	1.18
11/16	1	8.62
	2	1.62
Mean		5.89
Std. Dev.		4.89
SD/Mean		0.83

III Dechlorination Experiments

a. Isolation Studies

After the addition of soil and sediment to a medium containing biphenyl or 3-chlorobenzoic acid, cultures were grown for 4 to 6 weeks and then an aliquot of the culture was transferred to fresh medium. The transfer process was repeated twice and an aliquot of culture was plated onto agar containing mineral salts and biphenyl or 3-chlorobenzoic acid. The plates were incubated for 7 days and then examined for cell growth and

morphology of the different isolates. One to 2 isolates were identified on each plate after 7 days of growth (Table B). Longer incubation (4 weeks) did reveal additional isolates. An isolate which appeared on all plates was a coccus. The exception was those experiments in which thyroxine was used as the sole carbon source. In these experiments we observed 3 to 10 isolates per plate. However, when aliquots of these cultures were plated onto plates containing biphenyl or 3-chlorobenzoic acid only 1 to 2 isolates could be identified. This suggests that biphenyl and 3-chlorobenzoic acid is toxic to the cells and/or the cells cannot utilize these substrates as a sole carbon source. It was apparent that larger numbers of microorganisms in soil and sediment can utilize thyroxine as a sole carbon source, which suggests that they contain deiodinating but not, dechlorinating activity. It is noted that the addition of glucose to the media before plating or to the agar plated medium along with the organic substrates did not increase the number of isolates on the plates.

TABLE 8

NUMBER OF DIFFERENT ORGANISMS ON AGAR PLATES

SUBSTRATES

SAMPLE NO.	BIPHENYL		3-CHLOROBENZOIC ACID		THYROXINE	
	Aer.	Anaer.	Aer.	Anaer.	Aer.	Anaer.
1	1	0	0	0	7	8
2	2	2	1	2	5	6
3	2	1	0	0	4	8
4	2	0	0	0	4	6
5	1	2	2	1	7	9
6	2	0	0	0	5	5
7	0	1	0	0	5	7
8	2	0	0	0	7	5
9	1	2	0	0	3	8
10	2	0	0	0	5	9
12	0	1	0	0	6	8
13	0	0	2	2	4	7
14	0	1	0	0	5	4
15	2	2	0	0	7	9
16	0	1	0	0	5	5
17	0	0	1	1	4	6
18	2	1	0	0	6	8
19	2	1	0	0	10	6
20	0	1	0	0	5	8
New Hope Pond	2	2	0	1	8	10

Aer. = plates grown aerobically, Anaer. = plates grown anaerobically.

b. PCB Depletion and Dechlorination Studies

After cells were grown to stationary phase they were harvested by centrifugation and then sonicated to give a cell-free mixture. The Aroclor 1254 was added to a vial containing the sonicated cell fragments and the sealed vial was incubated at room temperature under aerobic or anaerobic conditions for 96 hours. At 0-time and 96 hours an aliquot of the mixture was removed and added to Triton X-100. After mixing, hexane was added and an aliquot was removed and mixed with Florisil. The PCB concentration in the hexane extract was determined by gas chromatography. The percentage of PCB depleted by cells grown under aerobic and anaerobic conditions in the presence or absence of biphenyl or 3-chlorobenzoic acid is shown in Table 9. As shown the concentration of PCB was reduced by 0 to 52% after 96 hours of incubation. The greatest

reduction in PCB levels for aerobic bacteria were observed when cells were isolated in biphenyl, which was not the case for the anaerobes. The ranges in the depletion of PCB by aerobes grown on biphenyl or 3-chlorobenzoic acid were 0 to 52% and 0 to 9%, respectively. In contrast, the ranges in the depletion of PCB by anaerobes grown on biphenyl or 3-chlorobenzoic acid were 0 to 49% and 10 to 42%, respectively. When depletion was observed under both conditions in the group grown on 3-chlorobenzoic acid, anaerobic bacteria were 2 to 10 times more efficient in depleting PCB than the aerobic bacteria. This observation suggests that reductive pathways may be more efficient in depleting PCB than the oxidative pathways.

TABLE 9

THE PERCENT DEPLETION IN PCB CONCENTRATIONS
BY AEROBIC AND ANAEROBIC ORGANISMS

SUBSTRATES				
BIPHENYL			3-CHLOROBENZOIC ACID	
GROWTH CONDITIONS				
SAMPLE NO.	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC
	%	%	%	%
1	36	--	--	--
2	0	49	0	23
3	24	13	--	--
4	37	--	--	--
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	--	--

-- cells were not isolated (see Table 8)

In classical dechlorinating experiments of PCBs attempts are made to correlate changes in substrate with the formed product. In the present studies dechlorination was determined by comparing peak heights of the isomers of 1254 in both control and experimental samples. There are 13 to 15 isomers identified in 1254 and the chlorine content in each isomer is shown in Table 10. As shown the chlorine content in these isomers ranges from 4 to 7. Thus, if dechlorination occurred we would anticipate changes in those isomers with a relatively high chlorine content to those with a relatively low content. Furthermore, isomers with a chlorine content of less than 3 (which are not observed on chromatograms of 1254) should be observed in the experimental samples.

TABLE 10

CHEMICAL COMPOSITION OF AROCLOR 1254
(CHLORINE CONTENT 54 %)

*RRT	MEAN WEIGHT PERCENT	NUMBER OF CHLORINES
21		2
32		3
37		3
40		4
The above isomers not observed in 1254		
47	6.2	4
54	2.9	4
58	1.4	4
70	13.2	4 25%
		5 75%
84	17.3	5
98	7.6	5
104	13.6	5
125	15.0	5 70%
		6 30%
146	10.4	6 30%
		6 70%
160	1.3	6
174	8.4	6
203	1.8	6
232	1.0	7

Total 100

* RETENTION TIME RELATIVE TO P.P'. DDE = 100

Figure 10 is a chromatogram of 1254 showing the peak heights from a control and experimental sample. It appears that the peak heights of several isomers in the experimental sample are lower than the control. It is also clearly evident that the peak height of isomer 98 is lower and the isomers 21 and 32 are present. The isomers 21 and 37 are not observed on chromatograms of 1254. The chlorine content of isomers 98, 21 and 37 are 5, 2 and 3, respectively which suggests dechlorination activity in this experimental sample. This suggestion is better illustrated in Figure 11. In calculating the total change in the concentration of each isomer minus the control (i.e. expressed in ng of isomer/ug of total PCB) it appeared that the decrease in concentration equaled the increase. For example, the average increase in concentration of the PCB isomers was 60 ng/ug total PCB and the decrease equaled 44 ng/ug total PCB. The isomers with of 2 to 4

chlorines accounted for 85% of this increase.

Similar results were observed in the experimental samples grown on biphenyl or 3-chlorobenzoic acid and then incubated with the Aroclor 1254 (Figure 12 and 13). The average increases and decrease in the isomers of PCB for aerobes and anaerobes grown on biphenyl were as follows; 19 and 22 ng/ug total PCB and 31 and 17 ng/ug total PCB, respectively. The average increases and decreases in the isomers of PCB for isolates grown aerobically or anaerobically on 3-chlorobenzoic acid were, 15 and 29 ng/ug total PCB and 29 and 26 ng/ug total PCB, respectively. These results suggest that the dechlorination process may not be affected by the growth conditions (i.e. carbon source and available oxygen levels). However as discussed above aerobes grown on 3-chlorobenzoic acid were less effective in depleting PCB which may suggest that the rates of dechlorination and mineralization for aerobes are slower than for anaerobes. It is noted that the increases in the isomers of PCB for the aerobes are about half the values observed for the anaerobes (Figure 13).

CONCLUSIONS

Extraction of PCB from soil.

Initial studies on extraction of PCB from soil indicated that a modification of the procedure of Bedard et al. (see Appendix A) would fulfill the necessary conditions for selective partitioning and complete extraction. However, it later became apparent that other factors were involved in binding of PCB to soil after extended incubation. The results suggested that there is some slow binding process in the soil, and that assessments made on freshly prepared soil samples or slurries may not reflect the kinetics of reaction or metabolism of PCBs which have been in the soil for extended periods of time. Despite indications that extractability of PCB was lower in soils in which greater biodegradative activity was observed, no significant difference was seen in the extractability of the labeled substrate from soil into hexane-ether as a function of bacterial cell concentration.

Modification of the extraction procedure improved the recovery of PCB from the soil. Specifically, substituting concentrated formic acid for 1 N HCl at the soil suspension step of the protocol yielded essentially quantitative recovery of TCB from incubated soils.

Assessment of mineralization

Results of mineralization experiments on lysimeter soil samples showed that little activity was observed in lysimeters 1-5, whereas more of the label was mineralized by soil from lysimeter 6, which had been enriched and supplemented with PCB-degrading organisms. The recovery of added substrate was also consistently lower from amended soils, implying that binding of PCB to soil was greater under conditions allowing better microbial growth.

Only a small amount of mineralization of either TCB or 4-CB as substrate was observed. The non-amended soil samples mineralized only approximately 1% of the available 4CB and about 0.2% of the available TCB. Amendment of the soil with mineral salts or yeast extract had no significant effect on mineralization during the 14-day period. Addition of glucose increased mineralization of 4CB by about 30%, but mineralization of TCB was not affected. Cultures of *Alcaligenes* A5 in mineral salts showed very little mineralization when the ¹⁴C-labeled PCB was the only carbon source, less than 0.03% of either 4CB or TCB. Addition of yeast extract increased mineralization of 4CB, but not above 0.1%, whereas addition of glucose increased the mineralization of 4CB by about ten-fold. Supplementation with either glucose or yeast extract did not increase mineralization of TCB.

These results indicate that although initial background PCB-degrading activity in the lysimeter soil is low, some PCB degradation does occur, and that nutritional supplementation may be effective in increasing the rate of mineralization by soil organisms. Increased mineralization in soil samples amended with glucose may reflect an increase in biomass of PCB-degrading organisms rather than a glucose-stimulated increase in the rate of degradation by the organisms initially present.

The variability in mineralization among replicates and repeat experiments was high with lysimeter 6 soil. The high level of variability is reflected by the high ratio of standard deviation to the mean (0.83). A comparison of this extent of variability with that observed with soil slurries might be informative in regard to the question of localization or sequestration of added PCB.

These considerations could be important in formulating recommendations for treatment of PCB-contaminated soils. It appears that nutritional enhancement of the soil and addition of PCB-degrading organisms and PCB as a stimulator of PCB degradation are both useful in increasing the potential of soil microbial populations to degrade resident PCB. However, these effects may be localized by the extreme insolubility of the substrate. If binding of PCB to soil is a major factor limiting the rate of degradation, effective treatment may require some procedure to slurry the contaminated soil, thus increasing contact between the PCB and the soil microbes, both of which appear to be relatively immobile.

Assessment of soil and sediment for microorganisms that dechlorinate and/or degrade PCB

The soil and sediment samples used in these studies contained aerobic and anaerobic microorganisms that dechlorinate and degrade PCB. However not all samples contain these microorganisms and thus, it would be necessary to add PCB degrading microorganisms to these soils. If these laboratory studies have application to field conditions it would be necessary to increase the population of PCB degraders (and thus the rate of PCB degradation) and the solubility of PCB in soil. Results of these studies also suggest that anaerobic conditions may increase the amount of PCB depleted in soil and sediment. Since methods for increasing the population of PCB degraders and the solubility of PCB in soil and sediment remain to be determined it is suggested that future research focus on these areas. It is clear from the present and previous studies that microorganisms in soil and sediment are capable of degrading PCB. However before the regulatory agencies accept the technology of in situ treatment of PCB in soil and sediment by microorganisms their will be a requirement for demonstrating

significantly higher rates of PCB degradation.
REFERENCES

1. Alexander, M. 1981. Biodegradation of chemicals of environmental concern. *Science* 211: 132-138.
2. Cook, A. M., H. Grossenbacher, and R. Huetter. 1983. Isolation and cultivation of microbes with degradative potential. *Experientia* 39: 1191-1198.
3. Ahmed, M., and D. D. Focht. 1973. Degradation of polychlorinated biphenyls by two species of Achromobacter. *Can. J. Microbiol.* 19:45-52.
4. Fedorak, P. M., and D. W. S. Westlake. 1984. Microbial degradation of alkyl carbazoles in Norman Wells crude oil. *Appl. Environ. Microbiol.* 47: 858-862.
5. Johnson, L. M., and H. W. Talbot, Jr. 1983. Detoxification of pesticides by microbial enzymes. *Experientia* 39: 1236-1246.
6. Kong, H. L., and G. S. Saylor. 1983. Degradation and total mineralization of monohalogenated biphenyls in natural sediment and mixed bacterial culture. *Appl. Environ. Microbiol.* 46: 666-672.
7. Tabak, H. H., S. A. Quave, C. I. Mashni, and E. F. Barth. 1981. Biodegradability studies with organic priority pollutant compounds. *J. WPCF* 53: 1503-1518.
8. Johnston, L. E. 1985. Decontamination and disposal of PCB wastes. *Environ. Health Perspect.* 60: 339-346.
9. Dotson, G. K., R. B. Dean, W. B. Cooke, and B. A. Kenner. 1972. Land spreading, a conserving and non-polluting method of disposing of oily wastes. Report PB 213 749, Ohio Basin Region, Federal Water Pollution Control Administration.
10. Francke, H. C., and F. E. Clark. 1974. Disposal of oil wastes by microbial assimilation. Report Y-1934, Oak Ridge Y-12 Plant, Oak Ridge, Tenn.
11. Kincannon, C. B. 1972. Oily waste disposal by soil cultivation process. Report EPA-R2-72-110, Shell Oil Co. and USEPA.
12. Furukawa, K., and F. Matsumura. 1976. Microbial metabolism of polychlorinated biphenyls. Studies on the relative degradability of polychlorinated biphenyl components by Alkaligenes sp. *J. Agric. Food Chem.* 24: 251-256.

13. Massé, R., F. Messier, L. Ploquin, C. Ayotte, and M. Sylvestre. 1984. Microbial degradation of 4-chlorobiphenyl, a model compound of chlorinated biphenyls. *Appl. Environ. Microbiol.* 47: 947-951.
14. Sayler, G. S., M. Shon, and R. R. Colwell. 1977. Growth of an estuarine *Pseudomonas* sp. on polychlorinated biphenyls. *Microbial Ecology* 3: 241-255.
15. Shiaris, M. P., and G. S. Sayler. 1982. Biotransformation of PCB by natural assemblages of freshwater microorganisms. *Environ. Sci. Technol.* 16: 367-369.
16. Shields, M. S., S. W. Hooper, and G. S. Sayler. 1985. Plasmid-mediated mineralization of 4-chlorobiphenyl. *J. Bacteriol.* 163: 882-889.
17. Becker, C. D., D. A. Neitzel, J. E. Rogers, and D. J. Silveira. 1985. Evaluating hazardous materials for biological treatment. *Nuc. Chem. Waste Management* 5: 183-192.
18. Freitag, D., H. Geyer, W. Klein, A. G. Kraus, E. Lahanatis, and F. Korte. 1979. An approach for comparative screening of the environmental behavior of chemicals. *Ecotox. Environ. Safety* 3: 133-151.
19. Gerike, P., and W. K. Fischer. 1979. A correlation study of biodegradability determinations with various chemicals in various tests. *Ecotox. Environ. Safety* 3: 159-173.
20. Gerike, P., and W. K. Fischer. 1981. A correlation study of biodegradability determinations with various chemicals in various tests. II. Additional results and conclusions. *Ecotox. Environ. Safety* 5: 45-55.
21. Gilbert, P.A. 1978. Biodegradability and the estimation of environmental concentration. *Ecotox. Environ. Safety* 3: 111-115
22. Shelton, D. R., and J. M. Tiedje. 1984. General method for determining anaerobic biodegradation potential. *Appl. Environ. Microbiol.* 47: 850-857.
23. Roberts, P. V., P. L. McCarty, M. Reinhard, and J. Schreiner. 1980. Organic contaminant behavior during groundwater recharge. *J. Water Pollut. Contr. Fed.* 52: 161-172.
24. Biosystems, Inc. Undated brochure.

25. Flathman, R. E., and G. D. Githens. 1985. In situ biological treatment of isopropanol, acetone, and tetrahydrofuran in the soil/groundwater environment. pp. 173-185. In E. K. Nyer (Ed.), Groundwater treatment technology. Van Nostrand Reinhold, New York.
26. Gore, D. 1985. Investigation of subsurface gasoline contamination. pp. 133-146. In E. K. Nyer (Ed.), Groundwater treatment technology. Van Nostrand Reinhold, New York.
27. Heyse, E., S. C. James, and R. Wetzel. 1985. In situ biodegradation of aquifer contaminants at Kelly Air Force Base. Ann. Mtg., American Institute of Chemical Engineers.
28. Nyer, E. K. 1985. Biological treatment of a groundwater contaminated with phenol. pp. 161-172. In E. K. Nyer (Ed.), Groundwater treatment technology. Van Nostrand Reinhold, New York.
29. Wetzel, R. S., S. M. Henry, P. A. Spooner, S. C. James, and E. Heyse. 1985. In situ treatment of contaminated groundwater and soils, Kelly Air Force Base, Texas. U. S. Environmental Protection Agency. Land Disposal of Hazardous Wastes, Proceedings of the Eleventh Annual Research Symposium. Cincinnati, Ohio. p. 97-106.
30. Suflita J. M., Horowitz A., Shelton D. R., Tiedje J. M., 1982. Dehalogenation: A novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science (Wash. D.C.) 218:1115-1117.
31. Furukawa K., Tomizuka N., Kamibayashi A., 1979. "Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls". Appl. Environ. Microbiol. 38:301-310.
32. Hartmann J., Reineke W., Knackmuss H. J., 1979. Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a Pseudomonad. Appl. Environ. Microbiol. 37:421-428.
33. Shelton D. R., Tiedje J. M., 1985. Isolation and Partial Characterization of Bacteria in an Anaerobic Consortium that Mineralizes 3-Chlorobenzoic Acid. Appl. Environ. Microbiol. 48:840-848.
34. Bumpus J. A., Tien M., Wright D., Aust S. D., 1985. Oxidation of Persistent Environmental Pollutants by a White Rot Fungus. Science 228:1434-1436.
35. Gibson D. T., Roberts R. L., Wells M. C., Kobal V. M., 1973. Oxidation of biphenyl by a Bijerinckia species. Biochem. Biophys. Res. Comm. 50:211-215

36. Horowitz A., Suflita J. M., Tiedje J. M., 1983. Reductive dehalogenations of halobenzoates by anaerobic lake sediment microorganisms. Appl. Environ. Microbiol. 45:1459-1465.
37. Suflita J. M., Stout J., Tiedje J. M., 1984. Dechlorination of (2,4,5-trichlorophenoxy) acetic Acid by Anaerobic Microorganisms. J. Agric. Food Chem. 32:118-221.
38. Suflita J. M., Robinson J. A., Tiedje J. M., 1982. Kinetics of Microbial Dehalogenation of Haloaromatic Substrates in Methanogenic Environments. Appl. Environ. Micro. 45:1466-1473.
39. Murthy N. H. K., Kaufman D. D., Fries G. F., 1979. Degradation of pentachlorophenol (PCP) in aerobic and anaerobic soil. J. Environ. Sci. Health B14:1-14.
40. Rogers C. J., Kornel A., Chemical Detoxification of Hazardous Wastes using APEG Treatment. EPA, 1986.
41. Boyd S. A., Shelton D. R., Berry D., Tiedje J. M., 1984. Anaerobic Biodegradation of Phenolic Compounds in Digested Sludge. Appl. Environ. Micro. 46:50-54.
42. Hutzinger O., Roof A. A. M., 1980. Polychlorinated biphenyls and related halogenated compounds. Analytical Tech. Environ. Chem. J. Albaiges, ed. Pergamon Press, Oxford, pp. 167-184.
43. Ballschmiter K., Zell M., 1980. Analysis of polychlorinated biphenyls (PCB) by glass capillary gas chromatography. Fresenius Z. Anal. Chem. 302:20-31.

REMOVAL OF $^{14}\text{CO}_2$ FROM AQUEOUS SOLUTION

FIGURE 1

REMOVAL OF $^{14}\text{CO}_2$ FROM SOIL SUSPENSION

FIGURE 2

REMOVAL OF $^{14}\text{CO}_2$ FROM AQUEOUS SOLUTION

FIGURE 3

QUENCHING OF ^{14}C BY WATER

Effect of phenethylamine

FIGURE 4

MINERALIZATION OF PCBs BY BEAR CREEK COMPOSITE SOIL

FIGURE 5

MINERALIZATION OF 4-CB BY BEAR CREEK LYSIMETER SAMPLES

FIGURE 6

FRACTIONATION OF 4-CB FROM BEAR CREEK LYSIMETER SAMPLES

FIGURE 7

EXTRACTABILITY OF TOB FROM BEAR CREEK LYSIMETER SAMPLES

FIGURE 8

EXTRACTION OF TCB FROM SOIL

Effect of Incubation Time

FIGURE 9

Catabolism of Aro 1254 by microbial fragments

Elution time

Fig 10

Incubation of Arochlor 1254 with microbial cell fragments. Fig. 11

Dechlorination of Arochlor 1254 Fig. 12
by microbial cell fragments.

Dechlorination of Arochlor 1254
by microbial cell fragments. Fig. 13

APPENDIX A

ASSESSMENT OF MINERALIZATION OF PCBs
BY FIELD SAMPLES FROM PCB-CONTAMINATED SITES

PROTOCOL

DRAFT REVISION
JULY 23, 1987

Oak Ridge Research Institute

OBJECTIVES:

The objectives of this study are to:

- (1) Provide information on the potential of organisms in soil samples taken from a PCB-contaminated area to mineralize PCB congeners;
- (2) Provide preliminary information on potential enhancement of PCB biodegradation as a result of nutritional amendment of the samples;
- (3) Carry out material balance studies to provide some indications as to the potential relative rates of biodegradation and loss of PCB congeners in the treatment system;
- (4) Carry out analyses of successive lysimeter samples to determine whether field treatments have had an effect on the capacity of soil microbes to mineralized PCBs.

RATIONALE:

Several authors have reported biodegradation of PCBs by microbes from a variety of natural sources (Ahmed and Focht, 1973; Bedard et al., 1986; Clark et al., 1979; Furukawa and Matsumura, 1976; Kong and Sayler, 1983; Massé et al., 1984; Shiaris and Sayler, 1982; Tabak et al., 1981; Yagi and Sudo, 1980). Degradation typically proceeds via ring cleavage and subsequent production of chlorobenzoic acids, which are in turn metabolized. Complete metabolism of PCBs results in release of some of the constituent carbon as CO₂. Therefore the release of ¹⁴C-labeled CO₂ by test samples is a stringent test for the degradation of ¹⁴C-labeled PCB substrates. In this study, two ¹⁴C-labeled PCB congeners will be used as substrates, and the rates of mineralization by a group of PCB-contaminated field samples will be assessed.

SAMPLES TO BE ASSESSED:

Samples of soil from a PCB deposition area near Bear Creek are to be supplied to ORRI for assessment of potential for mineralization of PCBs. In addition, samples from a series of treatment lysimeters will be supplied at intervals for assessment of PCB biodegradation.

SUMMARY OF METHOD:

¹⁴C-labeled 4-chlorobiphenyl and 2,4,2',4'-tetrachlorobiphenyl will be added to the field samples. After a period of

incubation during which degradation can take place, the headspace above the samples will be collected and the samples will be extracted and analyzed as described below. Concentrations of the two congeners will be compared to determine the effectiveness of biodegradation.

MATERIALS:

Screw-cap sample vials, 2-dram
Teflon-lined septa and caps
21 ga. x 1 1/2" hypodermic needles
3 cc plastic syringes
Tygon tubing, rubber stoppers (OO)
scintillation vials

4-chlorobiphenyl (4CB) and ^{14}C -labeled 4CB
2,4,2',4'-tetrachlorobiphenyl (TCB) and
 ^{14}C -labeled TCB

$\text{Na}_2^{14}\text{CO}_3$

0.6 % glucose solution

0.1 % yeast extract solution

mineral salts medium (Shields et al., 1985):

Composition	(g/l)
NaNO_3	4.0
KH_2PO_4	1.5
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	0.0011
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.01
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.0005

pH = 7.0

Florisil

phenethylamine

1 N HCl

10% Triton X-100

hexane, hexane-ether (1:1)

anhydrous sodium sulfate

scintillation cocktail (Scintiverse E)

PROCEDURE:

Duplicate 1-gram amounts of each sample are placed in sterile 2-dram screw-cap vials equipped with Teflon-lined septum tops. Nutrient additions are made as appropriate, and 10 ul of substrate is added to each vial. The vials are sealed and shaken to mix the contents.

PCBs are added to the samples as concentrated acetone solutions. When added in this way, the PCBs are immediately adsorbed to the cells or soil particles. The amount of each congener added is 1 ug (1 ppm, 10 ul of a 100 ppm solution

containing 10 nCi ^{14}C /ul in 4CB or TCB). The samples are then incubated for 14 days at room temperature.

Nutrient additions are carried out by adding 60 ppm glucose (as a readily degraded carbon and energy source), 10 ppm yeast extract (as a source of vitamins and of organic acids for metabolism), a 1/10 dilution of a mineral salts medium ((Shields et al., 1985) as a source of nitrate, phosphate, sulfate, and trace metals), or individual mineral salts solutions.

A culture of Alcaligenes A5 (Shields et al., 1985) in mineral salts is included as a positive control. A mid-log phase culture growing in mineral salts medium with 4CB is harvested and washed twice with mineral salts medium without substrate. The cells are then suspended in the original volume of mineral salts medium, and 1 ml is placed in each control culture vial. ^{14}C -labeled substrates are added and mineralization is assessed as for the other samples. As negative controls, samples of each type are placed in culture vials and autoclaved before addition of substrate.

INITIAL SAMPLE FROM LYSIMITERS

SAMPLE CULTURES (TO BE DONE IN DUPLICATE)						
NO.	SAMPLE	4CB	TCB	SALTS	GLUC	YE
1	STER SOIL	+				
2	SOIL	+				
3	SOIL	+		+		
4	SOIL	+			+	
5	SOIL	+				+
6	STER <u>Alc.</u> A5	+		+		
7	<u>Alc.</u> A5	+		+		
8	<u>Alc.</u> A5	+		+	+	
9	<u>Alc.</u> A5	+		+		+
10	STER SOIL		+			
11	SOIL		+			
12	SOIL		+	+		
13	SOIL		+		+	
14	SOIL		+			+
15	STER <u>Alc.</u> A5		+	+		
16	<u>Alc.</u> A5		+	+		
17	<u>Alc.</u> A5		+	+	+	
18	<u>Alc.</u> A5		+	+		+

0-TIME CONTROLS FOR SOIL AND BACTERIAL SAMPLES

Following 14 days of incubation, the sample vial is attached

via hypodermic needle to a trap consisting of 0.5 g Florisil in a 3-cc disposable plastic syringe. The Florisil trap is connected by Tygon tubing to a trap consisting of a mixture of 6 ml Scintiverse E and 1.5 ml phenethylamine. A source of air is connected through the septum by inserting a needle or cannula long enough to reach the bottom of the vial. Mineralization by test samples is stopped by the addition by hypodermic syringe of 1 ml of 1 N HCl to soil samples or 0.1 ml of 2 N HCl to water samples. ^{14}C -labeled CO_2 is driven off by purging the sample with air at 8-20 cc/min, delivered by a multichannel peristaltic pump or other controlled source. Air is pumped through the sample for 40 min, as indicated by control experiments described below.

Volatile ^{14}C -labeled organic compounds and CO_2 are trapped by the Florisil column. To recover the trapped CO_2 , the Florisil trap is removed from the vial, and CO_2 is eluted from the column by addition of 2 ml of 0.1 N NaOH, which is drained into the phenethylamine trap. A tube is connected by a rubber stopper and hypodermic needle to the top of the Florisil trap, and air is pumped through the Florisil trap to drive out as much of the dissolved CO_2 as possible. The Florisil is then placed in a scintillation vial with 1.5 ml of phenethylamine and 6 ml of Scintiverse E and counted. ^{14}C -labeled compounds are then extracted from the sample suspension by adding 1/10 volume of 10% Triton X-100 to the suspension, followed by 4 volumes of hexane-ether mixture (1:1). Prior to mixing, a small amount of anhydrous sodium sulfate is added to the water samples, which may have little or no sediment, to prevent formation of a stable emulsion. Samples are shaken vigorously for 20 - 30 min. The phases are separated by centrifugation, and 0.5 ml of the aqueous fraction and 1 ml of the solvent fraction are transferred separately to vials with 1.5 ml of phenethylamine and 6 ml of Scintiverse E for counting.

COMMENTS

SUBSTRATES:

Experiments by Saylor's group and by others have shown that 4-chlorobiphenyl (4CB) can be degraded by microorganisms from diverse sources, and that degradation of 4CB proceeds readily when bacteria which have been adapted to PCBs are added to cultures containing 4CB. Because 4CB is relatively easily degraded, it will be used as a substrate to indicate the existence of biodegradative potential for PCBs. It is likely that if no biodegradation of 4CB can be detected, the potential for biodegradation of a mixture of PCBs will be very low.

In general, the ability of an organism to metabolize PCBs

seems to decrease as the extent of substitution with chloro groups increases (Furukawa and Matsumura, 1976; Furukawa et al., 1978). Therefore, as a more stringent test of biodegradative potential, the ability of the test samples to mineralize a tetrachlorobiphenyl will be assessed. The compound used will be 2,4,2',4'-tetrachlorobiphenyl, with ^{14}C label in both rings. It is not anticipated that hexachlorobiphenyls will be routinely used, because they are extremely recalcitrant to biodegradation.

TIME COURSE:

Experiments by Saylor et al. (Kong and Saylor, 1983; Massé et al., 1984; Saylor et al., 1983; Shields et al., 1985) have shown that significant biodegradation of 4CB by adapted bacteria can be observed in as little as three days of incubation, and Bedard et al. (1986) recommend 24-hr incubations for the more easily degraded congeners. Because the extent of adaptation or ongoing metabolism by the test samples in this project is not known, incubation will be carried out for a total of two weeks. Because control experiments have shown that 4CB is readily volatilized by purging with air, no test vials will be purged until the end of the incubation period.

At 14 days (7 days for some bacterial control samples), the sample will be acidified to release CO_2 bound as carbonate or bicarbonate. Volatile organics and CO_2 will be measured, and the aqueous sample suspension will be extracted with a mixture of Triton X-100 in hexane-ether (Bedard et al., 1986), and the total remaining polar and nonpolar radioactivity will be measured, as will the residual radioactivity bound to the walls and seal of the culture vial.

ANALYSIS OF FRACTIONS:

Assays used to measure PCB degradative competency by the disappearance of Aroclors (commercial PCB mixtures) often produce false-positive results due to volatilization, adsorption, or absorption losses. Incomplete gas chromatographic resolution and uncertain identification of Aroclor peaks often leave the chemical nature of the microbial breakdown of PCBs unclear. In addition, chromatographic procedures are slow and require many control procedures. These problems will be avoided in the present study by using defined ^{14}C -labeled PCB congeners and measuring the release of ^{14}C -labeled CO_2 as the end product of degradation. To ensure that all of the substrate is accounted for, we will employ incubation and extraction methods that minimize the physical loss of PCBs, and we will determine the distribution of metabolites and substrates in the volatile, aqueous, and solvent-extractable phases.

ADSORPTION LOSSES:

PCB congeners have been shown to bind extensively to soil, glass, and plastic. The extraction of residual PCBs from culture tubes may be variable, making quantitation of biodegradation difficult. However, a number of precautions can be taken to reduce adsorption losses of PCBs. Polyethylene components of the culture system readily absorb PCBs (Bedard et al., 1986). The use of Teflon seals reduces adsorption of PCBs to plastic tube caps. In addition, the Florisil trap is to be connected directly via a metal hypodermic needle to the headspace, reducing the contact of volatile PCBs with plastic tubing. Appropriate solvents can elute most of the bound PCB from glass culture tubes. Furukawa et al. (1978) have reported recovery of 88-96% of PCBs added to cultures when the medium was brought to a pH of 1-2 and then extracted with ethyl acetate. Bedard et al. (1986) reported extraction efficiencies from 53% to 94% (depending on the specific congener) using hexane as a solvent, but the efficiency was increased to 93-97% when the solvent contained 1% Triton X-100. Therefore, the procedure to be used in this study will include acidification of the culture and addition of Triton X-100 during the extraction.

FLORISIL TRAPS:

Florisil is the matrix recommended by the USEPA (1982) for cleanup of extracts containing PCBs. According to the method description, when PCBs in hexane are passed through Florisil, they are quantitatively retained, and can be eluted by addition of 6-50% ether in hexane. However, our control experiments have indicated that hexane will elute PCBs from Florisil without the addition of ether. The USEPA method does not specifically state the binding capacity of Florisil for PCBs, so it will be necessary to ensure that breakthrough does not occur under the conditions of the experiment. Appropriate control experiments will be included with the study data to demonstrate the effectiveness of the Florisil traps.

PHENETHYLAMINE TRAPS:

Phenethylamine is widely used as a trap for CO_2 in metabolic assessments. It is an efficient trapping compound, and is readily miscible with scintillation cocktails, providing a high counting efficiency for the trapped CO_2 . Adequate control experiments will be included in the study to demonstrate that CO_2 is quantitatively transferred from the culture vials to the phenethylamine traps and that CO_2 is not lost by trapping in the Florisil traps.

To determine the time required for quantitative transfer of CO_2 from the culture vial to the trap, 10 μl of a solution of $\text{Na}_2^{14}\text{CO}_3$ (10 nci/ μl) will be placed in a culture vial containing sample but no substrate, the vial will be sealed and connected to

the trap, and 1 ml of 0.1 N or 1 N HCl will be added. The headspace will be purged with air at 2 or 10 cc/min, and the trap will be changed at intervals to determine the optimal time of bubbling.

COUNTING EFFICIENCY:

Because the component analytical fractions in this experiment have significantly different solvent matrices, it will be necessary to determine counting efficiencies in each and make the appropriate corrections when calculating the distribution of radioactivity.

Counting efficiencies will be determined by placing known amounts of radioactively labeled CO_2 , substrates, or surrogates in scintillation vials along with the same solvents and scintillants as will be present when these fractions are counted. The counting rate will be compared to the theoretical radioactivity to calculate the counting efficiency. Control experiments have indicated that quenching of ^{14}C radioactivity by water is reduced by the addition of phenethylamine, so all samples will be counted in a mixture of phenethylamine and Scintiverse E.

Quench curves will be constructed by use of channel ratios and external standard ratios. In the case of Florisil and phenethylamine traps, where the sample matrix should not be heterogeneous and total compound should make the main contribution to quenching, a series of vials will be prepared with a constant amount of ^{14}C -labeled compound and increasing amounts of non-labeled compound. These will be counted and the results will be used to construct a quench curve.

The aqueous extract of soil and sediment cultures will be more heterogeneous in composition from sample to sample. For these matrices, a quench curve will be constructed by placing a constant amount of water-soluble ^{14}C -labeled compound in each of a series of scintillation vials and adding a constant final volume of various ratios of water or settled soil suspension. Scintillant containing phenethylamine will be used to count these samples. Curves relating count rate to channel ratios, external standard ratios, and water-extract ratios will be constructed for use in determining the disintegration rate in experimental samples.

TRAPPING EFFICIENCY:

CO_2 - 10 μl of a solution of $\text{Na}_2^{14}\text{CO}_3$ (10 nci/ μl) is added to each of a series of scintillation vials containing field sample but no ^{14}C -labeled substrate. The vials are sealed and connected directly to the phenethylamine traps or to the Florisil-phenethylamine trap series, and HCl is injected as described above. The vials are purged with air and the contents

are extracted as described, and the amounts of ^{14}C in the phenethylamine, Florisil, solvent, and aqueous fractions are determined. The vials are emptied and rinsed with hexane, and any residual bound CO_2 is measured by adding scintillant and counting the vials.

^{14}C -labeled substrates

0-time control - 10 μl of a solution containing ^{14}C -labeled substrate in acetone (100 ppm, 10 nci/ μl) is added to each of a series of scintillation vials containing field samples. The vials are sealed with Teflon liners and connected to the Florisil-phenethylamine trap series, and HCl is injected immediately. The samples are then purged and extracted as described above, and the distribution of ^{14}C -labeled substrate in each fraction determined. The vials are emptied and rinsed with hexane, and the residual bound substrate is measured by adding scintillant and counting the vials.

Florisil trap loading - 10 μl of a solution containing ^{14}C -labeled 4CB in acetone (100 ppm, 10 nci/ μl) is added to each of a series of screw-cap culture vials. The acetone is allowed to evaporate (to prevent elution of the sample from Florisil by the acetone), and the vials are sealed and connected to the Florisil-phenethylamine trap series. The vials are purged with air for 60 min at 10 cc/min. The vial contents are then extracted with hexane-ether containing Triton X-100, and the Florisil traps are eluted with hexane-ether as described above. The distribution of ^{14}C -labeled 4CB in the vials and traps is determined. ^{14}C -4CB in a phenethylamine trap will be taken as an indication of breakthrough from the associated Florisil trap.

REFERENCES:

- Ahmed, M., and D. D. Focht. 1973. Degradation of polychlorinated biphenyls by two species of Achromobacter. Can. J. Microbiol. 19:45-52.
- Bedard, D. L., R. Unterman, L. H. Bopp, M. J. Brennan, M. L. Haberl, and C. Johnson. 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. 51: 761-768.
- Clark, R. R., E. S. K. Chian, and R. A. Griffin. 1979. Degradation of polychlorinated biphenyls by mixed microbial cultures. Appl. Environ. Microbiol. 37: 680-685.
- Furukawa, K., and A. M. Chakrabarty. 1982. Involvement of plasmids in total degradation of chlorinated biphenyls. Appl. Environ. Microbiol. 44: 619-626.
- Furukawa, K., and F. Matsumura. 1976. Microbial metabolism of polychlorinated biphenyls. Studies on the relative degradability of polychlorinated biphenyl components by Alkaligenes sp. J. Agric. Food Chem. 24: 251-256.
- Furukawa, K., K. Tonamura, and A. Kamibayashi. 1978. Effect of chlorine substitution on the biodegradability of polychlorinated biphenyls. Appl. Environ. Microbiol. 35: 223-227.
- Kong, H.-L., and G. S. Sayler. 1983. Degradation and total mineralization of monohalogenated biphenyls in natural sediment and mixed bacterial culture. Appl. Environ. Microbiol. 46: 666-672.
- Marks, T. S., A. R. W. Smith, and A. V. Quirk. 1984. Degradation of 4-chlorobenzoic acid by Arthrobacter sp. Appl. Environ. Microbiol. 48: 1020-1025.
- Massé, R., F. Messier, L. Ploquin, C. Ayotte, and M. Sylvestre. 1984. Microbial degradation of 4-chlorobiphenyl, a model compound of chlorinated biphenyls. Appl. Environ. Microbiol. 47: 947-951.
- Sayler, G. S., H.-L. Kong, and M. S. Shields. 1983. Plasmid-mediated biodegradative fate of monohalogenated biphenyls in facultatively anaerobic sediments. pp. 117-135. In G. S. Omenn and A. Hollaender (Eds.), Genetic Control of Environmental Pollutants. Plenum Press, New York.
- Sayler, G. S., M. Shon, and R. R. Colwell. 1977. Growth of an estuarine Pseudomonas sp. on polychlorinated biphenyls. Microbial Ecology 3: 241-255.

- Shiaria, M. P., and G. S. Sayler. 1982. Biotransformation of PCB by natural assemblages of freshwater microorganisms. Environ. Sci. Technol. 16: 367-369.
- Shields, M. S., S. W. Hooper, and G. S. Sayler. 1985. Plasmid-mediated mineralization of 4-chlorobiphenyl. J. Bacteriol. 163: 882-889.
- Tabak, H. H., S. A. Quave, C. I. Mashni, and E. F. Barth. 1981. Biodegradability studies with organic priority pollutant compounds. J. WPCF 53: 1503-1518.
- USEPA. 1982. Test methods for evaluating solid waste. Physical/chemical methods, 2nd Ed. Document EPA SW-846, 2nd Ed., US Environmental Protection Agency, Washington, D.C.
- Yagi, D., and R. Sudo. 1980. Degradation of polychlorinated biphenyls by microorganisms. J. Water Pollut. Control Fed. 52: 1035-1043.

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