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I am submitting herewith a dissertation written by Michael Peter Shiaris entitled "Interaction of Polychlorinated Biphenyls (PCB) with Natural Microbial Populations in Freshwater Environments." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology.

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INTERACTION OF POLYCHLORINATED BIPHENYLS (PCB) WITH NATURAL MICROBIAL POPULATIONS IN FRESHWATER ENVIRONMENTS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Michael Peter Shiaris

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ABSTRACT

The interaction between polychlorinated biphenyls (PCB) and natural aquatic microbial populations was investigated with respect to the impact of PCB on microbial growth and heterotrophic activity as well as the biodegradative fate of PCB. PCB substrates employed in these studies included Aroclor 1254, (^{14}C)PCB (54% chlorine by weight), 2-chlorobiphenyl, and 2,4'-dichlorobiphenyl. Hexane batch extraction and Tenax-GC column extraction of PCB were evaluated for use with PCB biodegradation assays. Biodegradation was assessed in terms of the environmental removal of PCB from natural waters, PCB metabolism and mineralization, and uptake of PCB by naturally-occurring heterotrophic bacteria. Laboratory and in situ degradation studies demonstrated significant temporal and spatial differences in PCB biodegradation among samples collected seasonally from an oligotrophic reservoir. PCB biodegradation was accomplished primarily through equilibrium partitioning-cellular uptake of PCB and decomposition of at least the lesser chlorinated biphenylic residues, indicated by the accumulation of chlorobenzoic acid and chlorobenzoyl formic acid as well as a dechlorinated acidic residue. Terminal decomposition of PCB to carbon dioxide was not an important component of Aroclor biodegradation as determined by a mineralization assay developed for low water soluble compounds. In virtually all cases, PCB stimulated the growth of heterotrophic reservoir bacteria at substrate concentrations ranging from approximately 4 $\mu g\ l^{-1}$ to 100 $\mu g\ l^{-1}$. Growth stimulation was accompanied by PCB induced production of extra-cellular

slime which may relate to alterations of microbial community structure and PCB accommodation by heterotrophic microorganisms. The overall short-term effects of Aroclor (100 $\mu g\ l^{-1}$) on the glucose uptake and mineralization activity of natural microbial populations was insignificant; however, significant stimulation and inhibition was observed on a sample to sample basis.

Freshwater, microbial populations are capable of degrading PCB mixtures, although at exceedingly slow rates. The extent of degradation is governed by a variety of interacting environmental parameters; however, the size of the heterotrophic bacterial population and the ambient PCB concentration are dominant variables. Biodecomposition in nature conforms to established biphenyl catabolic pathways and recalcitrant metabolites can be expected to accumulate in the environment at low concentrations. Higher chlorinated mixtures, however, are not extensively metabolized by natural microbial communities; therefore, bioaccumulation and cellular binding are the likely primary paths of biodegradation.

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I. INTRODUCTION

Polychlorinated biphenyls (PCBs) are complex industrial mixtures of randomly chlorinated biphenylic residues. Although domestic and foreign production and sales of polychlorinated biphenyls have been severely curtailed, the past widespread industrial usage has resulted in inputs of PCB to the environment. These compounds are chemically inert, acid-alkali resistant (86, 161) and extremely hydrophobic (31). Due to these refractory, bioaccumulatory, and apparent toxic properties (96), PCBs have been recognized as environmental contaminants of global distribution and concern (41, 53, 94, 140, 141, 164-166, 172).

The high degree of chlorine substitution renders PCB mixtures resistant to biodegradation (32, 56-58). Some investigators speculate that many PCB components are virtually non-degradable in the environment (14). The ability of many plants, animals, and bacteria to metabolically transform PCB has been reviewed (158). Evidence of PCB transformation and mineralization by isolated bacteria has been reported (1, 56-58, 93, 150). In addition, PCB biodegradation by enriched mixed bacterial cultures has been demonstrated (32, 95, 160, 162). Consequently, biodegradation may be important in long-term removal of PCB from aquatic systems (151), but little information of naturally occurring freshwater and marine PCB biodegradation exists (28). Most investigators have conducted qualitative studies of PCB biodegradation by bacterial isolates or enriched mixed cultures under laboratory conditions. Biodegradation can be defined as the biologically mediated removal of a compound from the environment by cellular binding, biochemical

transformation, and ultimately mineralization. The nature of PCB degradation with respect to this definition was investigated.

From an ecological perspective, the fate of PCB cannot be evaluated without understanding the impact of the pollutant on the heterotrophic microbial community. The perturbation by the pollutant may affect the decompositional activity of the microflora and, therefore, the fate of the PCB itself. In addition, there is virtually no knowledge of the effect of PCB on other microbial activities such as decomposition of organic materials and nutrient cycling.

In order to assess the potential environmental hazards of a chemical, information is needed on the impact and degradability of the substance in natural habitats. Such a task requires knowledge of the interaction of the pollutant with the abiotic factors, the microbial community, and other biotic components of the environment. Laboratory studies with pure cultures and defined media are valuable for ascertaining potential environmental consequences, however, it is generally difficult to extrapolate results from the controlled laboratory situation to the complex environment. Therefore, information gained from systems simulating environmental conditions, or if feasible, from in situ studies, is needed in accurately predicting the impact and fate of pollutants in the environment.

To this end, it is also necessary to employ, within analytical limits, levels of test contaminant that are realistic with respect to the actual environmental concentrations of the contaminant. In addition, the actual chemical complexity of the contaminant should be part of the experimental design in order to accommodate synergistic

effects that might be encountered in nature. Finally, a specific habitat representing natural unstressed aquatic environments represents, perhaps, the best source of natural communities that will respond to experimental environmental stress without the confounding problem of unrecognized physical or chemical stress.

With these prerogatives in mind, a comprehensive study was undertaken to assess the PCB-bacterial population interaction in freshwater environments. The specific goal of this investigation was to elucidate basic principles governing the PCB-microbe interaction and to provide specific information necessary to predict the fate and effects of PCB in aquatic environments that could be used for developing a model to assess the fate and effect of similar environmental contaminants.

II. REVIEW OF LITERATURE

The fate of aromatic organochlorine pollutants and their effects on microbial community structure and function in aquatic ecosystems is governed by a multitude of complex environmental parameters (36). These parameters can be divided into physico-chemical factors and biological factors, which together comprise the functional unit termed the ecosystem (126). An awareness of the possible effects and interactions of the environmental variables is necessary in order to make accurate predictions of the fate and effects of the pollutant in natural waters.

Physical-chemical factors including temperature, pH, hydrostatic pressure, redox, and salinity have well established effects on the state of the pollutant molecule as well as the functional role of the microbial community (5, 25, 36, 142). In a treatise on the biodegradative fate of recalcitrant molecules in the environment, Alexander (4) stressed an often overlooked point that the environmental conditions must favor the proliferation of degradative organisms as well as the operation of their respective biodegradative enzymes. Due to the interdependency of these factors, subtle changes in one or two parameters will in turn affect the biological component and ultimately the fate of an organic pollutant.

Oxygen availability is among the most important physical factors affecting the biodegradative fate of aromatic compounds. Alexander states (6) "in contrast with degradative processes catalyzed by microbial communities in the presence of O_2 which usually result

ultimately in the conversion of the substrate acted on to CO_2, water and other inorganic products--nonmicrobial processes rarely lead to total conversion of a complex compound to an inorganic state." Complete decomposition of contaminants by microorganisms occurs in the absence of oxygen as well, but the biodegradative metabolites and final products are usually quite different. Biodegradation of the benzene ring is a most poignant example. Aerobic, bacterial, benzene degradation involves molecular oxygen-mediated hydroxylation via catechol followed by cleavage of the benzene ring to form β-ketoadipate, a dicarboxy acid (129) which is further oxidized to acetate or acetyl phosphate plus CO_2 and water (44). In contrast, under anaerobic conditions, benzene can be reductively converted to cyclohexane, cleaved to aliphatic acids and eventually converted to methane (49). Therefore, the degree and rate of degradation, the nature of the metabolites, and the structure of the participating microbial community are dependent on the availability of O_2.

Light is also important to the fate of pollutants in two respects. First, photodecomposition of aromatic pollutants may be the major route of loss of the compound from the aquatic environment (38). Secondly, some photochemical reactions may be of importance to the environmental input of contaminants as well as removal. PCB, for example, can be formed from chlorinated benzenes (163) or from 1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene (115) and is, therefore, a source of the pollutant.

In aquatic systems, countless solid-liquid interfaces (or surfaces) can directly affect the fate of organic compounds (113).

Zobell (184) recognized that sorption of cells to colloidal surfaces such as clays can inhibit microbial activity. Alternatively, stimulation of bacterial growth has been observed by the addition of surfaces like glass beads (75) or chitin (88) in dilute organic media. This aspect is especially important to water-insoluble organochlorine compounds that readily partition to more hydrophobic suspended solids (69, 147, 156).

Other interactions of pollutant molecules with chemical components of aqueous systems have been described. In a process opposite in effect to phase partitioning, aquatic organic materials such as humic and fulvic acids have been reported to solubilize organochlorine pesticides (169). On the other hand, lipophilic hydrocarbons such as PCB can form droplets, limiting their accessibility to microbial biodegradation (4).

The interplay of biological factors on the physico-chemical interactions further complicates the study of the fate and effect of organochlorines in aquatic ecosystems. Biodegradation by pure, mixed, and bacterial cultures have received the most attention and will be reviewed in more detail below with respect to PCBs. However, realistic evaluations or organochlorine fate and effect in nature cannot be deduced from laboratory experiments alone (6). Micro-organism-microorganism interactions such as commensalism, mutualism, competition, antagonism, parasitism, and predation can affect the fate of pollutants (4). For example, Clark et al. (31) reported that mixed bacterial cultures biodegraded PCB mixtures more rapidly and efficiently than pure cultures. Co-oxidation and co-metabolism may

also be significant mechanisms of pollutant removal from the environment (83, 136). Gibson et al. (61) demonstrated co-oxidation of chlorinated toluene by a strain of Pseudomonas putida growing on unsubstituted toluene as a sole carbon and energy source. However, the extent of co-oxidation and co-metabolism of recalcitrant contaminants in nature is not known (136).

PCB Properties and Distribution in the Environment

First commercially produced in 1930, increasing amounts of polychlorinated biphenyls have been used through the 1970's (87), but it was not until 1966 that they were identified as environmental pollutants (89). It was soon realized that PCBs are among the most ubiquitous and persistent contaminants introduced by man (90, 91, 141). Since that time a vast amount of literature concerning the global distribution and environmental hazards of PCBs attests to the public and scientific concern of the PCB pollution problem. Several general reviews summarize this work (67, 77, 87, 94, 99, 134, 138, 161, 165).

Commercial PCB mixtures are sold under several trade names depending on the country of production (85): Kanechlor (Japan), Phenoclor (France), and Clophen (Germany). Monsanto Co. (St. Louis, Missouri), the only North American producer of PCBs, sells them under the trade name Aroclor. Aroclors are complex mixtures of randomly chlorinated biphenyl compounds designated by a four digit suffix (11). The first two digits identify the chemical structures in the mixture (e.g., 12 signifies PCBs) while the last two digits stand for the

average percent by weight chlorine incorporated into the product. For example, Aroclor 1254 is PCB mixture containing 54% chlorine.

Theoretically, 210 individual PCB compounds can exist, but only 100 or so are likely to be found in any single PCB mixture (87). Analysis of PCB mixtures by gas-liquid chromatography and mass spectrometry (79, 102, 146) has shown that each Aroclor mixture is predominated by relatively few PCB isomers. For example, over 75% of the mass of Aroclor 1254 is due to several tetrachlorobiphenyls isomers, 9.0% due to pentachlorobiphenyl, and 8.7% trichlorobiphenyl (79). Aroclor 1242, on the other hand, is predominantly composed of di- and trichlorobiphenyls.

In almost five decades of industrial utilization, the distribution and applications of PCBs have been extensive. Between 1930 and 1970, an estimated 500,000 tons of PCB were sold in the United States alone (87). In 1971, however, Monsanto Co. voluntarily curtailed PCB distribution to uses involving open systems. PCB use is now limited to sealed systems such as capacitors and transformers (127).

PCBs possess properties highly desirable for many industrial applications. They have been used as plasticizers for hotmelt adhesives due to their chemical stability and adhesiveness (94). They possess superior dielectric properties rendering them as ideal electrical insulators in transformers and capacitors (127). Due to their low vapor pressures they were used as lubricants, hydraulic fluids, cutting oils and vacuum diffusion pump oils (94). In addition, PCBs were employed as flame retardants and immersion oils as well as additives in pesticide formulations, varnishes, waxes and printing inks (86, 87).

While the collective chemical properties of chlorinated biphenyls render them valuable for many industrial uses, these same characteristics are highly undesirable upon distribution in the environment. As a result of their chemical stability and halogen content, polychlorinated biphenyls are highly biorecalcitrant and subsequently are stable in the environment. Analysis of environmental PCB residues has indicated that the higher chlorinated forms are particularly stable (14), with gas-chromatographic patterns closely resembling Aroclor 1254 or a combination of Aroclor 1254 and Aroclor 1260 (48, 82, 166).

The hydrophobic nature of PCB also leads to a set of environmental problems. PCBs are virtually insoluble in water (42, 68, 70, 161, 180). Solubility of individual PCB compounds increases with decreasing chlorination (42, 68). Estimates of Aroclor 1254 solubility range from 56 $\mu g\ 1^{-1}$ in distilled water (70) and 300-3000 $\mu g\ 1^{-1}$ in freshwater (180) to 22.7-28.1 $\mu g\ 1^{-1}$ for filtered seawater (171). Therefore, upon introduction into aqueous systems, PCBs readily sorb to sediments (69, 156) and suspended solids (147), including the aquatic biota (34, 133). This sorption phenomenon can result in the concentration of PCB in aquatic food chains (81, 86, 90, 91).

The concentration of ambient PCB contaminant in the water column is typically low. Tatsukawa (159) reported surface seawater levels between 10 and 910 parts-per-billion (ppb) PCB in Suruga Bay, Japan, a relatively polluted estuary. Greichus et al. (65) found 2 ppb PCB in African reservoir surface waters. Veith and Lee (166)

determined PCB levels in the Milwaukee River between 0.02 to 2.07 ppb PCB. Concentrations greater than 250 ppb (18, 45, 81, 105) were detected in water contaminated by sewage effluents. Harvey and Steinhauer (73) determined that pelagic ocean PCB levels ranged between 0.2 to 8.0 parts-per-trillion. As a result of PCB partitioning between the ambient water and solids (42, 133, 156), the concentration of PCB is typically several orders of magnitude higher in the sediments than the overlying water column. For example, Frank et al. (53) reported 252 ppb in Lake Erie sediments and 19 ppb in Lake St. Clair. A U.S. Geological Survey study (87) reported from less than 10 ppb to 50 ppb in 11 Pennsylvania lakes and between 10 to 3200 ppb PCB in 7 Florida lake sediments. Lake Superior sediments were reported to average 170 ppb PCB (46). In addition to aquatic environments, PCB has been recovered from rain and atmospheric samples (46, 116), human tissue and human milk (54, 117, 145, 181), animal tissues (12, 33, 34, 59, 90, 91, 98, 135), plant tissues (131), and soils (157, 159).

Effects of PCB on Bacteria

Due to the concern over PCB toxicity to the human population, the preponderance of PCB toxicological investigation has involved animal models. High concentrations of PCB have been shown to promote hepatocarcinogenesis (97, 140), to inhibit microsomal enzyme synthesis (78) and to produce skin and liver lesions in rats (179). Acute and chronic PCB toxicity towards higher animals (86, 94) have been thoroughly reviewed. PCB contamination of humans, and the Japanese "Yusho" case (77) in particular, have also received considerable attention.

Most of the research on PCB-microbial interactions has been conducted with phytoplankton because of their apparent sensitivity to PCB. Mosser et al. (121) demonstrated that certain algal species were sensitive to PCB at 10 μg l^{-1}, and it was subsequently shown that low PCB concentrations altered phytoplankton species composition (52, 119, 122) and reduced phytoplankton size and production (27, 100, 125). Cell respiratory and photosynthetic apparatus appear to be inhibitory targets (71, 104, 154) although in one case cellular division but not photosynthesis was inhibited (51).

Of the few reports present in the scientific literature concerning bacterial PCB effects, a whole spectrum of bacterial responses have been observed. Wong and Kaiser (174) observed no effect on the growth of lake bacteria in the presence of 500 μg Aroclor 1254 l^{-1}, while Aroclor 1221 and 1242 at the same concentrations were significantly stimulatory. Keil et al. (96) reported a stimulatory response in the growth of Escherichia coli by 10 and 100 μg Aroclor 1242 l^{-1} and a coincident increase in uridine uptake, although total nucleic acid content was unaffected. Sayler et al. (150) demonstrated a slight stimulatory effect on the growth of an estuarine pseudomonad at 10 μg Aroclor 1254 l^{-1} but no effect at 100 μg l^{-1}. Oxygen uptake was similarly stimulated by 200 and 500 μg Aroclor 1254 but not by 1000 and 2000 μg l^{-1}. No effect was observed on glucose utilization by natural bacterial populations in the presence of 100 μg Aroclor 1254 l^{-1} (150). Lipid synthesis by pure cultures of E. coli and Bacteriodes fragilis (64) was not affected in the presence of 10 and 100 μg Aroclor 1242 l^{-1}, although the dry weight harvest of B. fragilis

was almost doubled in the presence of 10 µg PCB 1^{-1}.

In contrast to these studies, Borquin et al. (24) screened 100 estuarine bacterial and fungal isolates for sensitivity to high concentrations of Aroclor 1242. Twenty-six Gram positive and Gram negative bacteria demonstrated varying inhibitory responses in absorbant sensitivity disc assays. The majority of sensitive organisms were amylase and gelatinase producers. A Bacillus sp. was inhibited at 1 mg 1^{-1} and a Pseudomonas sp. was completely inhibited at 5 mg 1^{-1}. Bourquin and Cassidy (23) screened an additional 85 estuarine bacterial isolates. Four sensitive strains, an Achromobacter sp., Bacillus sp., Corynebacterium sp. and a Pseudomonas sp., were further examined in broth containing 10 mg Aroclor 1242 1^{-1}. Extended lag periods in the presence of PCB indicated a bacteriostatic mechanism of inhibition for all strains. These authors did not, however, report possible stimulatory phenomena.

Blakemore and Carey (21) exposed 17 marine bacterial isolates to 1 to 100 µg Aroclor 1254 1^{-1}. Only 2, a pseudomonad and a tetrad-forming coccus, remained sensitive upon subculturing in 10 µg PCB 1^{-1}. Inhibition of growth or cell division was reversibly dose-dependent. Growth inhibitory PCB concentrations did not inhibit respiration. Further investigation of the PCB effect on the pseudomonad (20) indicated adenine uptake and incorporation into DNA by the cells, and not protein synthesis, was inhibited. Examination of adenine pools suggested impaired transport of the DNA precursor into the cell.

In a different vein, Wyndham et al. (176) reported that 4-chlorobiphenyl was highly mutagenic for the frameshift mutant

Salmonella typhimurine strain TA1538 as employed in the Ames bacterial test for mutagenesis (10). Mutagenicity was shown also to increase with decreasing chlorination. Consequently, Aroclor 1254 was shown to have only weak mutagenic characteristics.

PCB Biodegradation

Biodegradation, in addition to photodecomposition, is potentially the most important process of PCB removal from the global ecosystem (138). With respect to biodegradation, two areas, animal metabolism and microbial metabolism have received considerable attention. Several recent reviews (86, 114, 158, 177) adequately summarize the abounding literature concerning PCB biodegradation by animals. Briefly, PCBs are readily absorbed by most animals. The less chlorinated forms are metabolized, via hydroxylation, while the higher chlorinated components accumulate in lipid tissues. Concern exists over the potential toxicity of metabolites and storage of PCBs in the body.

A related area of interest that merits some mention is the PCB induction of specific liver enzymes. Alveres et al. (7) first recognized that PCBs are potent inducers of the cytochrome P-448- and P-450-associated, drug-metabolizing enzymes in animals. It is now well established that small amounts of PCB, introduced even by skin contact, can greatly elevate drug-metabolizing hydroxylases in a variety of animals (8, 26, 63, 112, 173).

Microbial metabolism of PCBs has received attention because of the relationship of metabolism to environmental breakdown of PCB

in soils, sediments and waters. Ahmed and Focht (1) first demonstrated bacterial decomposition of PCB by two species of Achromobacter isolated from PCB-enriched sewage effluent. Subsequently, several bacterial strains have been shown to degrade polychlorinated biphenyls. Gibson et al. (60) employed an active biphenyl-degrading Beijerinckia mutant to study the metabolic pathway of biphenyl decomposition. Active degradation and mineralization of hexachlorobiphenyl by a Pseudomonas sp. was reported by Sayler et al. (150). Furukawa et al. (56, 57) evaluated the ability of an Alcaligenes sp. and an Acinetobacter sp. to degrade 31 different PCB compounds. Baxter et al. (15) presented evidence of Aroclor 1242 degradation by Nocardia sp. and Pseudomonas sp. Aroclor 1242 degradation was also observed by Kaiser and Wong (93) employing an unidentified bacterium isolated from a freshwater harbor.

Concern of PCB fate in natural environments and interest in developing PCB-degrading wastewater systems has stimulated studies of PCB degradation by mixed bacterial culture. Clark et al. (32) investigated Aroclor 1242 degradation by three different PCB-enriched mixed cultures isolated from soil and river sediment. These cultures, composed predominately of Alcaligenes odorans, Alcaligenes denitrificans, and an unidentified Gram-negative bacterium, actively degraded less chlorinated components of the Aroclor. A co-metabolic degradation mechanism was suspected when acetate was added to cultures. Ballschmiter and co-workers (13) isolated a mixed bacterial culture from PCB-enriched garden soil capable of converting PCB compounds to their respective chlorinated biphenylols (123) and chlorinated benzoic acids (13).

For a more direct assessment of the PCB fate in wastewaters, several investigators have evaluated the PCB-degrading activity of activated sludge. Tucker et al. (160) reported considerable biodegradation of Aroclor mixtures utilizing non-PCB-enriched activated sludge. In a 48-hour period, observed degradation of Aroclors 1221, 1242, and 1254 was 81, 26, and 15% at initial PCB concentrations of 1 mg l^{-1}. Similarly, Tulp et al. (162) detected metabolic products of 4,4'-dichlorobiphenyl degradation by activated sludge, however, detectable PCB decomposition was suppressed by the addition of alternative carbon sources. Therefore, they concluded that PCB biodegradation is not significant in wastewater facilities. This view is supported by other investigators. Kaneko et al. (95) could not detect appreciable biodegradation of Kaneclor 500 added at low concentrations (1.5 and 10 µg l^{-1}) to PCB-acclimated activated sludge although O_2-uptake was greatly stimulated. Similarly, Herbst et al. (75) did not detect pentachlorobiphenyl (200-400 µg kg^{-1} activated sludge) biodegradation in a 6-hour incubation; however, 1% biodegradation of trichlorobiphenyl was observed. Much of the conflicting evidence concerning activated sludge PCB biodegradation is most likely due to the wide range of PCB concentrations and formulations as well as the variable nature of the activated sludge and incubation times. Therefore, it is difficult to compare independent studies.

A paucity of information exists concerning the biodegradation of PCB by natural bacterial populations. Wong and Kaiser (174) presented preliminary evidence that Aroclor 1221 and 1242, but not 1254, was utilized as a sole carbon and energy source by natural lake

microorganisms. More conclusive evidence of aerobic (^{3}H)Aroclor 1254 degradation by natural marine bacterial populations was reported by Carey and Harvey (28). Trichlorobiphenyl was degraded to the same extent, 1.4%, whether 0.39 μg l^{-1} or 1.6 mg l^{-1} were employed but no biodegradation was observed in anaerobic sea muds. Finally, in studies related to PCB biodegradation in nature, Sayler et al. (151) enumerated potential PCB-degrading organisms in estuarine and marine environments. Possible degraders were isolated from all samples, but did not correlate with concentrations of in situ PCB pollution.

Concern over the environmental consequences of PCB metabolite accumulation has stimulated investigation of the catabolic fate of PCB. A conventional pathway of aromatic hydrocarbon degradation (40, 60) based on metabolic intermediates isolated from PCB biodegradation has been proposed. Ahmed and Focht (1) isolated benzoic acid and chlorobenzoic acid metabolites from biphenyl and 4-chlorobiphenyl biodegradation, respectively. Subsequent investigations employing pure (56-58, 128) and mixed bacterial cultures (13, 162) have verified chlorinated benzoic acid accumulation during PCB biodegradation. In addition, Furukawa et al. (58) identified di-, tri-, and tetrachlorinated benzoic acids from the correspondingly chlorinated PCB compounds.

Other metabolites isolated from PCB degradation include chlorinated biphenyldiols (162) and chlorinated biphenyls (123). Ohmori et al. (128) identified γ-benzoyl butyric acid as a metabolite of 2-chlorobiphenyl. Furukawa and Matsumura (56) demonstrated that PCB degradation to chlorinated benzoic acid proceed via a chlorinated

derivative of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, which suggests that degradation proceeds through 1,2-meta-ring fission, a known pathway for biphenyl catabolism (29, 110).

Environmental PCB residues display a general pattern of predominance by compounds containing 5 or more chlorine atoms per biphenyl molecule (48, 82, 98) even though the majority of PCB preparations sold in the United States contained mainly the lesser chlorinated PCB compounds (87). This is compelling evidence that the higher chlorinated PCB residues are more resistant to environmental degradation than the lesser chlorinated molecules (160). This view is strongly supported by a variety of pure (15, 56-58, 93) and mixed bacterial culture (32, 160, 161, 174) work which demonstrate that bacteria preferentially degrade the less chlorinated compounds.

In addition to the effects of the number of chlorines on PCB biodegradation, Furukawa et al. (58) conducted a detailed investigation on the effect of the steric chlorine position on PCB biodegradation. PCBs containing two chlorines on either of the ortho positions of one ring or an ortho position on each ring were less biodegradable than other dichlorobiphenyl isomers. Also, ring fission occurred preferentially on the non-chlorinated or lesser chlorinated ring. Clark et al. (32) presented confirming evidence that the chlorine position is critical to bacterial biodegradability. The rate of PCB biodegradation by mixed bacterial culture was significantly affected by the isomeric form of the PCB molecule.

Only a handful of investigators have attempted to apply natural conditions, although the long-range objectives in most of these

studies was to predict the fate of PCB in nature. Crawford et al. (37) added (^{14}C)hexachlorobiphenyl to soil samples in order to evaluate natural PCB mineralization activity. Significant $^{14}CO_2$ evolution was not detected in any natural samples. On the other hand, Carey and Harvey (28) detected PCB metabolites in marine samples containing (^{3}H)Aroclor 1254 incubated in the laboratory. Similarly, Wong and Kaiser (174) reported biodegradative removal of Aroclors 1221 and 1242, but not 1254, from laboratory-incubated lake samples.

In a different experimental approach, Sayler et al. (151) determined the occurrence and distribution of potential PCB degraders in marine and estuarine environments. PCB degraders were isolated from all samples examined and presumptively identified as either Pseudomonas spp. or Vibrio spp. PCB resistant, or degrading organisms correlated with nutritional factors governing microbial growth but not with ambient PCB concentrations. It was suggested that many nutritional and physical variables play a role in governing the size and composition of microbial populations capable of degrading PCB.

III. OBJECTIVES

It is apparent from the diversity of information on the fate and effects of PCB in natural systems that PCB's do interact with a variety of natural microbial populations. However, much of this information is inadequately related to or applied to natural systems themselves. Consequently, an accurate prediction of the true environmental fate and effects of PCB cannot be made from previous investigations which have sought to examine, primarily, the "potential" fate and effects of PCB contamination in natural environments.

The objectives of this study were designed to integrate this diffuse body of data and to place it in a real world perspective with respect to environmental contamination. The specific objectives of this investigation were five-fold and included:

(1) A determination of the environmental rate of PCB degradation in freshwater environments.

(2) An assessment of <u>in situ</u> PCB biodegradation.

(3) An elucidation of important environmental parameters affecting PCB biodegradation.

(4) The determination of biodegradative fate of PCB.

(5) The determination of the impact of PCB on the growth and heterotrophic activity of microbial populations.

IV. MATERIALS AND METHODS

Sampling

The majority of field and laboratory studies were performed with natural microbial populations obtained from Center Hill Reservoir (CHR), located in central Tennessee (Fig. 1). CHR was chosen for its relatively oligotrophic condition and virtual relief from industrial and domestic wastes. In addition, Tech Aqua Biological Field Station, complete with laboratory facilities, is conveniently located near the center of the reservoir. A U.S. Army Corps of Engineers impoundment of the Caney Fork River, CHR lies within the eastern highland rim of Tennessee at an elevation of 199 m above sea level. Maximum reservoir depths approach 77 m. The reservoir has a relatively undeveloped and restricted shoreline exceeding 640 km in length and drains a 5.38 x 10^5 hectare basin. Most of the drainage basin is composed of a second-growth deciduous forest.

Samples were collected from 7 sample sites (Fig. 1) on CHR between February 1977 and August 1978. Sample sites, ranging a distance of 12 km across the reservoir and encompassing both major arms of the reservoir, were selected to represent different reservoir microhabitats. A brief description of each site is given:

Site 1. at the fork of the Falling Water River and Taylor Creek (farthest upstream site), 1.2 km above Cookeville Boat Dock.

Site 2. the downstream Falling Water River site, 4.4 km below Cookeville Boat Dock.

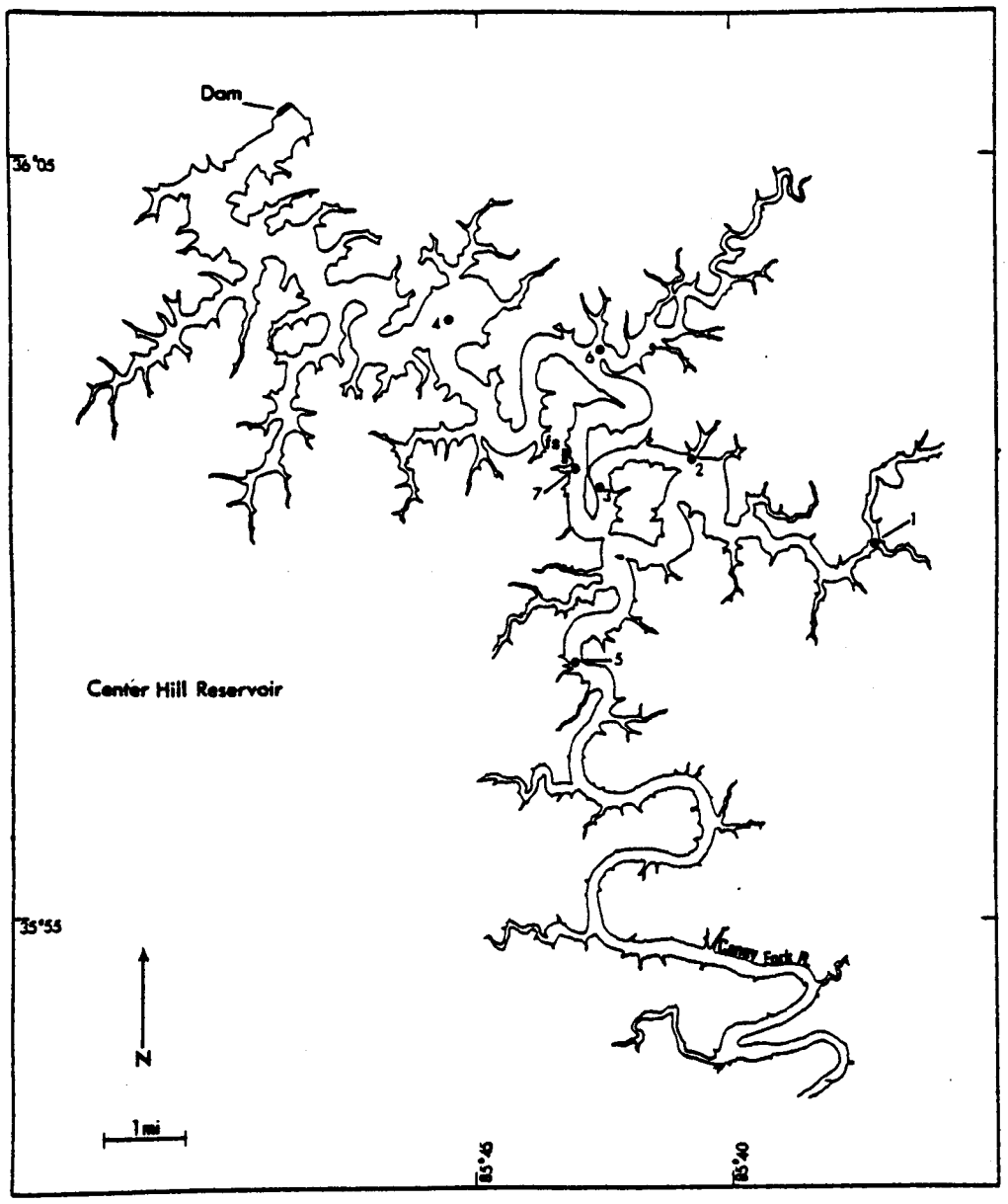

Fig. 1. Center Hill Reservoir, Tennessee site locations for samples collected from February to December, 1977. Field station (fs) facilities are located adjacent to Site 7.

Site 3. A deep (> 30 m) mid-reservoir site 3 m from edge of shear shale cliff on island, approximately 1.5 km from Tech Aqua Station.

Site 4. A deep (> 30 m) site with a relatively open area, downstream from all other sites (represents a composite site), 7 km below Hurricane Bridge.

Site 5. Sligo site at mouth of cove on the Caney Fork River, 1 km above Sligo Boat Dock.

Site 6. Mine Lick Creek site at mouth of creek and main body of reservoir.

Site 7. A small, relatively protected cove fed by a small stream within 300 m of the field station.

Sampling was performed from a gasoline-powered motor boat allowing rapid access to the different sites. All samples were taken 1 meter below the water surface using a Niskin sterile bag sampler (General Oceanics, Miami, Fla.). The samples, ranging in size between 1 to 2 liters, were returned to the shore-based laboratory facilities (Fig. 1) and sample processing was initiated within 1 hour of sample collection. In the interim, samples were maintained at ambient water temperature.

Fort Loudoun Reservoir (FLR), located in Knoxville, Tennessee, was also used as a source of a natural mixed bacterial populations. In contrast to CHR, FLR is a eutrophic body of water, which is moderately polluted by urban, industrial, and agricultural sources. The only samples collected were taken off a dock in Bicentennial Park on the northern bank of the reservoir in the same manner described

above. Sample processing was begun within 15 minutes of sampling.

Analytical Methods

Environmental Parameters

Various field sampling parameters were taken at the time of sampling. Water temperature and conductivity were determined with a Model 33 YSI conductivity meter (Yellow Springs Instrument Co., Yellow Springs, Ohio). Dissolved oxygen (DO) was determined with a YSI DO meter. The O_2-permeable membrane was routinely replaced prior to sampling. An index of water transparency was obtained with the aid of a Secchi disk (Wildlife Supply Co., Saginaw, Mich.). The Secchi disk was secured to a calibrated line and lowered into the water beyond visibility. Upon retrieval (in the shade), water transparency was defined as the depth at which the disk was again visible to the naked eye. The pH measurements were obtained with a portable Orion Model 407A pH meter (Orion Research, Inc., Cambridge, Mass.).

Phosphorus, nitrate, suspended sediments, and dissolved organic carbon (next section) were determined in the laboratory. The colorimetric, ascorbic acid reduction method for total phosphorus was performed according to EPA standard methods (47). Nitrate concentrations were measured with the aid of an Orion 93-07 nitrate specific ion electrode.

For suspended solids determination, 0.45 μm pore-size Nucleopore (Nucleopore Corp., Pleasanton, Ca.) membranes were conditioned by prefiltering with approximately 100 ml volumes of distilled water.

The filters were dried in a 100 C oven, cooled in a dessicator, numbered and weighed on an analytical pan balance (tare weight). Triplicate 100 ml samples of reservoir water were filtered through the membranes to separate the solids from the water. The filters were redried and weighed. Total suspended solids were calculated by subtracting tare weight from final weight.

Determination of Dissolved Organic Carbon

Heterotrophic bacterial growth and activity are greatly dependent on dissolved organic carbon (DOC) concentrations (148). Therefore, it was felt that DOC might be important in controlling PCB biodegradation and interacting with microbial populations. Routine field samples were prefiltered through a 0.45 μm pore-sized, Nucleopore, membrane filter to remove particulate matter. The filtrates were collected in sterile 13 x 100 mm screw-cap test tubes and stored in a freezer. DOC was determined with the aid of a Model 215A Total Organic Analyzer (Beckman Instruments, Inc., Fullerton, Ca.) equipped with an infra-red detector. The instrument (sensitivity range, 1-100 mg DOC l^{-1}) is based on detection of carbon as carbon dioxide from a high temperature combustion column (total carbon) and a low temperature column (total inorganic carbon). The high temperature column was calibrated with a standard stock solution of sodium biphthalate (100 mg l^{-1}) and the low temperature column was calibrated with a standard stock solution of sodium bicarbonate (100 mg l^{-1}). To determine the DOC content of the reservoir samples, duplicate injections with a Hamilton CR700 Constant Rate syringe (Hamilton Co., Reno, Nev.) were performed in the total organic carbon column

(combustion temperature, 500 C) and the inorganic carbon column (temperature, 50 C). DOC was calculated as follows:

DOC = total organic carbon - inorganic carbon

Due to instrument down-time, the DOC determinations for the soil extract and spent culture medium used in the Tenax extraction studies were performed by a modification of the wet persulfate combustion technique as described by Gilmour et al. (62). The combustion was performed in 70 ml glass serum bottles equipped with red-rubber serum stoppers. The stoppers were fitted with plastic center wells (Kontes, Vineland, N.J.). A 45 ml DOC sample was added to each bottle, followed by 0.15 ml concentrated H_2SO_4 and 0.2 ml of 0.4% $AgNO_3$. Each bottle was heated to approximately 60 C on a hot plate, at which time 1.0 g potassium persulfate was added to the sample and 0.5 ml of 2 N NaOH was added to the center well. The samples were then boiled for 30 minutes, liberating CO_2 to the NaOH trap.

Carbon dioxide resulting from the persulfate oxidation of DOC was determined by pH titration with an Orion Model 407A pH meter. The center well NaOH was transferred to a 50 ml beaker (facilitated by distilled water rinsing). Next, the pH of the NaOH trap was reduced to pH 8.35 by the addition of 0.2 N H_2SO_4 thereby converting all inorganic carbon to bicarbonate. A second stage titration, employing 1/12 N H_2SO_4 to reduce the pH from 8.35 to 4.6, converted the bicarbonate to free CO_2. One ml of 1/2 N H_2SO_4 is equivalent to 3.67 mg of CO_2 or 1.0 mg of carbon.

Assessment of Bacterial Populations and Media Employed

A low nutrient medium was desired for enumeration of the heterotrophic reservoir bacteria. Other investigators have reported that oligotrophic bacteria (i.e., bacteria isolated on low nutrient agar) are the most numerous bacterial group in low nutrient or non-eutrophic, aquatic systems (111). Therefore, yeast-extract peptone glucose (YEPG) agar, containing only 0.34% (w/v) available organic carbon, was selected for CHR bacterial counts. A second medium, PCB agar, was also employed (149). Because 1 g of Aroclor 1254 was incorporated to a liter of PCB agar, it was assumed that PCB agar counts represented PCB resistant bacteria as well as potential PCB-degrading organisms. The formulation of all media utilized in these studies is described in Appendix A.

Reservoir samples were serially diluted in quarter-strength Ringer's buffer (Appendix A) and spread plated according to accepted microbiological technique. YEPG agar and PCB agar plates were incubated at 25 C and counted at 2 and 4 weeks unless otherwise noted. Only plates containing between 30 to 300 colonies were counted.

Chemicals

For reasons previously discussed, Aroclor 1254 (Monsanto Co., St. Louis, Mo.) was the PCB mixture of choice for all experimentation unless stated otherwise. At room temperature, Aroclor 1254 is a colorless, translucent, viscous oil. It has a specific gravity of 1.505 at 15.5 C (86). It is soluble in a wide variety of organic solvents including alcohols and esters but not in water, glycerine or 40%

formaldehyde (86). For more detailed chemical specifications, Hutzinger et al. (86) provide a thorough summary.

Stock acetone solutions of Aroclor 1254 were routinely prepared prior to utilization in order to prevent photodecomposition and evaporative losses. Stock solutions for gas chromatography were made in hexane rather than acetone. The solutions were prepared in aluminum foil-covered volumetric flasks and dispensed during experiments via 10 or 100 µl pipettes.

All organic solvents (i.e., acetone, amyl acetate, ethyl acetate, methanol, and benzene) were pesticide-grade (Fisher Scientific Co.) except for hexane. Glass-distilled, pesticide-grade hexane from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.) was found to be superior in purity upon examination by electron-capture gas chromatography and was, therefore, employed for all PCB extraction and glassware cleanup.

Two analytical polymeric resins were employed for PCB extraction and absorption. Tenax-GC (Applied Sciences Laboratories, Inc., State College, Pa.) 60/80 mesh polymeric beads were used as an extraction resin for residual PCB. Tenax-GC, a diphenylphenylene oxide polymer, was rinsed with acetone and hexane prior to use. XAD-4 (Supelco, Inc., Bellefonte, Pa.), a styrene/divinyl benzene co-polymer bead, was employed in PCB mineralization experiments. Before use, the XAD resin was powdered with a mortar and pestle, washed five times in methanol, and rinsed exhaustively with distilled water. Stock XAD was stored in distilled water (17.1%; w/v) to assure wetability.

Other materials employed in mineralization experiments were:

Kaolinite, an acid cleaned kaolin clay (Fisher); microglass beads, 10-30 μm diameter (Cataphote Corp., Jackson, Miss.); Sephadex G-25 Fine, 20-80 μm particle size (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.); and polyurethane foam. The polyurethane foam was prepared by mincing (approximately 1 mm squares) and washing 6 times in methanol and distilled water.

Two pure chlorobiphenyl compounds, 2-chlorobiphenyl and 2,4'-dichlorobiphenyl (Analabs, Inc., North Haven, Conn.), were used as biodegradation substrates and standards. Their chemical purity (> 98%) was confirmed by electron-capture gas-liquid chromatography. Benzoylformic acid (Aldrich Chemical Co., Inc., Milwaukee, Wisc.), para-, meta-, and ortho-chlorobenzoic acid (Pflatz and Bauer, Stanford, Conn.), and 4-chloro-4-biphenylol and 4,4-dichloro-3,3-biphenydiol (RFR Corp., Hope, R.I.) were employed without purification as standards for thin layer chromatography and mass spectrometry.

Several radiochemicals were employed in mineralization, bioaccumulation, and heterotrophic uptake studies. (U-^{14}C)polychlorinated biphenyl (54% chlorine by weight), specific activity of 31.3 mCi mmol^{-1} (New England Nuclear, Boston, Mass.), was employed in mineralization studies, while (U-^{14}C)polychlorinated biphenyl (50% chlorine by weight, specific activity of 37 mCi mmol^{-1}) was used for bioaccumulation studies. (1-^{14}C)Naphthalene (Amersham), specific activity of 39.1 mCi mg^{-1}, was employed for naphthalene mineralization studies. The (U-^{14}C)glucose (NEN or Amersham), specific activity ranging from 24.5 to 1820 mCi mg^{-1}, was the substrate for heterotrophic uptake studies.

It was necessary to purify the hydrocarbon radiochemicals

prior to use. Florisil^{R} (Fisher), a standardized magnesium-silica gel, has been used successfully in the past for purification of both PCB (86) and hydrophobic aromatic compounds (74). Florisil was activated by heating to 650 C for 1 h. A 3 inch layer of activated Florisil topped with a 1 inch anhydrous sodium sulfate layer was packed between 2 glass wool plugs in a Pasteur pipette. (^{14}C)PCB or (^{14}C)naphthalene in hexane was placed on the top of the pipette column and diluted with 5 ml of hexane. Polar impurities were adsorbed to the Florisil while the PCB or naphthalene flowed through with a hexane rinse. Chemical purity was confirmed by gas chromatography.

Finally, to insure chemical purity, all glassware employed in PCB studies was thoroughly washed with detergent, rinsed three times with tap water, three times with deionized water, twice with acetone, twice with hexane and dried overnight in a 300 C forced-air oven.

Scintillation Counting

All scintillation counting was performed in plastic, disposable, scintillation vials containing 15 ml of scintillation cocktail. Aqueous samples were placed in 0.8% Omnifluor (NEN) in dioxane and non-aqueous samples were placed in 0.4% Omnifluor in toluene. Vials were counted in a Packard Tricarb liquid scintillation counter or a Tracor Analytic Model 6892 scintillation counter. A counting error preset was used to assure less than 1% counting error. Counting efficiency (75% to 94% for both counters) was determined from a standard, channels-ratio, quench curve prepared by the internal standard technique for each scintillation cocktail.

Thin Layer Chromatography

Thin layer chromatography (TLC) was employed as a preparative system in the PCB metabolite study. It has been well documented that hydrophobic PCB compounds are biologically converted to polar metabolites by plants, animals, and bacteria (86, 114, 158, 177). The purpose of the TLC system, therefore, was to separate potential PCB metabolites from each other as well as from PCB. Aroclor 1254, 2-chlorobiphenyl, chlorobenzoic acid, 4-chloro-4-biphenylol, and 4,4'-dichloro-3,3'-biphenydiol were used as standards. A solvent system was developed which achieved maximum separation of standard compounds. A solution of benzene:90% methanol:acetic acid (90:9:1) was found to give adequate resolution and was used for further chromatography.

PCB metabolite extracts and chemical standards dissolved in 0.1 to 1.5 ml volumes of ethyl acetate were applied to TLC plates with the aid of a Kontes TLC plate spotter. Kontes LQF Chromoflex^{R} pre-coated fluorescent TLC plates (20 x 20 cm) were used. Aromatic ring-containing PCB metabolites can be visualized as dark spots against the fluorescent background. TLC plates were developed inside a solvent-saturated, TLC, developing tank (Kontes), in a 2-hour processing time. After drying, possible metabolite spots were scraped off and redissolved in 0.1 ml ethyl acetate for mass spectrometry.

Mass Spectrometry

Suspected metabolites were characterized by direct probe mass spectrometry employing a Hewlett-Packard 5980A mass spectrophotometer. The compounds were impacted with 70 eV electrons. Mass spectra were

recorded on a computer disk for later analysis against a National Institute of Health (NIH) library of mass spectra. Spectra of authentic standards were obtained under identical instrument conditions and matched to metabolite spectra for confirmation of identity.

Statistical Analysis

Raw sampling data, plate count data, heterotrophic uptake velocity data, and biodegradation data were subject to computer analysis using a DEC 10, IBM 360/5-370/148 computer system. Statistical analysis subprograms for analysis of variance, multiple correlation and linear regression were derived from Statistical Package for the Social Sciences (124). Significance limits were set at the 95% level.

Analysis of PCB Residue

Solvent Batch Extraction

Concern over the environmental hazards of PCB has stimulated the development of a large array of analytical techniques for the extraction and concentration of aquatic PCB residues. Some of these techniques include solvent batch extraction (166), membrane filter adsorption (103), urethane foam plug extraction (17), XAD resin extraction (35), and Tenax-GC resin extraction (106). For aqueous samples, hexane batch extraction is the preferred method, even though it can be quite cumbersome (86).

Since extraction efficiency and precision are most important for biodegradation assays, batch extraction with hexane was employed.

Equal volumes of hexane were added directly to degradation flasks, vigorously shaken 1 hour on a wrist-action shaker, and placed in a biodegradation flask and reextracted with another equal volume of hexane. The two hexane phases were combined in a Kuderna-Danish reflux apparatus (Kontes) and reduced to approximately 10 ml by gentle refluxing in a 70 C water bath. The extract was further concentrated to 2 ml by heating in a graduated Kuderna-Danish concentrator tube containing between 0.2 to 0.5 g anhydrous sodium sulfate. No further cleanup was required prior to gas chromatography. Extracts were refrigerated in 13 x 100 mm, Teflon-lined screw-capped tubes.

Tenax Extraction

Although hexane batch extraction of PCB was effective, it was noted that a rapid, relatively inexpensive, yet efficient extraction procedure would greatly facilitate the processing of large numbers of biodegradation samples. Several reports have indicated that Tenax-GC may be of use in extraction of organic compounds (167) including aromatic pollutants (106). A Tenax-GC extraction technique was developed and evaluated for this purpose (153).

A 60/80 mesh, Tenax-GC, extraction column was prepared by packing a long stem (15 cm) glass filtration funnel with 10 cm of Tenax-GC (Fig. 2). The extraction polymer was held in place with pyrex glass wool plugs at either end. The final dimensions of the column were 10 cm x 0.4 cm. The column was inserted into a mini-adapter (Ace Glass, Inc., Vineland, N.J.) and fitted with a Teflon O-ring seal and vacuum adapter (threaded female port with a 4/40 ground class male

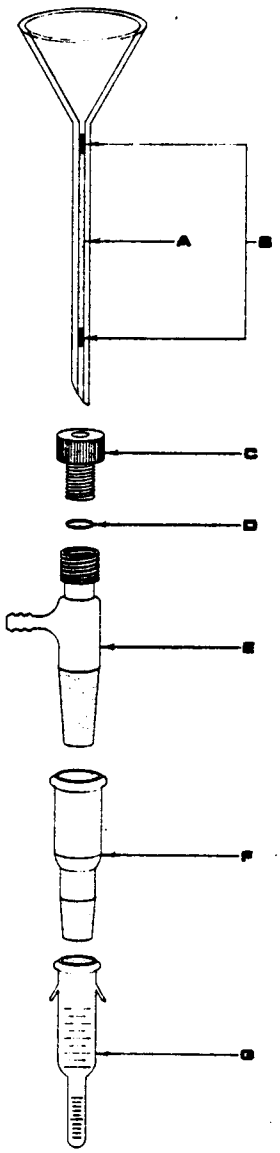

Fig. 2. Tenax extraction apparatus. Components: a, 10 cm X 4mm Tenax-GC column; b, pyrex glass wool plugs; c,d,e, nylon fitting, teflon O-ring, and vacuum adapter (mini-adapter, Ace Glass Co.); f, 24/40-19/22, ground glass adapter; and g, 15 ml concentrator tube (Kontes).

port). A 15 ml concentration tube (Kontes) with a 24/40 ground glass neck was used as a receiving vessel for the column eluate. A negative pressure of 5 psi was applied via vacuum pump to the column to facilitate the extraction procedure.

Preparation of Aroclor 1254-saturated water. In order to assess the PCB extraction efficiency of this technique, a saturated aqueous solution of Aroclor 1254 was prepared in the following manner: Acetone-washed 4.5 glass carboys were filled with 3.0 1 of distilled water. One gram of Aroclor 1254 was added to the carboy containing a Teflon stir bar. The carboy was sealed with an aluminum foil-covered rubber stopper and autoclaved for 1 hour at 120 C. The carboy was then placed on a magnetic stirring plate and stirred for a period of 2 months at room temperature. It was allowed to equilibrate unstirred for an additional 2 months at which time saturation was assumed complete. Care was taken to avoid exposure of the carboys to sunlight or intense fluorescent light.

Organic matter preparation. In order to study the effects of naturally occurring dissolved organic matter on the efficiency of Tenax extraction, two representative sources of dissolved organic matter were prepared. An extract of soil organic matter was prepared by adding 100 g soil to 1.0 liter of distilled water. The mixture was heated to 80 - 90 C for 1 hour with moderate stirring and residual soil was removed by clarification through cheesecloth. The clarified soil extract was prefiltered through Whatman #1 filter paper and passed through a 0.45 μm Gelman cellulose-acetate membrane filter

(Gelman Instrument Co., Ann Arbor, MI). The resulting extract was sterile and dark golden in color.

A second source of dissolved organic matter was prepared from a spent bacteriological culture medium. Five hundred ml of YEPG broth was inoculated with 10 ml of raw Fort Loudoun Reservoir water. The mixed bacterial culture was incubated for 48 hours at 20 C with constant agitation. The bacterial suspension was pelleted by centrifugation at 3000 x g for 10 min and the spent culture supernatant was filtered through a 0.2 µm Nucleopore membrane filter. The DOC concentrations for the soil extract and the culture media extract were 390 mg C l^{-1} and 970 mg C l^{-1}, respectively.

Tenax extraction efficiency and substrate recovery. Radioactive PCB was employed to determine the net recovery and extraction efficiency of the Tenax extraction technique. Filter sterilized lake water (100 ml) in 250 ml screw-capped Erlenmeyer flasks was dosed with 5 µg of (U-^{14}C)PCB. Following mixing and equilibration, a 1.0 ml aliquot was removed and placed in a scintillation vial for counting. The remaining sample was extracted via the Tenax column and the extracted aqueous sample was again subsampled and counted. The Tenax column was eluted with two 5.0 ml volumes of hexane and an aliquot was removed from each eluate and counted prior to and after evaporative concentration to 1.0 ml.

Effects of aqueous sample loading and dissolved organic matter on Tenax extraction. A series of experiments were performed in order to determine the effects of aqueous sample size and dissolved organic

matter on the recovery of Aroclor 1254 by Tenax extraction. Three volumes, 50, 100, and 200 ml, of PCB-saturated water were extracted using the prescribed technique. Each eluate of triplicate samples for each volume extracted was concentrated to 1.0 ml and was analyzed via GLC for quantitating substrate recovery. For assessing the influence of dissolved organic matter (DOM) on Tenax extraction efficiency, 0.01%, 0.1%, and 5.0% solution of either soil extract (SE) or culture medium extract (CME) were prepared in 100 ml of PCB-saturated water. Extraction, concentration, and quantitation techniques employed were identical to those described vide supra.

Gas-Liquid Chromatography of PCB

Ultra-violet spectrophotometry, high-performance liquid chromatography, ultra-violet spectrofluorimetry, and neutron activation are among the methods employed to quantify PCB, but electron capture gas-liquid chromatography (EC-GLC) is still the most sensitive and practical technique available (146). Difficulties with the procedure do exist. One difficulty is a lack of accuracy. This deficiency arises because EC-GLC is not based on complete resolution of all the chlorinated biphenyl compounds and the quantitive detector responses to the different peaks are unknown (86). This problem, however, is of little consequence in these studies, because precision, and not necessarily accuracy, was critical. One suggested approach to increasing precision is perchlorination which yields solely decachlorobiphenyl (86), but the error from manipulation and preparation time outweigh the advantages. Another difficulty is the choice of

procedures used to quantitate PCB gas chromatograms. Chau and Sampson (30) compared individual peak analysis, mean of all peaks as Aroclor 1254, and total peak height of all peaks as Aroclor 1254, and concluded that the first two methods gave acceptable results. Since a Perkin-Elmer M_2 integrating calculator could be interfaced with the gas chromatograph, the second method was selected as the most precise and expedient.

Aroclor 1254 as well as individual PCB compounds were quantified by injecting 1 to 10 µl of condensed hexane extract into a Perkin-Elmer 3920 gas chromatograph equipped with ^{63}Ni electron capture detector (ECD). The ECD is an extremely sensitive, highly specific detector for applications such as the analysis of organochlorines. It has a linear dynamic range of 10,000:1 and a sensitivity of approximately 1 pg for Aroclor 1254.

The glass column (9 ft. x 1/4 in. O.D. x 2 mm I.D.) was packed with a high resolution liquid phase (5% SE-30) coated on an inert solid supporting phase, Chromosorb WHP 100/120 mesh (Supelco, Inc., Bellefonte, Pa.). SE-30, a methyl silicon fluid, is a general purpose liquid phase with widespread PCB application. The column was conditioned before use by flushing with nitrogen gas at 250 C for 24 hours.

The following parameters were determined after preliminary optimization to achieve baseline stability, minimal drift and maximum PCB peak resolution: Injector temperature, 290 C; column temperature, 240 C isothermal; and ECD temperature, 300 C. Oxygen-scrubbed nitrogen was employed as the carrier gas at an inlet pressure of 70 psi and a flow rate of 60 ml min^{-1}.

An Aroclor 1254 calibration curve was performed routinely as a quantitative standard curve and to assure a linear ECD response. Aroclor 1254 (0.5, 1.0, 5.0 and 10 ng) in hexane was employed as the standard.

PCB Biodegradation

Biodegradation can be defined as the biologically mediated loss of a compound from the environment by cellular binding, biochemical transformation and, ultimately, mineralization. The following experiments were designed to assess the rate of PCB degradation, determine what environmental parameters are most important to PCB biodegradation, and to investigate the nature of PCB biodegradation by natural bacterial populations with respect to above definition.

Laboratory Biodegradation

A laboratory biodegradation assay was designed to evaluate the potential of natural reservoir microorganisms to degrade PCB and to evaluate what effect various environmental parameters might have on the biodegradation. It was assumed that loss of PCB due to biological activity is explained by biodegradation. By adding PCB to raw and sterile reservoir water, biodegradation can be determined by comparing the PCB residuals between the two treatments. This approach to PCB biodegradation has been previously used by Clark et al. (32) employing mixed bacterial cultures, Tucker et al. (160) employing activated sludge, and by Sayler et al. (150) and Baxter et al. (15) utilizing bacterial isolates.

PCB biodegradation assay. From each sample site, triplicate 100 ml samples were placed in sterile, 250 ml Erlenmeyer flasks equipped with Teflon-lined, screw caps, Celite (Fisher), a diatomaceous earth, was previously added at 0.01% (w/v) to act as an inert carrier thereby reducing losses of PCB by volatilization. Ten µl of acetone stock solution containing 1 mg Aroclor 1254 ml^{-1} were added to each flask to give a final PCB concentration of 100 µg l^{-1}. The flasks were dark incubated for 1 month at 25 C and extracted with two 100 ml volumes of hexane as described previously.

Spatial and temporal biodegradation. In order to determine whether potential PCB biodegradation was affected by varying environmental parameters and seasonal effects, PCB biodegradation was assessed between February and December 1977. Twenty-four total samples were examined and up to six different sites were sampled during a single sampling period. A typical sampling period lasted 2 days with 3 sites processed the first day and the rest on the second day. Triplicate autoclaved controls were employed for each sampling site. All PCB recoveries were compared to sterile controls and reported as relative percent biodegradation.

Effects of varying PCB concentration and pH on biodegradation. Biodegradation assays were run as above with minor modifications. For determination of pH effects on biodegradation, the pH of reservoir water samples was aseptically adjusted with sterile 0.1 N NaOH or HCl to the desired pH prior to incubation. The effects of PCB concentration on biodegradation were evaluated by adding varying amounts of PCB

(delivered in 10 µl acetone) to the water samples. Triplicate sterile control flasks were included for all biodegradation assays at each pH or PCB concentration employed.

In situ Biodegradation

A major objective of the study was to determine if PCB biodegradation can occur in nature. Jordan et al. (92) have employed closed bottle assays to evaluate the rate and extent of in situ aromatic hydrocarbon degradation. A major disadvantage of this system, however, is the lack of material exchange between the degradation chamber and the environment. Biochambers, open to the surrounding environment via filter membranes, have been used to overcome this problem (120). With this approach, bacterial cells, algae, and large suspended solids are retained within the biochamber, while dissolved chemicals and nutrients are in equilibrium with the environmental menstruum. However, biochambers are highly susceptible to contamination and physical damage. In addition, the typically heavy growth of microorganisms on the biochamber surfaces can strongly influence experimental results.

The biochambers illustrated in Fig. 3 have been previously described in detail (120). Briefly they are 25 cm long x 7.5 cm diameter polycarbonate tubes closed off at both ends with 90 mm Nucleopore membranes (0.2 µm pore-size) and sealed with silicone rubber sealant. The outer surfaces of the membrane were covered by urethane foam pads to retard PCB loss to the external environment. Control chambers were aseptically filled to 720 ml capacity with autoclaved reservoir water and test chambers were filled with raw reservoir water.

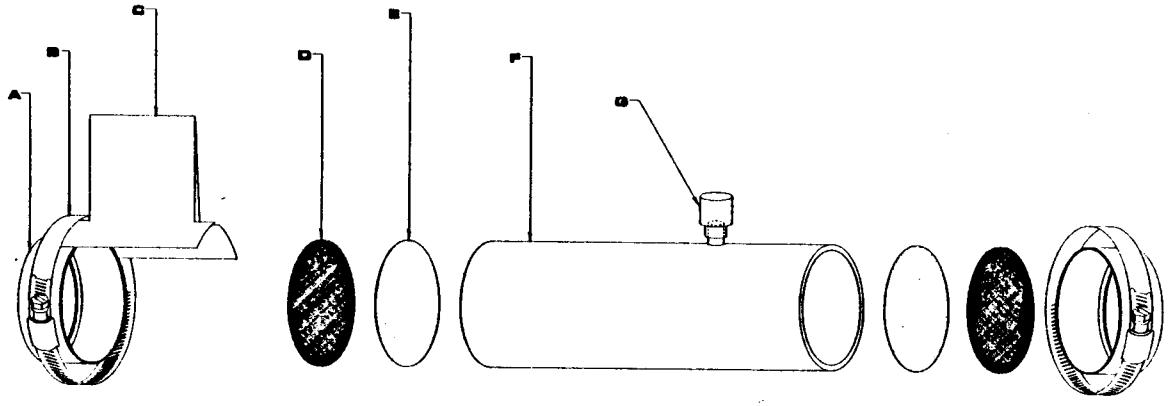

Fig. 3. Flow-through environmental chamber used for in situ PCB biodegradation assay. (A) Polyethylene retainer ring; (B) Stainless steel band clamps; (C) Aluminum sheet metal stabilizer fin; (D) Polyurethane foam pad; (E) 0.2 μm Nucleopore polycarbonate filtration membrane; (F) Clear polycarbonate cylinder; (G) Rubber sampling system. Overall dimensions: Length 25 cm, diameter 75 mm.

The chambers were dosed with 100 µg Aroclor 1254 from a 10 mg PCB ml^{-1} acetone stock solution to give a final concentration of 139 µg Aroclor 1254 l^{-1}. In August, 1977, the chambers were secured with weights and floats at Site 7 on Center Hill Reservoir at an average depth of 1 meter. In situ biodegradation assessment was performed after 1 week and 1 month incubation periods. Following incubation, each chamber was processed by removing the aqueous phase and extracting it twice with 100 ml volumes of hexane (see PCB batch extraction). The chambers were rinsed with 40 ml of hexane and this fraction was also collected. The membrane and foam pads were placed in separate Erlenmeyer flasks and extracted with 50 ml and 100 ml hexane, respectively. Each fraction was condensed by heating at 70 C in a Kuderna-Danish reflux apparatus. It was necessary to remove polar impurities and interfering residues from the extracts using Florisil column chromatography before analysis by EC-GLC.

(^{14}C)PCB Mineralization

In an effort to more fully elucidate the potential metabolism of PCB, natural microbial population studies were undertaken to quantify CO_2 evolution from the terminal decomposition of PCB. It is assumed that $^{14}CO_2$ is released from the aerobic bacterial oxidation of (^{14}C) substrates added to the medium. The hydrophobic nature of PCB, however, presents a serious obstacle to the application of this technique with aqueous systems. The technique is based on the quantitation of radiolabeled CO_2 evolved from a labeled substrate and, therefore, requires a mechanism for separating the CO_2 from the substrate. Due

to the very high water activity coefficients of PCB compounds (as a direct result of their hydrophobic properties), they are rapidly volatilized from the water column (156). Therefore, in the case of the mineralization assays, a significant amount of the aqueous PCB can be co-volatilized with the carbon dioxide and end up in the carbon dioxide trap. Other investigators employing (^{14}C)hydrocarbons have circumvented this problem by tediously separating the hydrocarbon from CO_2 in a specially designed apparatus (16, 92). This was not feasible, however, for a system requiring large-scale experimentation. Therefore, a method was developed to prevent PCB volatilization from the water phase and thus allow direct quantitation of radiolabeled CO_2 by liquid scintillation counting.

Development of mineralization assay. The PCB mineralization studies were carried out in 70 ml-capacity glass serum bottles equipped with rubber serum stoppers. A plastic center well (Kontes) containing a 1 x 5 cm, fluted, paper wick was fitted on each stopper. The bottles were autoclaved and a 10 ml aqueous sample was aseptically pipetted into each bottle. A 10 µl aliquot of (^{14}C)PCB stock solution in acetone was placed in each bottle to give a final concentration of 3.75 µg l^{-1}. The bottles were incubated in darkness at 25 C. To terminate the incubation and gasify aqueous carbonate species, 2 ml of trichloroacetic acid was injected through the stopper into the aqueous phase. In the same manner, 0.4 ml of 0.2 N KOH was added to the center well. The bottles were shaken for 2 hours to allow complete $^{14}CO_2$ uptake by the KOH trap. The center wells were removed and placed in scintillation vials for counting.

The following compounds were examined as possible materials for the absorption of (^{14}C)PCB: Celite, Kaolinite, Florisil, Amberlite XAD-4 beads, Sephadex G-25, microglass beads, polystyrene beads and polyurethane foam. Ten mg of each compound was placed in reaction vials prior to sterilization. A positive control (no compound addition) and negative control (no PCB addition) were included. Triplicate bottles were incubated for 3 days.

After XAD was chosen for further experimentation, the effect of varying XAD concentration on PCB and naphthalene volatility was determined. The experiment was performed as above except 0.5, 1.0, 5.0, 10 and 50 mg XAD were added to the reaction bottles. Ten mg XAD per bottle was selected for all further experiments.

(^{14}C)PCB and (^{14}C)naphthalene mineralization. Center Hill Reservoir water, obtained in December, 1978, was employed for mineralization studies. Eight triplicate experimental sets were performed in order that a time course study of PCB mineralization could be observed. In addition, two sets of sterile controls were employed; one by autoclaving and the other by adding 10 µg $HgCl_2$ per bottle.

Results of the (^{14}C)PCB mineralization by Center Hill Reservoir water prompted further investigation. Two likely possibilities resulted from a lack of $^{14}CO_2$ evolution. Either PCB was not readily mineralized by reservoir bacteria or the addition of XAD interfered with the interaction of the PCB and the microorganisms. In order to resolve this question, naphthalene, a more readily degradable compound with similar chemical properties as PCB was selected for examination.

The same experiment was performed with Fort Loudoun Reservoir water employing both (^{14}C)PCB and (^{14}C)naphthalene. Final naphthalene concentration was 1.25 mg l^{-1} and final PCB concentration was 150 µg l^{-1}.

Monitoring the effects of XAD and PCB on the growth of Center Hill bacteria further aided the interpretation of the mineralization results. A second set of bottles and controls was established concurrently with the Center Hill (^{14}C)PCB mineralization study. These controls included: (1) XAD control (no PCB addition) (2) XAD-acetone control (10 µl of acetone, no PCB addition), (3) PCB control (no XAD addition), and (4) positive control (no PCB or XAD additions). At a selected time corresponding to the (^{14}C)PCB mineralization time course, 0.1 ml was sampled from each bottle, serially diluted, and plated on YEPG agar.

Isolation and Identification of PCB Metabolites

Studies on the biodegradation and mineralization of PCB were further supported by the isolation and characterization of PCB metabolites. Lesser chlorinated biphenyls were chosen as model substrates for these studies because they are more susceptible to biodecomposition than the higher chlorinated forms (32, 56-58, 93, 150, 160). Furthermore, the isolation and identification of suspected metabolites was facilitated by employing pure PCB isomers rather than mixtures.

A protocol for the extraction of polar PCB metabolites as described by Furukawa, Tonomura, and Kamibayashi (58) was employed.

Site 4, Center Hill Reservoir (Fig. 1, page 21) was sampled in May, 1978 and 500 ml aliquots were placed in 1 liter, cotton-stoppered, Erlenmeyer flasks containing 50 mg Celite. The flasks were dosed with 10 µg of a 2-chlorobiphenyl or 2,4'-dichlorobiphenyl solution to give a final concentration of 40 mg PCB l^{-1}. Control flasks were autoclaved prior to PCB addition. All flasks were incubated in darkness at 25 C. After a 3-month incubation for 2-chlorobiphenyl and an 8-month incubation for 2,4'-dichlorobiphenyl, flasks were adjusted to pH 1.5 with concentrated HCl and twice extracted with 500 ml volumes of ethyl acetate. The organic phases were combined, condensed to 10 ml by roto-evaporation, and further reduced to approximately 1 ml by passing a gentle stream of nitrogen gas over the solvent surface. Extracts were then processed by thin layer chromatography.

PCB Bioaccumulation

The potential for cellular accumulation of PCB as a mechanism for biodegradation was examined to more fully delineate the PCB-microbe interaction. A bacterial isolate, PP1, was chosen for this experiment rather than a mixed culture to reduce possible complex variables as well as experimental error. On the other hand, experiments were performed in filter-sterilized CHR water so a more accurate evaluation of the results could be made among the various biodegradation studies.

PP1, a Gram-negative rod, was isolated on PCB agar from the PCB-induced flasks of the bacterial populations study. This unidentified organism is an orange chromogen, oxidative, non-motile, and does not produce acid from carbohydrates (Appendix B). For bioaccumulation

studies, PP1 was grown in YEPG broth (25 C) and shaken at 100 rpm. When the culture became turbid, cells were centrifuged at 1000 x g and resuspended in phosphate-buffered saline (Appendix A). The suspension was divided into 2 aliquots after another washing. One aliquot was autoclaved (killed cells), while the other aliquot received no further treatment (live cells). Cells were enumerated on YEPG agar.

Serum vials (160 ml volume) were filled with filter-sterilized CHR water and capped with butyl, crimp-seal caps. At time zero, 1 ml of cell suspension (live or killed) and 10 µl of (^{14}C)PCB in acetone solution were added to vials in triplicate (final PCB concentration, 16 µg l^{-1}). Controls did not receive cell additions. Vials were dark incubated (25 C) and an 11 ml sample was withdrawn from each vial via syringe at predetermined intervals. One ml was added directly to a scintillation vial and the remainder filtered through a 0.45 m pore-size membrane filter. A filtrate subsample (1 ml) was placed directly into a scintillation vial. The filter containing cells was rinsed with a 3 ml distilled water filtration and placed into a separate scintillation vial. Tests indicated that water was equally effective as a cold PCB/water solution in rinsing loosely bound (^{14}C)PCB off the filter.

Effects of PCB on Bacterial Populations

Effects of PCB on Growth of Reservoir Bacteria

The effect of PCB on the growth of heterotrophic microorganisms was examined. Essentially a scaled-up version of the PCB biodegradation

assay with PCB-dosed and undosed cultures was employed and the growth response of the organisms was examined with time.

One liter samples of CHR water (Site 4, December, 1977) were placed in 1.5 liter, screw-capped, Erlenmeyer flasks containing 100 mg Celite. The caps were lined with aluminum foil to prevent PCB sorption onto the caps. The following treatments were employed: (1) experimental, PCB added; (2) sterile control, phenylmercuric acetate added; (3) PCB-induced, PCB added and inoculated with a 1 ml mixed bacterial culture enriched in PCB medium; and (4) PCB control, no PCB additions. All flasks were incubated for 25 weeks of 25 C and sampled periodically for plating on PCB agar and YEPG agar.

PCB additions were made by dosing flasks with 10 µl of a 1 mg Aroclor 1254 l^{-1} acetone solution to give a final PCB concentration of 100 µg l^{-1}. Control flasks were established by adding 50 mg l^{-1} phenylmercuric acetate in dioxane to the reservoir water. A PCB-induced (PI) mixed culture was prepared by incubation reservoir water for 3 months in the presence of 100 µg PCB l^{-1}. Final cell densities were approximately 5 x 10^5 CFU ml^{-1} (determined from plate counts on YEPG agar). A variety of colonial morphologies indicated the presence of several bacterial types.

Scanning Electron Microscopic Study of Reservoir Growth Flasks

Scanning electron microscopic (SEM) studies were initiated to examine qualitative differences between treatments of the PCB effects study (preceding section). Following a 25 week incubation, samples of Celite were randomly pipetted from the bottom of the flasks and

collected on a 0.2 μm pore-size membrane (Nucleopore) by applying a negative pressure of 5 psi in a Millipore filtration apparatus (Bedford, Conn.). The filters were fixed in 2.5% gluteraldehyde in 0.1 m cacodylate buffer (pH 7.4) for 1 hour and immediately dehydrated 10 min each in a graded ethanol series: 10, 25, 50, 75, 90, and 100% ethanol. This was followed by two 10 min dips in amyl acetate. Before final gold-coat processing on SEM specimen studs, the filters were dried by either of two methods. The first was conventional critical point drying in CO_2 with the aid of a critical point dryer. The second method called for placement of the filters in glass petri dishes containing liquid nitrogen. The petri dishes were placed inside on a vacuum-type dessicator and a negative pressure of 5 psi was applied to evaporate the nitrogen. The samples were examined by an ETEK Autoscan scanning electron microscope (operating voltage 20,000V) and micrographs were recorded on Polaroid type 55 positive/ negative film.

Effects of PCB on Heterotrophic (^{14}C)Glucose Uptake by Reservoir Bacteria

The critical role of microorganisms as the basic agents of carbon cycling and transformation of all classes of nutrient is well known (5, 25, 126). Glucose uptake velocities were chosen as a parameter for measuring the effects of PCB on heterotrophic potential. Hobbie and Crawford (80) have indicated that the assay provides a rough index of gross heterotrophic metabolism when respiratory corrections are included. Heterotrophic uptake analysis of (U-^{14}C)glucose was performed according to the methods of Parsons

and Strickland (132) as modified by Wright and Hobbie (175). A brief description of the method is given here. The complete protocol for this group of experiments has been described elsewhere (149).

Reaction vials were 60 ml serum bottles fitted with a suspended plastic center well. For each sample collected, two heterotrophic uptake studies were performed in triplicate. Samples included a natural control study with raw lake water and 10 µl of acetone and lake water with the addition of 100 µg of Aroclor 1254 l^{-1}, delivered in 10 µl of acetone. Appropriate sterile controls, using autoclaved lake water or lake water containing 1.0 mg of $HgCl_2$ per ml were included for all experiments.

The measurements were performed six times between June, 1977 to August, 1978 with Center Hill Reservoir samples. Experimental vials were filled with 10 ml of reservoir water. Control samples received no PCB additions, while the experimental vials were dosed to a final PCB concentration of 100 µg l^{-1}. After a 1 hour acclimation period, the vials received a (^{14}C)glucose addition. The reaction vials were sealed and incubated from 1 to 24 hours at room temperature. Incubation was terminated by adding 1.0 ml of 2 N H_2SO_4 to the aqueous phase. β-phenethylamine (0.4 ml) was injected into the center well and vials were allowed to stand for 1-2 hours.

The center wells were removed from the reaction vials and placed directly in scintillation vials. Cell-bound (^{14}C)glucose was determined by withdrawing a 3.0 ml sub-sample from the aqueous phase and separating the cells from the medium by membrane filtration. The filters were washed with distilled water and placed in scintillation vials for counting.

V. RESULTS

Comparison of PCB Extraction Methods for Biodegradation Assays

Solvent Batch Extraction

The efficiency of hexane batch extraction of PCB upon prolonged incubation of the PCB biodegradation flasks was examined. Two series of triplicate flasks containing autoclaved reservoir water received 10 µg Aroclor 1254 per flask. One set was promptly extracted and the other set was incubated for 1 month prior to extraction. Residual PCB was quantified by GLC. PCB recoveries were 95.4 ± 6.1% and 98.4 ± 2.1% for the time zero flasks and the 1 month flasks, respectively. It was concluded that prolonged incubation did not affect PCB recovery.

Tenax-GC Column Extraction

The results of the Tenax PCB extraction and efficiency studies are given in Table 1. Approximately 89.4% of total added (U-^{14}C)PCB was recovered in a subsample of the aqueous phase and another 6.8% was adsorbed to the surface of the reaction vessel for a combined recovery of 96.2%. Following extraction, 67.2% and 2.5% of the total radioactivity was recovered in the first and second 5.0 ml hexane eluates, respectively, which accounted for a 78% extraction efficiency relative to the PCB recovered in the aqueous phase. Approximately 16.6% of the (^{14}C)PCB was not extracted by the column and was recovered in the aqueous phase after extraction. Upon concentrating the first hexane elution from 5.0 ml to 1.0 ml, the net recovery of PCB in the eluate dropped from 67.2% to 34.4%, approximately a 50% loss upon concentration.

Table 1. Analytical recovery of (U-^{14}C)PCB, following Tenax extraction.

Sample	Mean Recovery[a] (%)
Aqueous Phase (prior to extraction)	89.4 ± 6.7
Aqueous Phase Residual (after extraction)	16.6 ± 7.4
1st Eluate (prior to concentration)	67.2 ± 6.8
1st Eluate (after concentration)	34.4 ± 6.6
2nd Eluate	2.5 ± 2.7
Reaction Vessel Residual	6.8 ± 2.4

[a]Relative to the known total dpm added to the reaction mixtures.

Effects of Organic Matter and Sample Size on PCB Extraction by Tenax-GC

Three volumes of Aroclor 1254-saturated water (50 ml, 100 ml, and 200 ml) were extracted via Tenax-GC extraction and eluted with 5.0 ml. Each eluate was concentrated to 2.0 ml and quantitated by GLC. The net recovery of PCB from 50 to 200 ml solutions extracted appeared linear (Table 2). Sample size did not affect PCB extraction, except for a noticeable increase in the variability of triplicate samples at 200 ml volume.

The effect of organic matter, either soil extract (SE) or spent culture medium (CM), significantly ($\alpha = 0.05$) decreased the Tenax PCB extraction efficiency (Table 3). All concentrations of SE added to the saturated water caused a significant reduction ($\alpha = .05$) in extraction efficiency. Upon increasing the concentration of soil extract, however, there was no significant ($p > 0.05$) loading effect on Tenax extraction. The soil extracts caused an average 41.6% reduction in the recovery of extractable PCB as compared with unamended PCB-saturated water (Table 2).

The effect of aged bacterial growth medium on PCB extractability was not as pronounced as with soil extract. The 27.8% reduction was significant ($\alpha = 0.05$) and, as with soil extract, the amount of spent medium added to the PCB solution did not have a significant dose effect.

The organic matter in both treatments dramatically increased the variability of triplicate extractions. The coefficient of variation (C.V.) for extraction of 100 ml PCB-saturated water was 6.1

Table 2. Effect of aqueous sample size on Tenax extraction efficiency or Aroclor 1254 and phenanthrene saturated solutions.

Substrate[a]	Recovery[b] (µg)	S.D.	Aroclor 1254[c] Concentration (µg l^{-1})
Aroclor 1254			
50 ml	4.0	0.28	80.0
100 ml	7.3	0.45	73.0
200 ml	17.9	3.67	89.5

[a]Aqueous samples taken from 3.0 l stock solution of Aroclor 1254-saturated distilled water.

[b]Mean recovery of triplicate samples and standard deviation.

[c]Calculated PCB concentration in water solution.

Table 3. Effect of dissolved organic matter on the Tenax-GC extraction efficiency of Aroclor 1254.

Organic Matter[a]	Aroclor 1254 Recovered[b] (µg)	Relative Recovery[c] Efficiency (%)
Soil Extract		
0.01%	2.8 ± 3.6	38.2
0.1%	2.5 ± 1.2	33.9
1.0%	2.6 ± 2.9	35.7
5.0%	3.1 ± 6.4	42.5
		Efficiency 37.6 ± 3.7
Culture Medium		
0.01%	3.5 ± 5.3	47.9
0.1%	3.7 ± 2.5	50.6
1.0%	3.4 ± 4.0	46.6
5.0%	3.0 ± 1.2	41.2
		Efficiency 46.5 ± 4.0

[a]Control Aroclor 1254 recovery was 7.3 ± 0.45 µg (Table 2, page 54).

[b]Mean recovery of triplicate samples.

[c]Extraction efficiency relative to the recovery of Aroclor 1254 from 100 ml Aroclor 1254 saturated water (Table 2).

(calculated from Table 2), while the soil extract increased the C.V. to 48-206 and the spent culture medium C.V. increased to 40-151 (calculated from Table 3).

PCB Biodegradation

Spatial and Temporal PCB Biodegradation

Environmental parameters were determined at the time of sampling (Table 4). Site depth ranged from 5 meters at Site 2 to greater than 30 meters at Sites 4 and 5. Temperature, dissolved oxygen, transparency, conductivity, and pH varied temporally but variation was slight from site to site on a given date. Total viable counts (TVC) ranged from 3.22 $\log_{10} ml^{-1}$ to 5.43 $\log_{10} ml^{-1}$ and PCB resistant counts ranged from 2.71 $\log_{10} ml^{-1}$ to 4.27 $\log_{10} ml^{-1}$. The highest bacterial counts for all sites were encountered in April; however, the month in which lowest counts were recorded varied with site.

PCB biodegradation was observed in all samples. Typical gas-chromatographic tracings of residual Aroclor 1254 are illustrated in Fig. 4. The area under the 11 to 12 major peaks of Aroclor 1254 was less in the "biodegraded" sample as compared to the sterile samples. These tracings were also representative in the sense that the relative peak heights of the sterile flask were not noticeably different from the experimental flasks (i.e., the shape of the curves was similar in all samples), indicating that specific Aroclor 1254 chlorinated biphenyl components were not preferentially biodegraded. In cases of more extensive biodegradation, only the six largest peaks were detectable in the GC tracings (Fig. 4).

Table 4. Site characteristics for samples collected from Center Hill Reservoir.[a]

Date	Site	DEPTH[b] (m)	TEMP (°C)	DO (mg l^{-1})	DOC (ppm)	SECC (m)	COND (umho)	PO_4 (mg l^{-1})	pH	NO_3^- (mg l^{-1})	S S (mg l^{-1})	TVC[c]	PCB[d]
2/77	2	21.0	3.5	13.8	22	2.5	90	.02	7.3	1.0	0.8	3.58	3.11
	4	30.0	4.5	13.2	32	4.0	80	.02	7.8	1.0	1.3	3.53	3.94
	5	30.0	3.9	13.4	54	3.2	80	.02	7.1	1.0	1.4	3.58	3.11
3/77	1	24.0	15.0	10.2	10	1.0	129	.05	8.6	1.0	27.8	3.85	3.61
	2	21.0	15.0	10.6	11	1.0	109	.02	8.3	1.0	24.6	4.11	3.04
	3	0.5	12.0	10.4	8	0.8	103	-	8.6	1.0	28.3	4.15	3.15
	4	30.0	11.5	10.5	7	1.5	95	.02	8.3	1.0	24.4	4.15	2.71
	5	30.0	16.0	10.0	8	0.5	80	.02	8.0	1.0	37.3	4.20	3.49
	6	30.0	14.5	10.6	8	1.3	103	.05	8.4	1.0	34.0	4.62	3.08
4/77	1	21.5	19.3	10.2	-	1.2	138	.09	9.5	1.1	4.2	5.43	4.08
	2	20.0	20.0	9.4	-	0.5	91	.03	8.5	2.5	9.1	4.95	3.89
	3	14.3	18.0	9.2	-	-	90	.06	8.8	2.7	5.6	4.88	4.08
	4	30.0	-	-	-	-	-	.03	-	4.3	6.5	4.57	4.27
	5	-	20.0	9.4	-	1.3	12	.05	-	2.4	0.5	4.95	3.80
	6	30.0	-	-	-	-	-	.04	-	2.5	13.7	4.73	3.66
6/77	1	-	27.5	2.2	-	1.3	130	.01	9.6	1.0	108.0	4.57	3.28
	2	25.0	27.0	-	-	1.8	132	.01	9.4	1.0	112.0	4.52	2.91
	3	3.2	28.0	-	-	1.7	108	-	9.2	1.0	3.70	4.54	3.00
	5	20.8	28.0	-	-	0.9	160	.01	9.0	1.0	81.7	4.78	2.85
	6	30.0	26.5	7.8	-	2.0	129	.05	9.3	1.0	124.7	4.57	3.00
8/77	4	30.0	30.0	7.2	-	3.0	131	.09	7.7	-	3.8	3.54	2.38
	7[e]	8.0	29.0	7.6	-	2.5	151	.01	8.4	-	2.9	3.38	2.38
9/77	4	30.0	28.5	7.8	-	4.0	280	-	6.2	-	-	3.47	1.56
	7	8.0	28.5	7.4	-	2.0	280	-	6.5	-	-	2.54	1.90
12/77	2	-	9.5	11.6	-	-	96	-	-	-	11.6	3.96	3.32
	4	30.0	13.0	10.0	-	1.0	117	-	8.0	-	-	3.22	2.74
	5	25.0	11.0	13.6	-	-	-	-	-	-	13.6	4.16	3.33
8/78	7	8.0	32.0	7.8	-	1.5	150	-	-	-	-	4.05	3.74

[a]Abbreviations: TEMP, water temperature; DO, dissolved oxygen; SECC, water transparency (Secchi disk); COND, conductivity; S S, suspended solid; TVC, total viable counts; PCB, PCB-resistant counts.

[b]Sample site depth, all samples were collected 1 m below the surface.

[c]Total viable count from plate counts on YEPGA medium.

[d]PCB resistant bacteria from plate counts on PCBA medium.

[e]Site characteristics at beginning of the <u>in situ</u> PCB biodegradation.

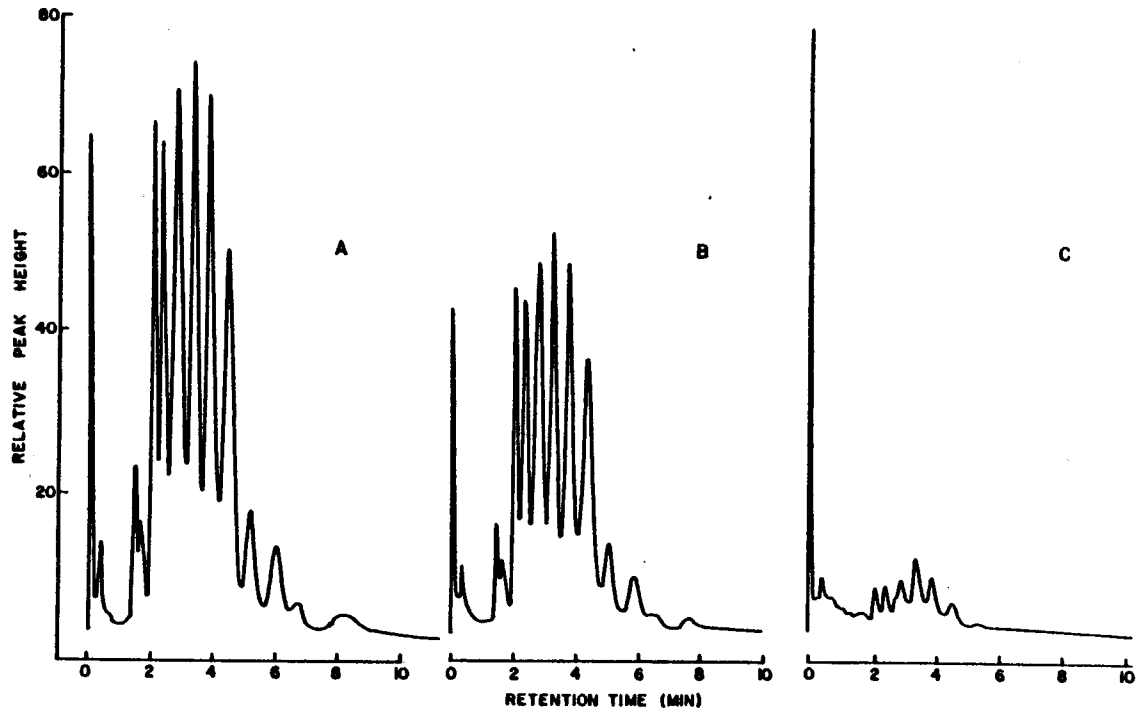

Fig. 4. Representative gas-chromatographic tracings of Aroclor 1254 extracted from biodegradation flasks. Center Hill Reservoir water was dosed with PCB (100 mg l^{-1}) and dark incubated at 25 C for 1 month. (A) Sterile control, (B) 30.1% biodegradation, and (C) 89.9% biodegradation.

The results of PCB biodegradation monitored throughout 1977 from Center Hill Reservoir are given in Table 5. The data are presented as mean percent biodegradation relative to sterile controls for each month. The monthly biodegradation ranged from 6.5% ± 10 on Site 4 in February to 81.2% ± 16 on Site 6 in March with an overall monthly mean of 46.5%. Analysis of the variance confirmed that the observed variance was due to both temporal and spatial differences (Table 6). Interaction between month and site effects was insignificant, and thus the majority of the explained variation was due to specific site effects (61%); the remainder of the variation was explained by the monthly effects. Consequently, a multiple regression analysis between PCB biodegradation (Table 5) and the sample site characteristics (Table 4) was performed (Table 7). As established by multiple coefficient of determination (R^2) value, 42.9% of the variation of relative biodegradation was explained by the given site characteristics. The computed regression equation, however, did not significantly ($\alpha = 0.05$) describe the observed variations, indicating that a greater sample size was necessary. Although total viable count (TVC) was the only independent variable that significantly correlated with PCB biodegradation. Dissolved oxygen and, to a lesser extent, suspended sediments and dissolved organic carbon also accounted for much of the explained biodegradation variation (14.82, 3.04, 2.95%, respectively). TVC accounted for 15.6% of the variability.

In situ Biodegradation in Center Hill Reservoir

Ultimately, it was necessary to determine whether PCB biodegradation occurs under actual reservoir conditions. The biochambers were

Table 5. Percent biodegradation of Aroclor 1254 and 25 C by the flask assay with Center Hill Reservoir water in 1977.

Site	% PCB Degradation on Month					
	Feb.	Mar.	Apr.	Jun.	Aug.	Dec.
1	ND[b]	62.6 ± 10	53.5 ± 7	70.3 ± 14	ND	ND
2	13.8 ± 7	35.0 ± 7	18.8 ± 15	51.3 ± 28	ND	43.6 ± 9
3	ND	47.7 ± 33	37.4 ± 18	69.8 ± 18	ND	ND
4	6.8 ± 10	11.4 ± 11	66.7 ± 30	ND	42.4 ± 21	37.3 ± 19
5	47.7 ± 28	10.8 ± 33	41.5 ± 39	53.8 ± 6	ND	69.8 ± 8
6	ND	81.2 ± 16	71.2 ± 20	70.4 ± 36	ND	ND

[a]Values expressed as % PCB degradation relative to sterile controls.

[b]ND = Not determined.

Table 6. Two-way analysis of variance of Center Hill Reservoir PCB biodegradation.[a]

Source of Variation	Degrees of Freedom	Mean Square	F[b]
Main Effects			
Site	5	692.5	3.783*[c]
Month	5	447.7	2.445*
Interaction			
Site x Month	13	256.2	1.399
Explained	23	490.3	2.678*
Residual	42	183.1	

[a]Percent PCB degradation data were transformed by arcsine function for ANOVA.

[b]F, F-statistic.

[c]*Significant F-statistic, $\alpha = 0.05$.

Table 7. Summary table of correlation coefficients and regression coefficients of PCB biodegradation.

Independent Variables	Multiple Regression[a] Coefficient (b)	Multiple Coefficient[a] of Determination (R^2)	R^2 Change	Simple Correlation[b] Coefficient (r)
PCB (X_1)	0.0425	0.0018	0.0018	-0.0425
TEMP (X_2)	0.0991	0.0098	0.0080	0.0991
DO (X_3)	0.3975	0.1580	0.1482	0.3848
DOC (X_4)	0.4331	0.1875	0.0295	-0.3461
SECC (X_5)	0.4493	0.2019	0.0144	-0.2841
COND (X_6)	0.4560	0.2079	0.0060	-0.0141
PO_4 (X_7)	0.4585	0.2102	0.0023	-0.0796
pH (X_8)	0.4887	0.2388	0.0286	-0.1548
NO_3 (X_9)	0.4920	0.2421	0.0033	0.0937
SS (X_{10})	0.5220	0.2725	0.0304	0.2687
TVC (X_{11})	0.6549	0.4289	0.1564*	0.4176

[a]Presented as a stepwise cumulative value.

[b]Product-moment coefficient.

*Significant ($\alpha = 0.05$).

incubated during August 1977 at Site 7, a small, isolated cove fed by a small tributary which was rarely frequented by boats or other recreational traffic, thus assuring minimal interference with the bio-chambers. Site characteristics at the beginning of the 30-day incubation are given in Table 4, page 57. The recovery of Aroclor 1254 from the sterile controls stabilized after 5 days in the reservoir (Table 8); thus, it was assumed a rapid equilibrium of PCB occurs between the reservoir water and the biochamber components resulting in approximately a 50% loss of PCB from the biochamber. Of the PCB remaining in the chambers, 67.3 ± 16% was removed by microbial activity in 30 days. No significant loss was detectable after only 5 days. PCB concentrations in Center Hill Reservoir (Site 7) are less than 1 ng PCB l^{-1}; therefore, background PCB contamination is negligible and not expected to affect the experimental results.

Effect of pH on PCB Biodegradation

The pH is a major environmental controlling parameter of biological activity. The effect of pH on PCB biodegradation is illustrated in Fig. 5. Percent extraction efficiency for all sterile controls ranged from 92 to 98% except for pH 3 sterile flasks (68 ± 12%). No explanation is given for this low recovery. The highest percent biodegradation (40.1 ± 4.2%) was observed at pH 8.0, which corresponded to the actual pH (pH 7.7) of Site 4 before acid or base adjustment. Relative biodegradation observed at pH 7.0 and 9.0 was insignificantly lower than at pH 8.0. No significant PCB biodegradation was observed at pH 3.0 or 5.0.

Table 8. *In situ* biodegradation of Aroclor 1254 at Site 7, Center Hill Reservoir during August, 1977.

Flask	Percent Total PCB Recovery[a]	Percent Relative PCB Degradation[b]
5 Day Sterile Control	53.5 ± 18[b]	--
5 Day Degradation	46.5 ± 12	13.1 ± 19
30 Day Sterile Control	46.2 ± 3	--
30 Day Degradation	15.1 ± 8	67.3 ± 16*

[a]Percent PCB of total added PCB (± S.D.).

[b]PCB loss compared to sterile control recovery.

*Significant ($p < 0.05$).

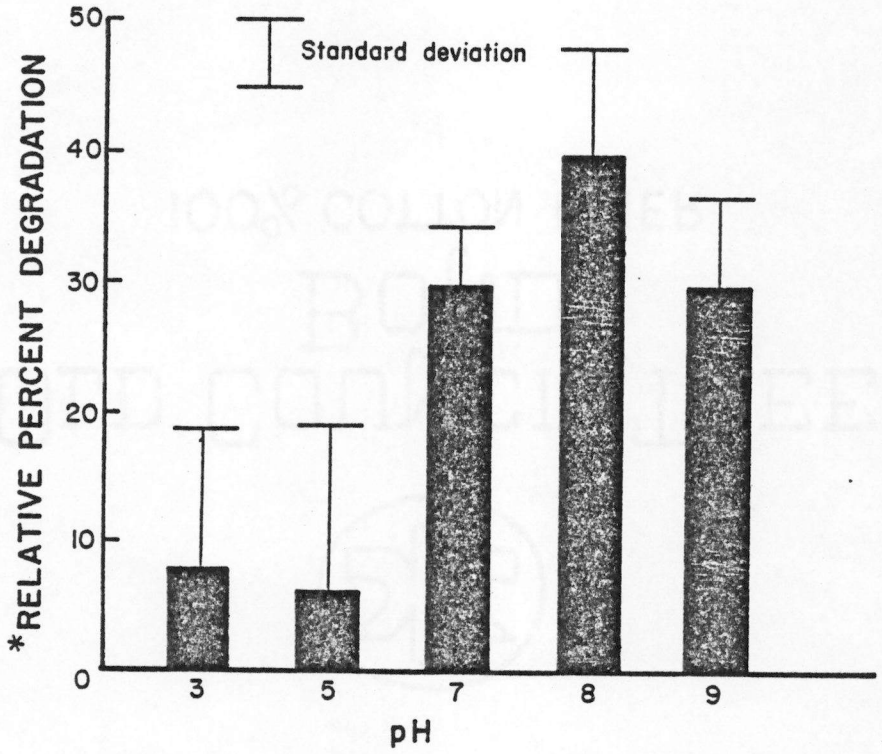

Fig. 5. Effect of pH on Aroclor 1254 biodegradation. Center Hill Reservoir water (pH 7.7) was adjusted with sterile HCl or NaOH, dosed with Aroclor 1254 and incubated at 25 C for 1 month. Relative biodegradation was computed from residual PCB compared to sterile control PCB at appropriate pH.

Effect of Aroclor 1254 Concentration on PCB Biodegradation

The concentration of Aroclor exerted considerable influence on the biodegradation rate (Table 9). At the PCB concentration used in the temporal biodegradation assay (i.e., 100 µg PCB 1^{-1}), 24.2 ± 8.8% of the added Aroclor 1254 was removed by biological activity, while biodegradation at a 10 fold lower PCB concentration was almost doubled, 43.2 ± 10%). The PCB biodegradation rate, however, increased from 4.4 µg 1^{-1} $month^{-1}$ to 20.2 µg 1^{-1} $month^{-1}$ upon increasing the Aroclor 1254 concentration from 10 µg 1^{-1} to 100 µg 1^{-1}, respectively. At higher concentration of Aroclor 1254 (1000 to 100,000 µg 1^{-1}), net PCB biodegradation was sufficiently low to be masked by random analytical error and could not be assessed.

(^{14}C)PCB Mineralization

Optimization of mineralization assay. Several compounds were screened as possible agents to prevent PCB volatilization in PCB mineralization assays. The compounds were added to water in serum vials, autoclaved, and followed by a (^{14}C)PCB addition. After a 3-day equilibration period, the water phase was acidified and KOH was added to the center wells. In accordance with the mineralization protocol, the vials were shaken for 2 hours and the center walls removed for scintillation counting. The final PCB concentration was 105 µg 1^{-1}.

The percent of PCB added to the vials that volatilized and was recovered in the CO_2-trap (center well) for each material examined is given in Table 10. The results indicate that, on a weight

Table 9. Comparative Aroclor 1254 biodegradation with varying PCB concentration.[a]

Aroclor 1254 Concentration ($\mu g\ l^{-1}$)	Net Aroclor 1254 Recovery (%)	Aroclor 1254 Biodegradation Rate[b] ($\mu g\ l^{-1} month^{-1}$)	Relative Percent Biodegradation (%)
10	56	4.4 ± 2*	43.2 ± 10*
100	80	20.2 ± 7*	24.2 ± 8.8*
1000	100	ND[c]	ND
10000	100	ND	ND
100000	100	ND	ND

[a]Incubated in darkness at 25 C for 30 days.

[b]Compared to sterile control at respective PCB concentration (*significantly different from control, $p < 0.05$).

[c]ND = Not detectable.

Table 10. Screening of compounds to reduce (^{14}C)PCB volatilization in mineralization assay.

Compound	(U-^{14}C)PCB Recovered in Center Well KOH	
	DPM	% of Added PCB
Control	20319 ± 853	29.9
Celite	21264 ± 808	31.3
Florisil	17710 ± 1417	26.1
Glass Beads	17618 ± 599	25.9
Kaolin	21537 ± 2198	31.7
Polyurethane	11888 ± 369	17.5
Sephadex	22334 ± 1920	32.9
XAD-4	5652 ± 2046	9.3

basis, XAD was the most efficient PCB adsorber since only 8.3% of the total added PCB was recovered in the CO_2-trap. Polyurethane also demonstrated substantial PCB retention but the rest of the compounds were not more effective than water alone. At this PCB concentration (105 $\mu g\ l^{-1}$) 29.9% of the PCB is volatilized and retained by the center well.

Before XAD was employed, it was necessary to determine the relationship of PCB volatility to XAD concentration. The mineralization assay was repeated with varying amounts of PCB. The initial (^{14}C)PCB concentration was 105 $\mu g\ l^{-1}$. The $\log_{10}$ of the PCB concentration varied linearly with the $\log_{10}$ of the XAD concentration (Fig. 6). Based on this relationship, a 10 mg XAD per vial concentration was selected for further experimentation. At this concentration, only 0.08% of the PCB added (Fig. 6) was lost to the center well, allowing considerable sensitivity in the detection of evolved $^{14}CO_2$.

(^{14}C)PCB mineralization. Vials containing CHR Site 4 water supplemented with (^{14}C)PCB (3.75 $\mu g\ l^{-1}$) were monitored for mineralization at regular intervals up to 96 days. Significant $^{14}CO_2$ evolution was not detected for the duration of the experiment. Less than 1% of the radioactivity of the added PCB was consistently recovered in the sterile control $^{14}CO_2$ trap throughout experimentation; hence, 1% is a reasonable estimate of the sensitivity of the assay. Approximately 98% of the total radioactivity was recovered in the aqueous menstruum of the reaction vessels.

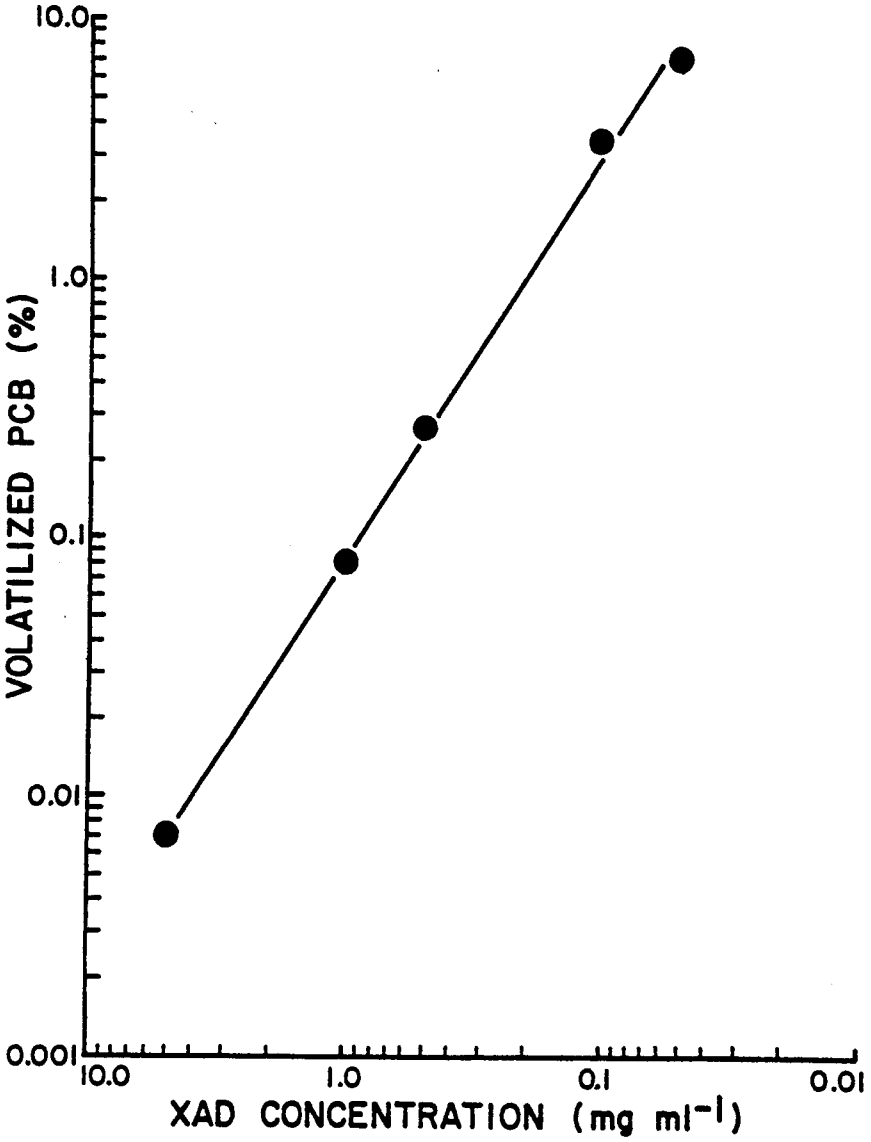

Fig. 6. Percent of (^{14}C)PCB volatilized with varying Amberlite XAD-4 concentrated in mineralization assay. Triplicate bottles of sterile water plus XAD were dosed with 3.75 µg l^{-1} (^{14}C)PCB and incubated for 3 days. Center well trap (KOH) activity was determined.

Another series of mineralization experiments was performed with Fort Loudoun Reservoir water. In addition to (^{14}C)PCB, (1(4,5,8)-^{14}C)-naphthalene was used as a substrate for mineralization by the reservoir bacteria. (^{14}C)PCB and (^{14}C)naphthalene were added to mineralization vessels at a final concentration of 150 µg l^{-1} and 1.25 mg l^{-1}, respectively. After a short lag period, naphthalene was readily mineralized (Fig. 7). In contrast, the evolution of PCB derived CO_2 was not detected. As before, the sensitivity of the assay was below 1%.

Bacterial growth response in mineralization assay. Although $^{14}CO_2$ evolution resulting from PCB decomposition was not detected, heterotrophic bacterial counts were significantly stimulated by the joint presence of XAD resin and PCB (Fig. 8). All treatments demonstrated an initial "bottle" effect resulting in increased total viable cell counts. This apparent stimulation, however, was negligible, for the natural control upon termination of the experiment. It was concluded that PCB alone was not stimulatory, but in conjunction with XAD, PCB significantly ($\alpha = 0.05$) increased heterotrophic counts above the stimulatory level of XAD alone.

Bioaccumulation Studies

A time course PCB accumulation study was conducted with a live and killed bacterial isolate, PP1, to differentiate between active PCB uptake and simple partitioning as a process of PCB biodegradation. PP1 was isolated on PCBA from the pre-enriched cultures as previously described. Fig. 9 depicts the loss of total (^{14}C)PCB activity during

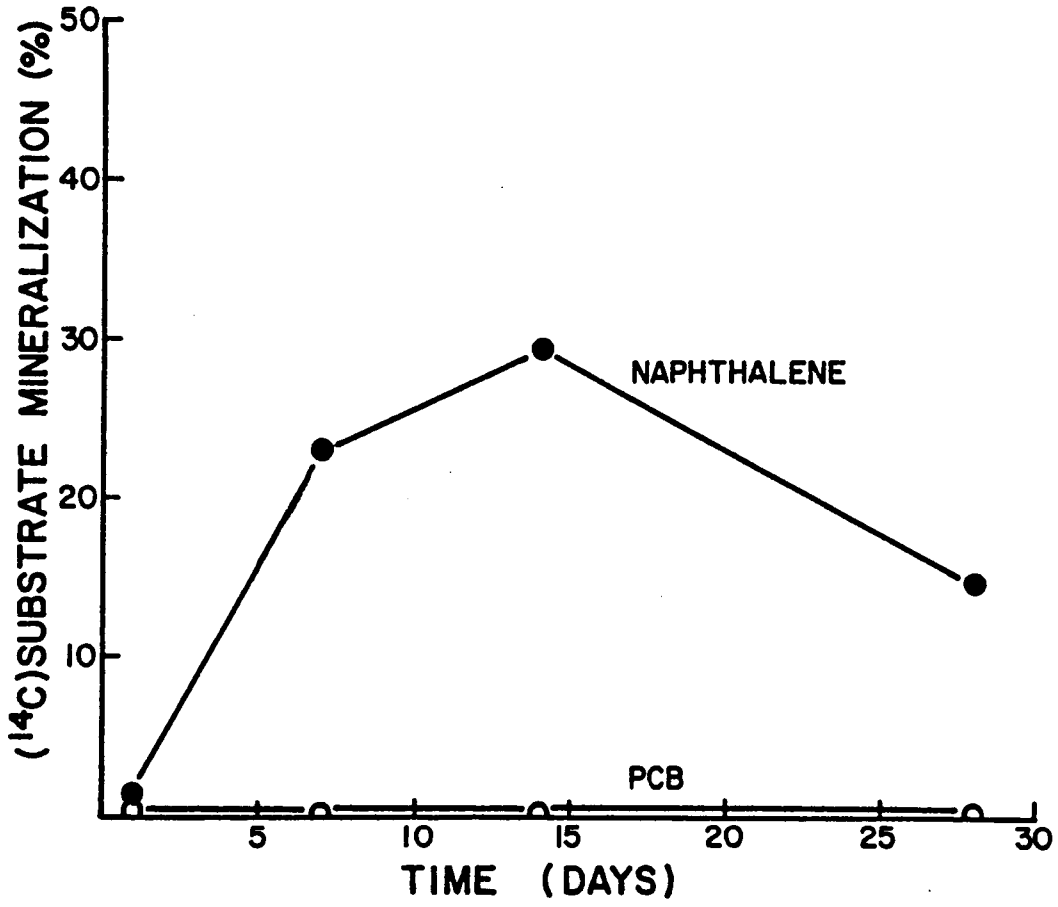

Fig. 7. Mineralization of (1-^{14}C)naphthalene and (U-^{14}C)PCB by Fort Loudoun Reservoir microorganisms. Triplicate samples were dosed to a final concentration of 1.25 l^{-1} naphthalene and 150 µg l^{-1} PCB and incubated in darkness at 25 C.

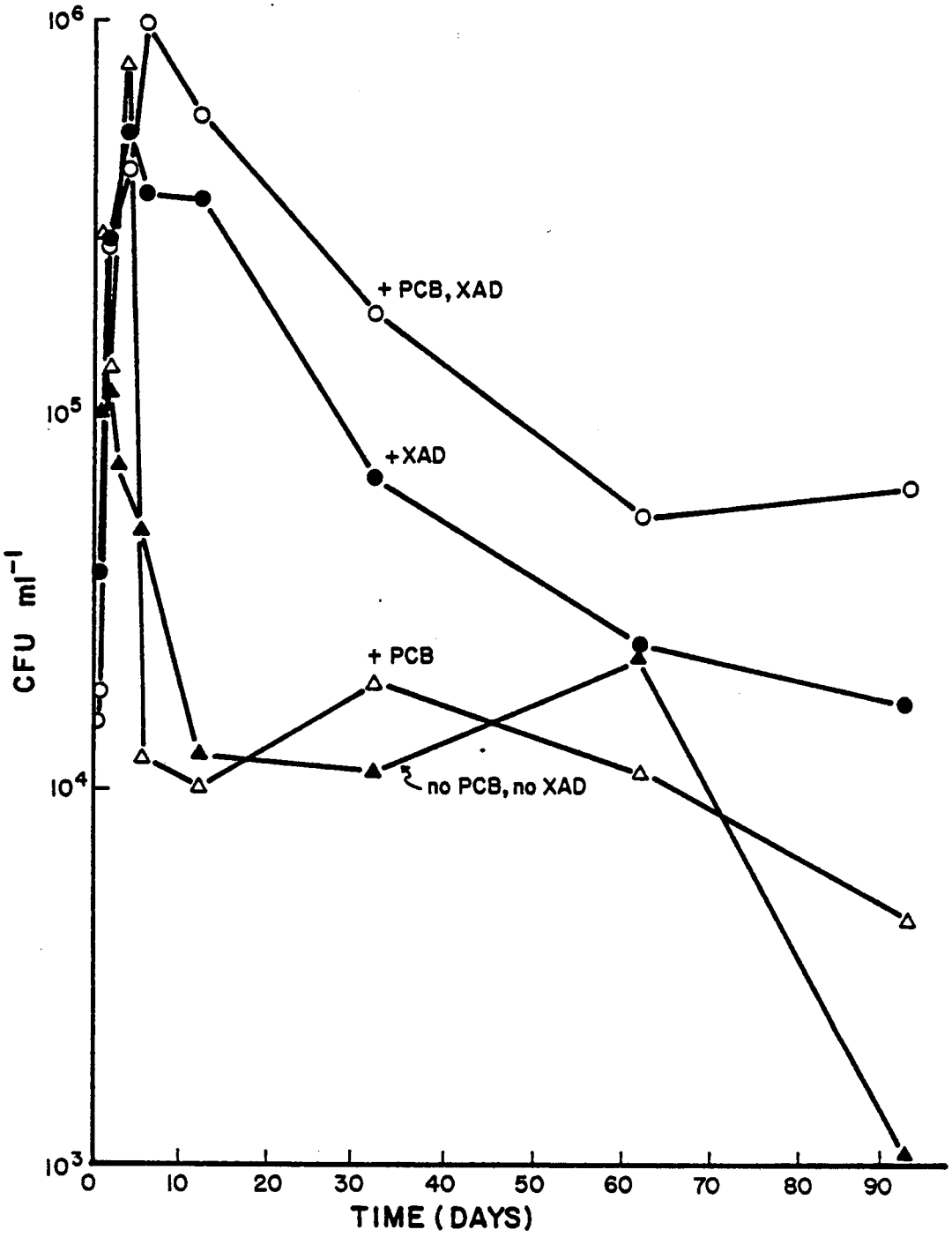

Fig. 8. Total viable bacterial counts of (U-^{14}C)PCB mineralization assay. Samples were plated on YEPGA and incubated at 25 C for 1 week. The XAD-acetone control curve was identical to XAD curve.

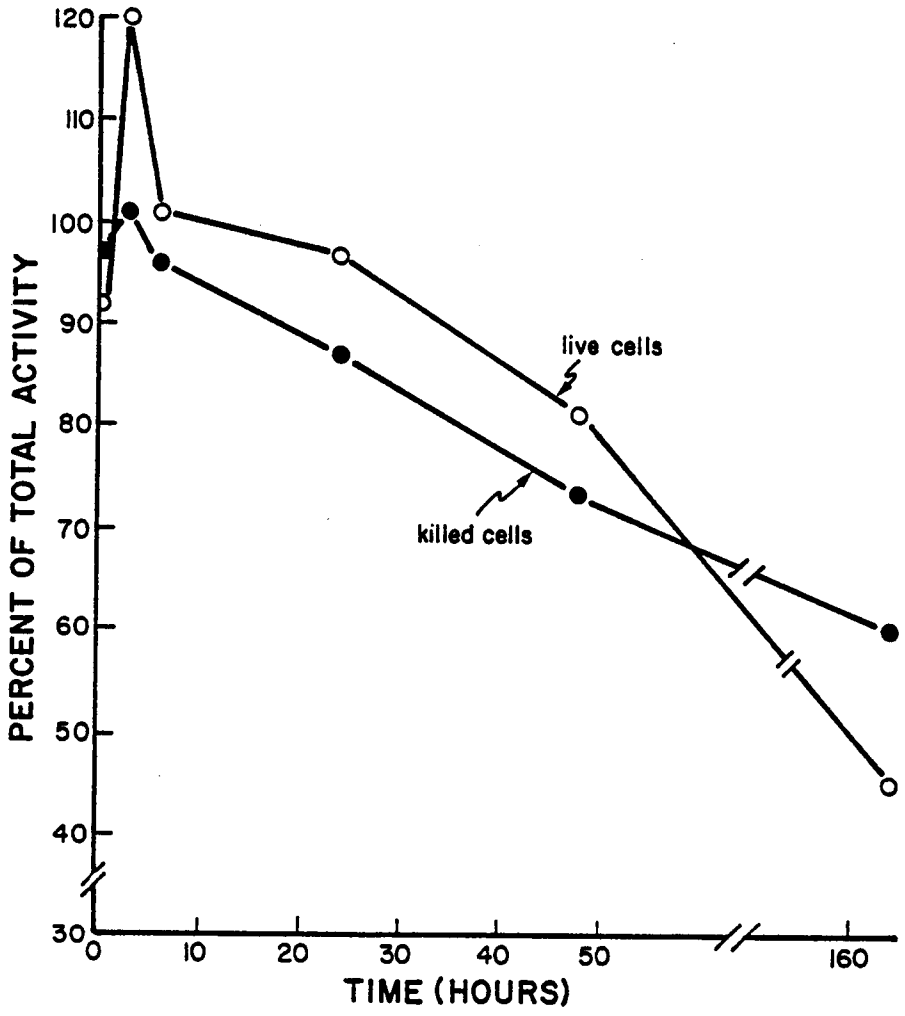

Fig. 9. Loss of (U-^{14}C)PCB activity from aqueous phase of bioaccumulation assay. Bacterial isolate PP1 was incubated in filter-sterilized Center Hill Reservoir water with (U-^{14}C)PCB (16 µg l^{-1}).

the course of a 164 hour incubation. A slow loss of activity took place in both live and killed cells, although at slightly different rates. At the termination of the experiment, killed cell PCB loss was 40.5% while live cell loss was 55.1% of the initial PCB. Adsorption by the butyl rubber caps and glass walls probably explains the majority of PCB loss.

The results of the bioaccumulation study are illustrated in Fig. 10. PCB was accumulated by the live cell fraction at all time points but not by killed cells. After 164 hours incubation, 59.4% of the activity was recovered in the live cell fraction and only 1.2% in the killed cell fraction. Correspondingly, the live cell filtrate retained only 22.9% of the ^{14}C activity while 67.1% of the total activity was recovered in the killed cell filtrate. Considerable variation, especially in the first 3 hours of incubation is noticeable for the live cell fraction. Theoretically, the cell fraction activity should total 100%; however, up to 45% can easily evaporate from the filtrate during the filtration procedure accounting for the observed discrepancy.

In order to examine microscopic effects on PP1 by autoclaving, cells were heat fixed on a glass slide and stained with crystal violet for microscopic comparison with untreated cells. Both live and killed cells appeared as intact short rods and no cell debris was observable. It was concluded, therefore, that either live PP1 cells actively take up PCB or cellular components that bind PCB are destroyed or solubilized upon autoclaving.

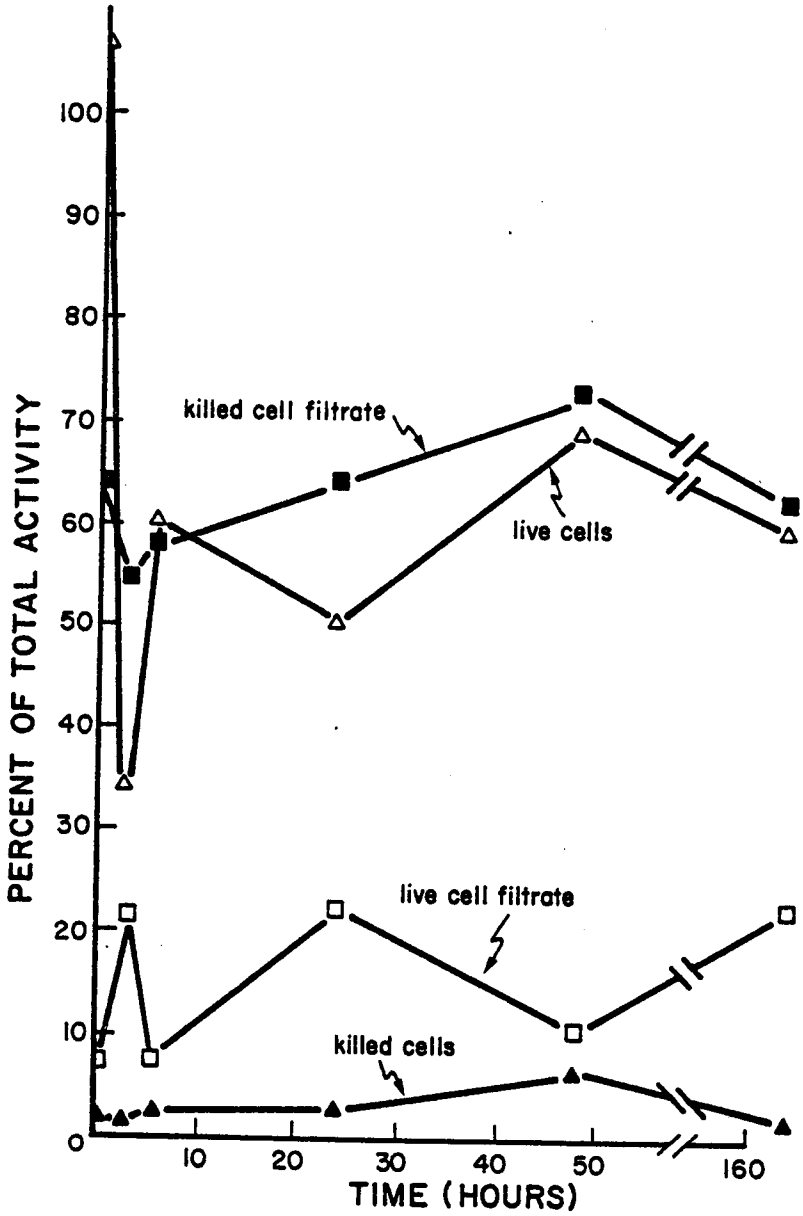

Fig. 10. Bioaccumulation of (U-^{14}C)PCB by live and killed (autoclaved) bacterium, PP1. PP1 was incubated in filter-sterilized Center Hill Reservoir water with (U-^{14}C)PCB (16 µg l^{-1}). The cell fraction was separated by membrane filtration. Total radioactivity was corrected at each time point for aqueous PCB loss.

The percent of labeled PCB associated with the live and killed cell fractions remained fairly constant with time (Fig. 10) which is interesting in view of the net loss of (^{14}C)PCB (Fig. 9). This observation is evidence for an equilibrium partitioning phenomenon between the cells and the ambient water and perhaps saturation of the absorption sites on the surface of the microbial cell.

PCB Metabolites

Evidence suggested that Aroclor 1254 was subject to biodegradation but not accompanied by mineralization. Therefore, an investigation of possible PCB decomposition was initiated. Analysis of acid extractable components following incubation of reservoir microflora with 2-chlorobiphenyl and 2,4'-dichlorobiphenyl suggested the lesser chlorinated biphenyls were indeed subject to microbial decomposition. Three spots were resolved on TLC plates during the analysis of 2-chlorobiphenyl extracts. The fastest migrating spot (R_{f_1} = 0.98) co-migrated with the 2-chlorobiphenyl standard. The identity was confirmed from the mass spectrum (M+, m/e 188). The other two spots (R_{f_2} = 0.68, R_{f_3} = 0.57) were not present in the sterile control extracts. The slowest moving spot co-migrated with para-, meta-, and ortho-chlorobenzoic acid standards on the TLC plate. This spot appeared as a single peak giving rise to a molecular ion at m/e 156. The identity of this metabolite was verified as chlorobenzoic acid by comparison to mass spectra of authentic chlorobenzoic acid standards (Fig. 11). No attempt was made to discern the isomeric form of the compound. The intermediate spot on TLC (R_f = 0.68) showed

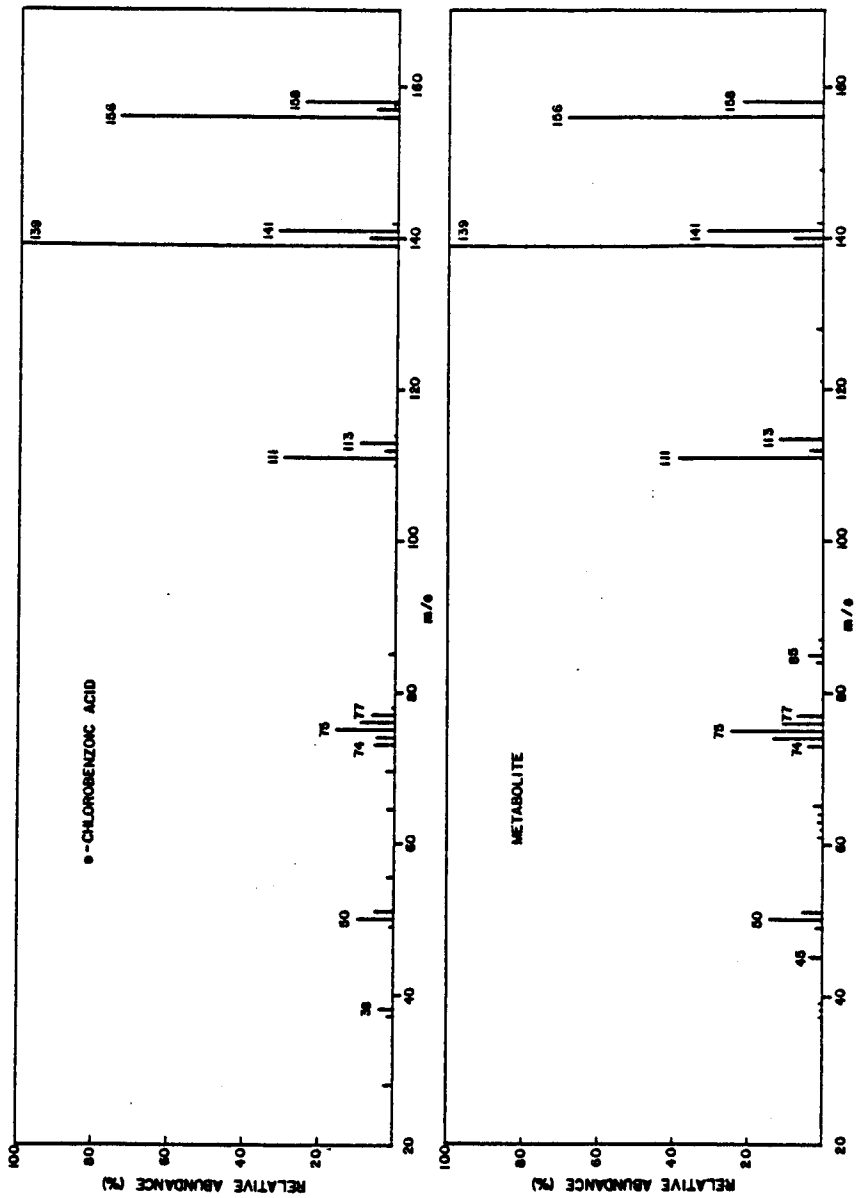

Fig. 11. Mass spectra of o-chlorobenzoic acid standard and the metabolite isolated from Center Hill Reservoir water amended with 2-chlorobiphenyl.

molecular ions at m/e 167, 139 and 111 with characteristic chlorine doublets (Fig. 12). The compound was tentatively identified as chlorobenzoyl formic acid based on the nature of the mass spectral fragmentation pattern and comparison with unsubstituted benzoyl formic acid (Fig. 13).

Components of the 2,4'-dichlorobiphenyl metabolism extract migrated as a single yellow spot on the TLC plates (R_f = 0.91), while the sterile control extract moved as a different single spot corresponding to parent 2,4'-dichlorobiphenyl (R_f = 0.99). The potential metabolite was resolved into two peaks by GC-MS. The first peak was identified as a dichlorobiphenyl from the mass spectrum (Fig. 14); presumedly it was the parent compound, 2,4'-dichlorobiphenyl (M+, m/e 222). The second peak (Fig. 15) was tentatively identified as an unchlorinated phthalate derivative (M+, m/e 278) based on matching with an NIH library of mass spectra. It is suggested that the failure of the dichlorobiphenyl to migrate as a single spot may be due to complex formation with the tentatively identified phthalate, and extractable cellular components.

Growth of Heterotrophic Bacteria in Biodegradation Flasks

In order to develop a more complete understanding of the biodegradation results and the PCB-microbial interaction, the heterotrophic growth response of Center Hill Reservoir bacteria was examined in the presence and absence of Aroclor 1254. In order to investigate the possibility of bacterial acclimation to PCB, a PCB-induced (PI mixed culture) was used as an inoculum in autoclaved reservoir water

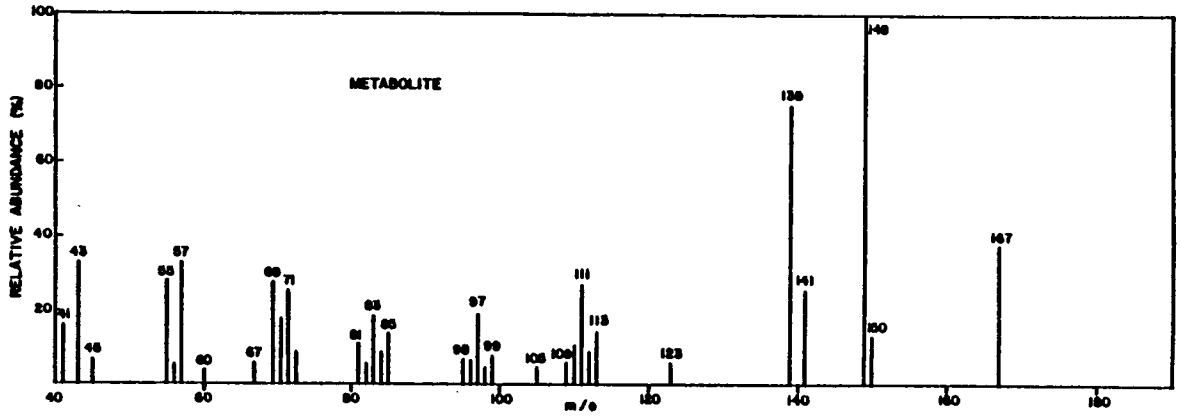

Fig. 12. Mass spectrum of chlorinated metabolite tentatively identified as chlorobenzoyl formic acid isolated from Center Hill Reservoir water amended with 2-chlorobiphenyl.

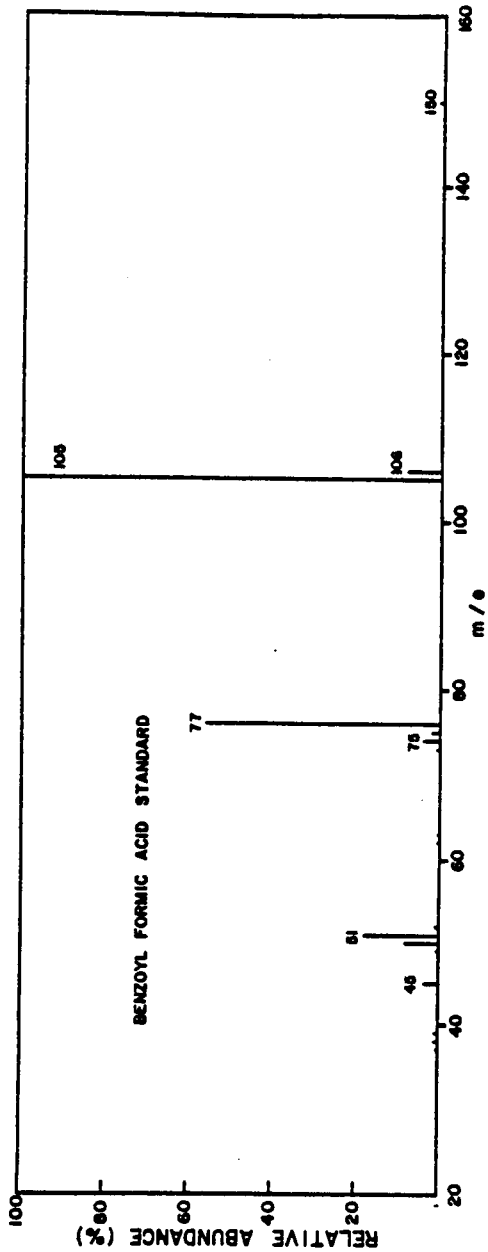

Fig. 13. Mass spectrum of authentic benzoyl formic acid (see Fig. 12).

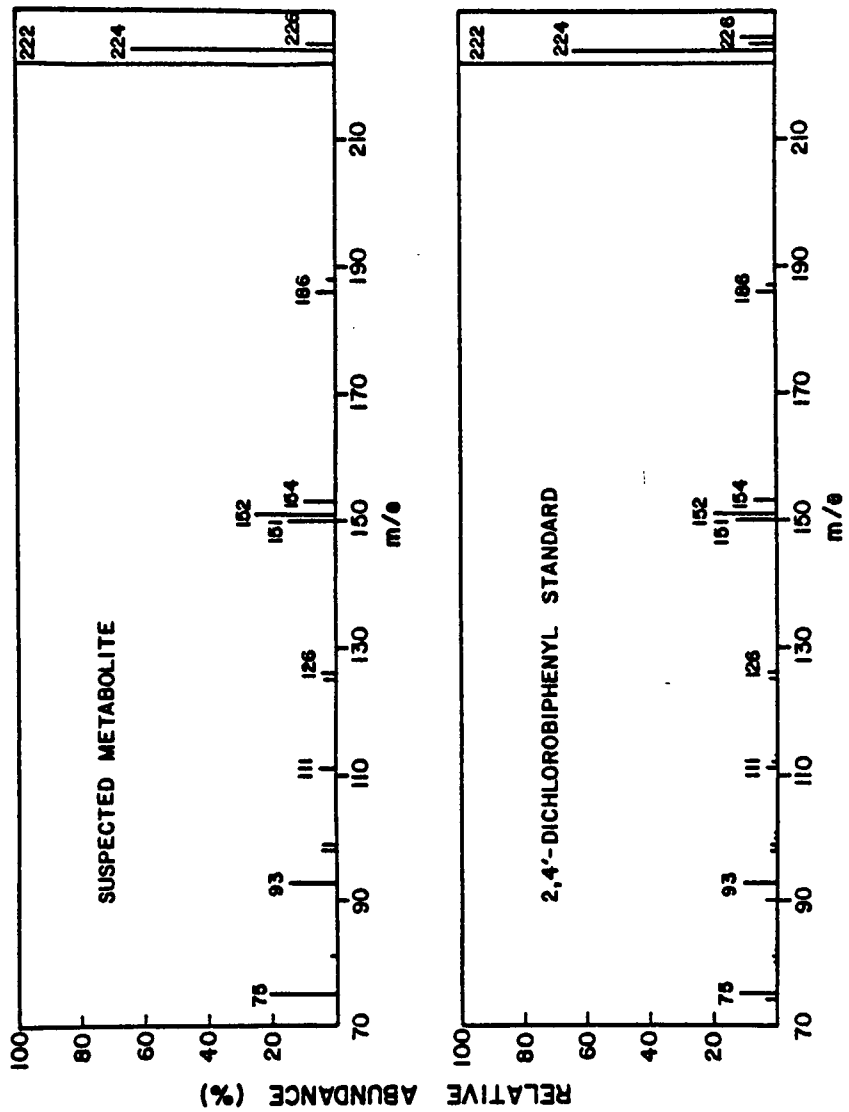

Fig. 14. Mass spectra of 2,4'-dichlorobiphenyl standard and of suspected metabolite isolated from Center Hill Reservoir water amended with 2,4'-dichlorobiphenyl.

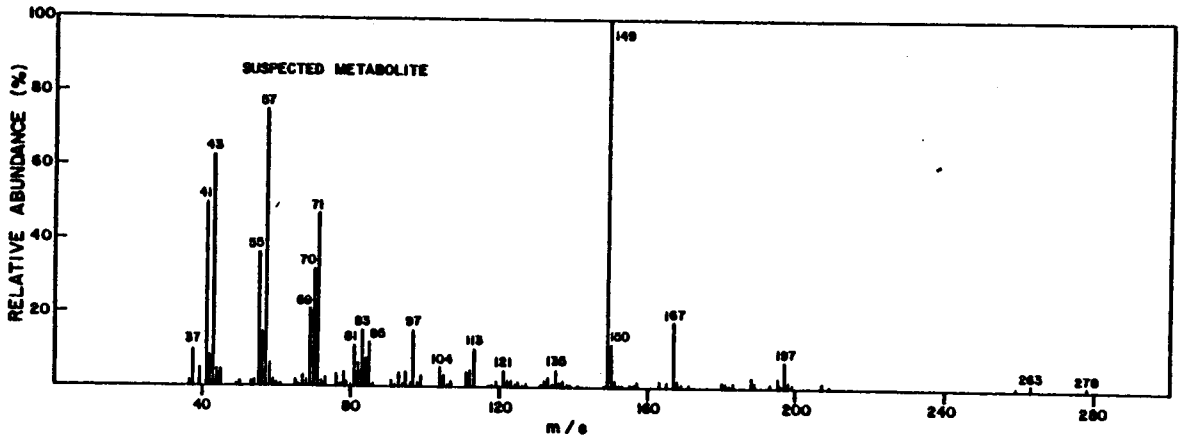

Fig. 15. Mass spectrum of unchlorinated compound tentatively identified as dioctyl phthalate isolated from Center Hill Reservoir water amended with 2,4'-dichlorobiphenyl.

dosed with Aroclor 1254. PCB stimulated aerobic heterotrophic bacteria as was previously observed in studies of PCB plus XAD (Fig. 16). The initial thousand-fold increase of PI counts is evidence of bacterial adaption to the presence of PCB. In the absence of PCB, a transient 6-fold increase of YEPGA count was followed by a 10-fold decrease. Only after 25 weeks did counts approach those in the PCB dosed flasks (9.5×10^4 CFU ml^{-1}). The PCBA counts (Fig. 17) displayed the same general trends. The PI counts once again demonstrated an adaptive response to PCB as indicated by the 500-fold increase in counts at week 1. The mixed culture dosed with Aroclor displayed a similar stimulatory response, proportionately less by an order of magnitude. In control vessels, the mixed culture counts declined throughout the first 12 weeks of incubation. Both YEPGA and PCBA counts indicated a direct PCB stimulation of heterotrophic bacteria. Unaccountable fluctuation was initially observed; however, after the second week of incubation, a consistent trend was established among the different treatments. The flasks without PCB exhibited higher PCBA/YEPGA count ratio throughout the incubation period as compared to the other two treatments (Fig. 18). The lowest ratios were consistently found in the PI culture exposed to PCB. These results suggest that previous exposure to PCB results in a PCB-adapted bacterial population, which on the other hand, is less resistant to the 10,000-fold higher PCB concentration incorporated in PCBA (i.e., 1 g Aroclor 1254 l^{-1}).

Gross differences among the flasks were observed during the course of incubation. The physical appearance of the PCB-induced

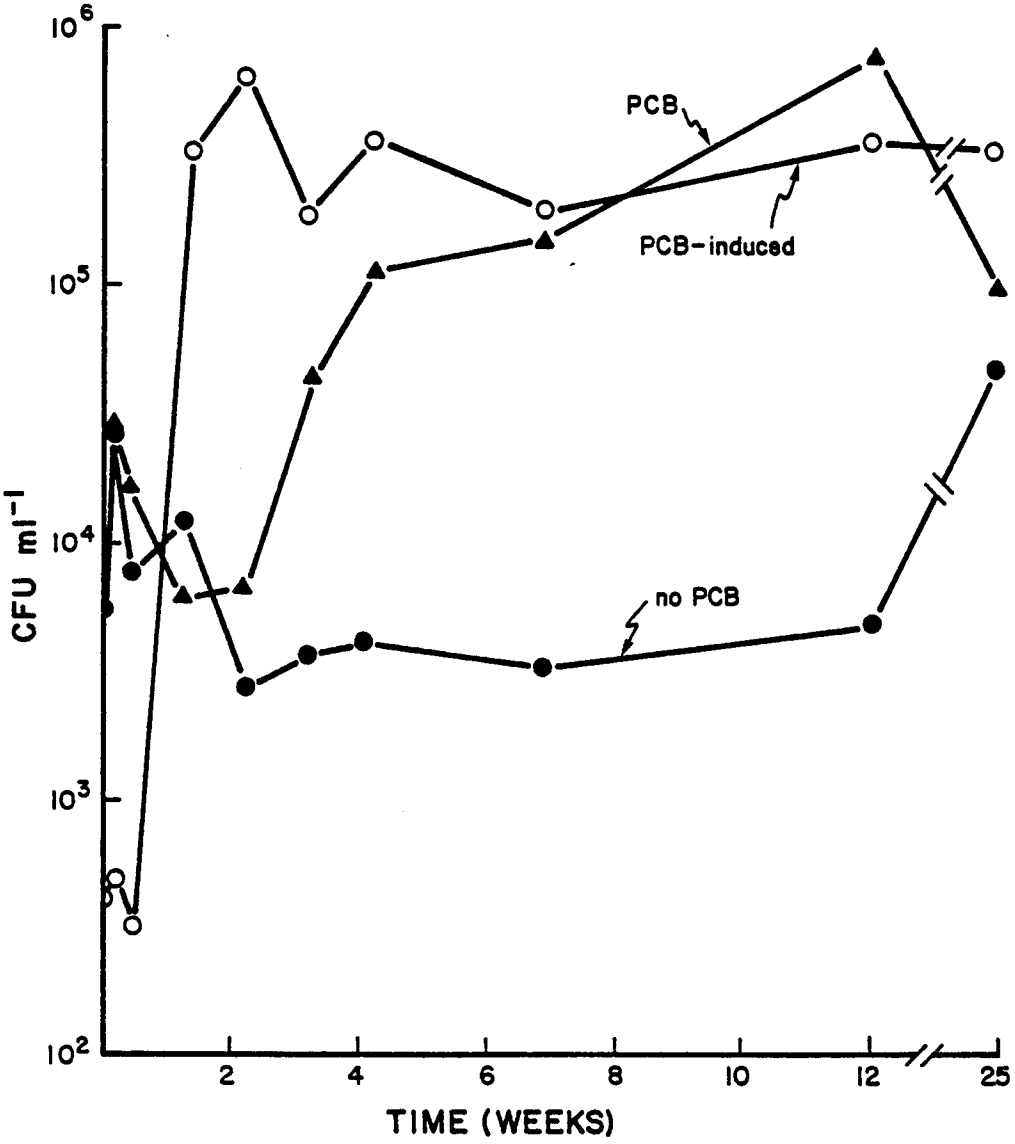

Fig. 16. Effect of 100 µg Aroclor 1254 l^{-1} on bacterial growth. Various treatments of Center Hill Reservoir water were dark incubated at 25 C and plated on YEPGA.

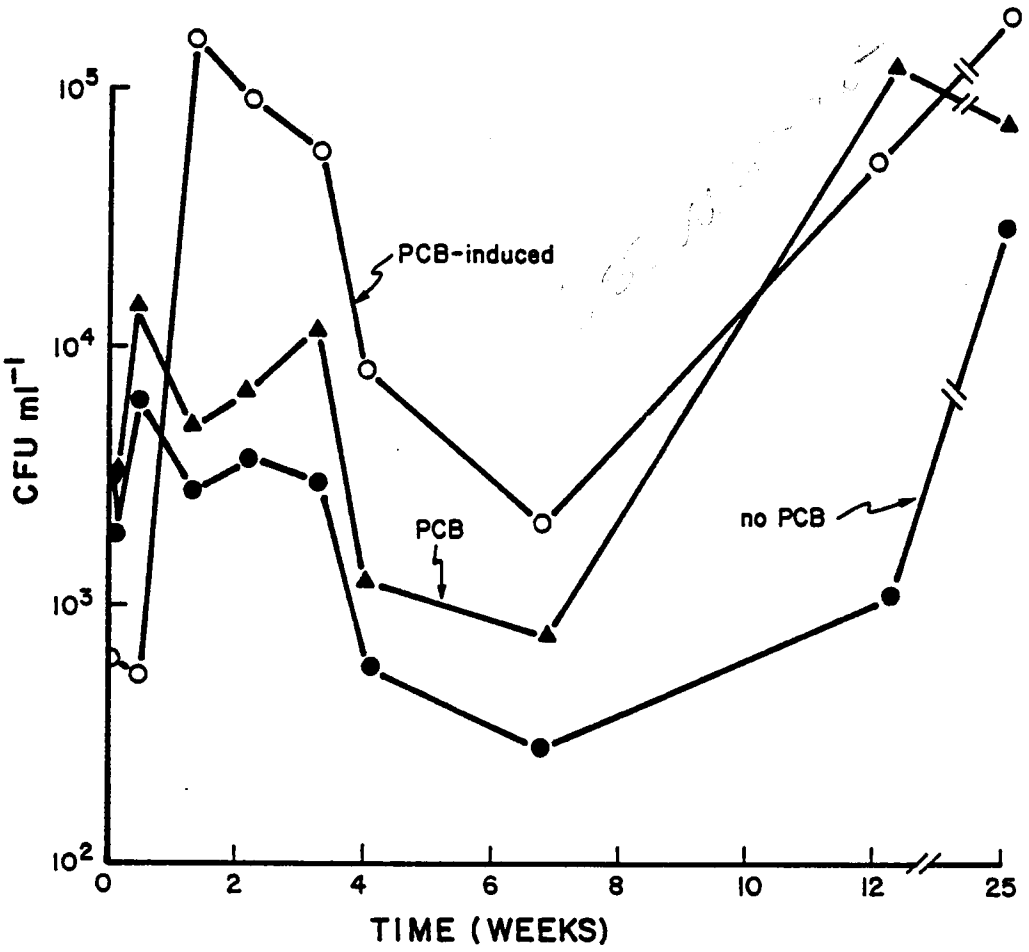

Fig. 17. Effect of 100 µg Aroclor 1254 l^{-1} on PCB-resistant bacterial growth. Various treatments of Center Hill Reservoir water were dark incubated at 25 C and plated on PCBA.

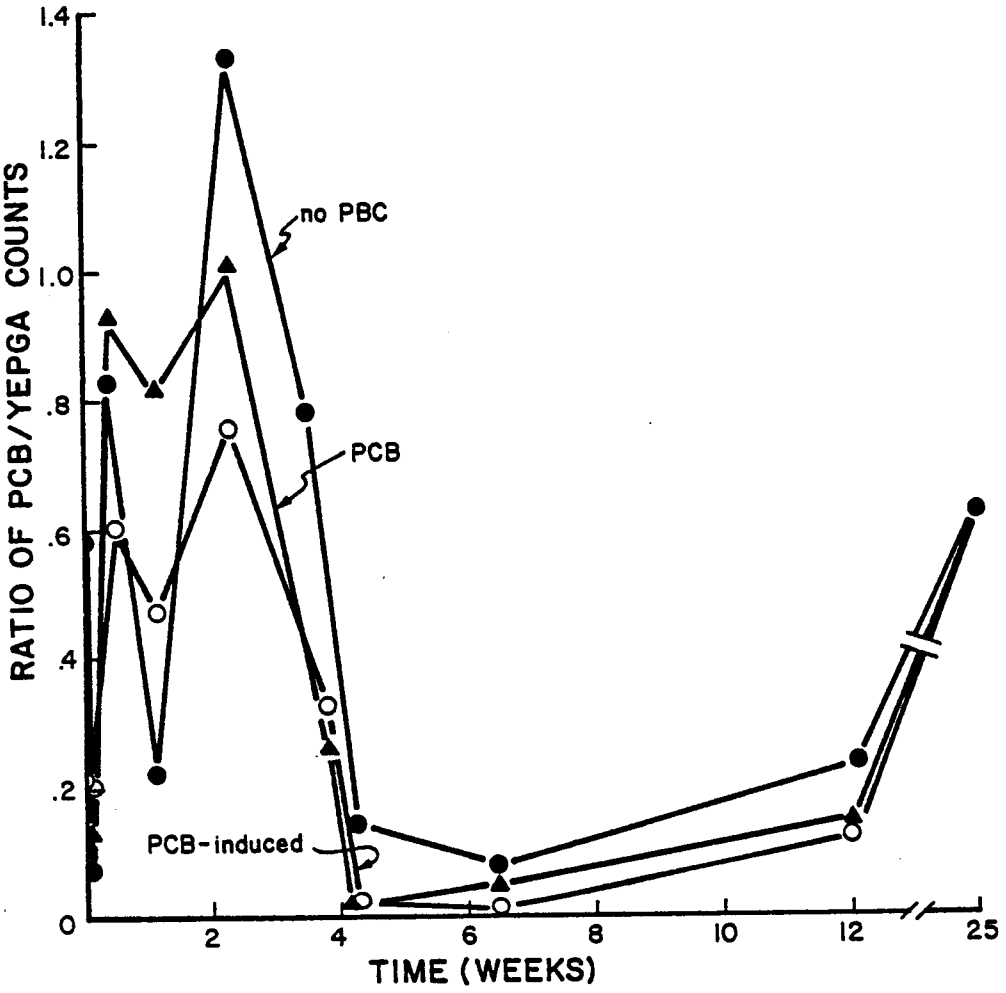

Fig. 18. Ratio of total viable counts on PCBA and YEPGA (see Figs. 16 and 17).

mixed culture flasks began to change at 4 weeks' incubation. A similar change in the flask containing PCB was detected during the fifth week of incubation. The appearance of all flasks before and after incubation is summarized in Table 11.

Physical alterations of the Celite in the biodegradation flasks prompted closer observation by scanning electron microscopy. The sterile control containing PCB (Fig. 19A) was identical to the septic control without PCB (Fig. 19B) in appearance. In both treatments, the diatom frustules of the Celite were loosely dispersed on the Nucleopore filter surface. Occasional microbial forms (not illustrated) were observed in the septic control upon closer scrutiny. None of these organisms appeared to be directly associated with the frustules, but rather displayed a random distribution.

The frustules in the PCB cultures were tightly clumped (Fig. 20). The nature of this clumping phenomenon became clear at higher magnifications (Fig. 20). Copious quantities of slime-like material with embedded rod-shaped bacteria covered the silicate frustules. Other, less numerous rod forms, possessing terminal strands of polymer-like material, spanned frustules of the gelatinous conglomeration. The PI culture (not illustrated) was identical to the PCB-dosed culture in appearance.

Effects of PCB on Heterotrophic Glucose Uptake by Reservoir Bacteria

Effects of PCB on Natural Glucose Turnover

Glucose turnover times for each of the eight samples examined are presented in Table 12. Natural control glucose uptake velocities approximated saturation kinetics in only one instance (Site 4,

Table 11. Visible characteristics of Celite in biodegradation flasks.

Treatments	PCB[a]	Incubation Time (Weeks)	Color of Celite	Celite Consistency[b]
Reservoir Water	+	0	White	Powder[d]
PMA-killed		25	White	Powder
Reservoir Water	+	0	White	Powder
		25	Yellow-brown	Clumped[e]
Reservoir Water	-	0	White	Powder
		25	White	Powder
PCB-enriched	+	0	White	Powder
Inoculum		25	Yellow-brown	Clumped

[a]Presence or absence of Aroclor 1254 in culture vessel.

[b]Visible appearance of Celite in flask.

[c]PMA, phenylmercuric acetate.

[d]Celite had the appearance of a fine particulate, settleable powder distributed evenly on the bottom of the flask.

[e]Celite was coagulated into a few, multilobed clumps with rubber-like texture.

Fig. 19. Scanning electron micrographs of Celite in PCB biodegradation controls. (A) Low-power micrograph of sterile control demonstrates loose nature of diatomaceous earth in sterile control. Bar represents 100 μm; (B) Septic control (no PCB) is identical in appearance at higher magnification. Bar indicates 20 μm.

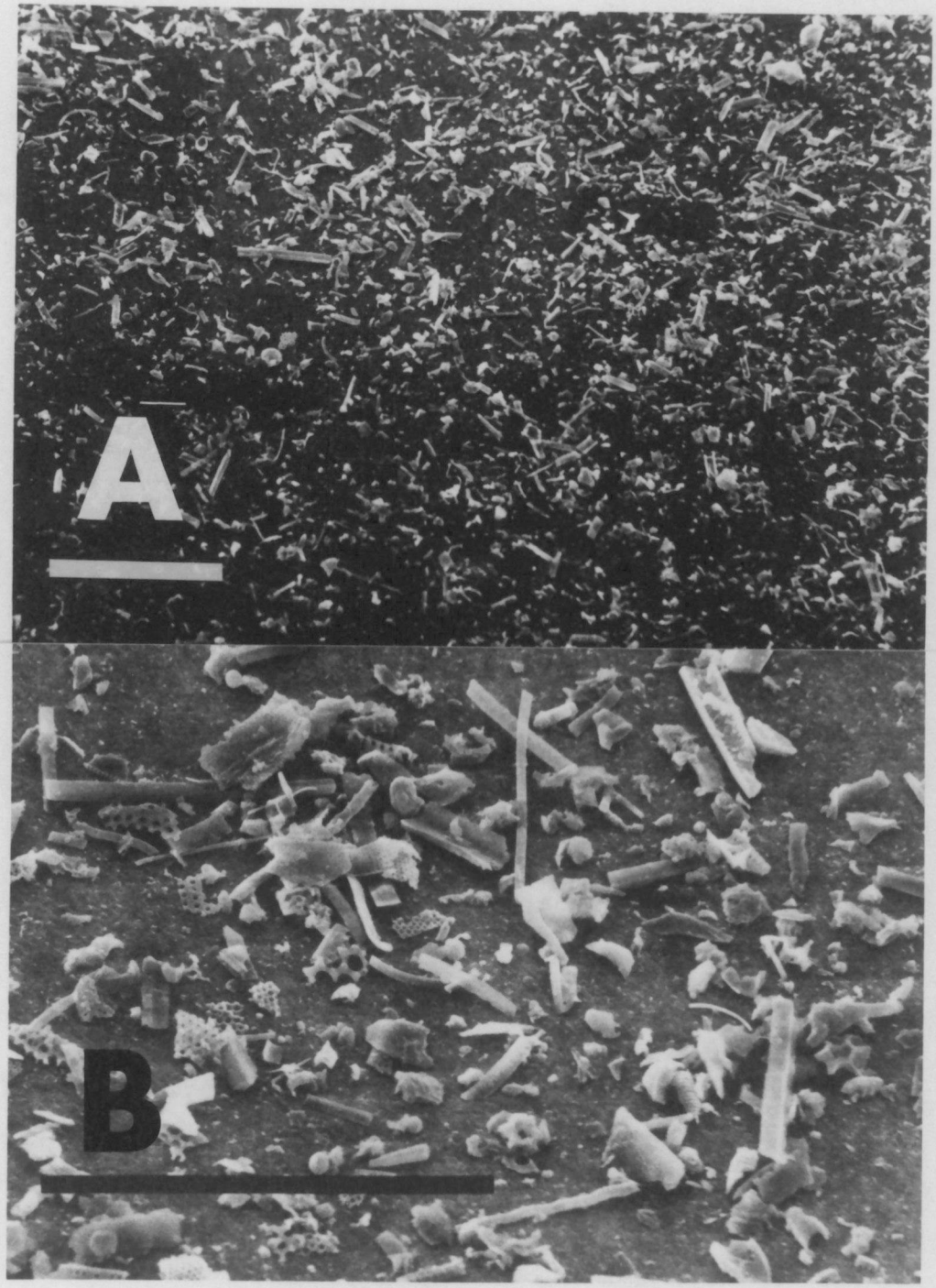

Fig. 19

Fig. 20. Scanning electron micrographs of Celite in PCB biodegradation flasks. (A) Low-power magnification of experimental flask dosed with Aroclor 1254 illustrates clumped nature of Celite. Bar represents 100 μm; (B) A higher magnification of experimental flask Celite reveals slime-like sheath with embedded bacteria enveloping the diatom frustules. Bar indicates 10 μm.

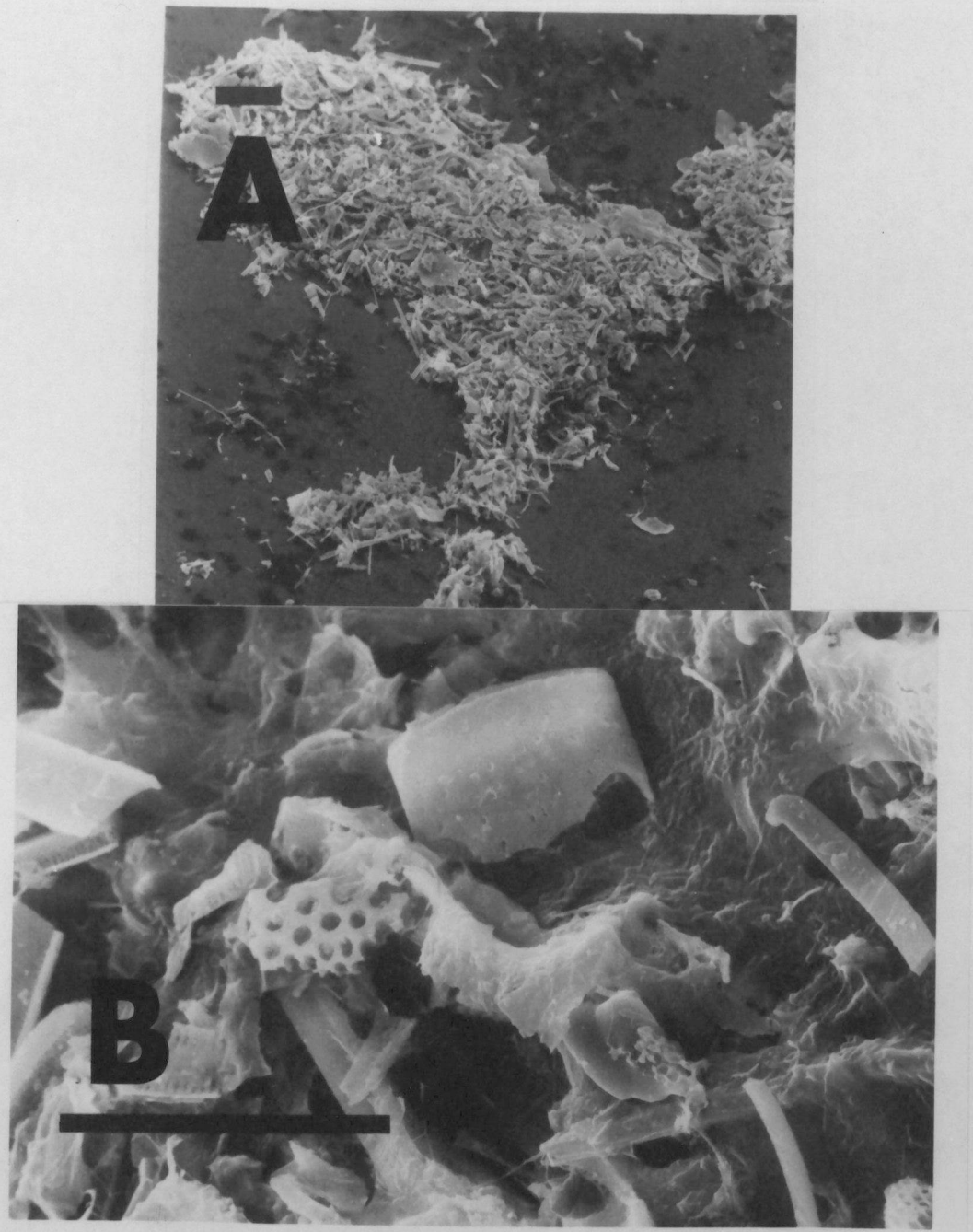

Fig. 20

Table 12. Comparative effect of PCB on natural glucose turnover among temporally and spatially isolated samples.

Date	Site[a]	Turnover Time[b] (h) Natural	PCB
6-77	2	2,500	355*
	4	270	675*
	5	260	645*
8-77	1	1,940	620*
	7	245	75*
9-77	4	1,430	120*
	7	1,295	1,230
12-77	4	690	550

[a]See Fig. 4, page 58.

[b]Turnover times were estimated from linear glucose uptake kinetics over a glucose concentration range of 5, 30, 60, and 120 μg l^{-1}.

*Values significantly different from natural samples ($p < 0.05$).

December, 1977). With minor exceptions, all other uptake velocities appeared linear with respect to increasing glucose substrate levels from 5 to 120 μg l^{-1}. Consequently, turnover times were calculated on the basis of linear uptake kinetics as described by Wright and Hobbie (175). Overall, no distinct patterns in glucose turnover were observed. The fastest rate of glucose turnover was observed at Site 7, August (245 h) and the slowest rate was observed at Site 2, June (2,500 h). In general, there were no temporal or spatial trends indicated by either turnover times or raw uptake velocities.

The presence of PCB resulted in both stimulation and inhibition of natural glucose turnover (Table 12). PCB stimulated glucose turnover in four samples and inhibited turnover in two samples. The PCB effects ranged from a 10-fold stimulation to a 3-fold inhibition of glucose turnover.

In December, 1977 (Site 4) and August, 1978 (Site 7), examination of the PCB stress on glucose turnover at elevated glucose concentrations was undertaken. Samples collected in December exhibited glucose turnover rates on order of magnitude higher than any samples previously examined (Table 13). There was a significant increase in glucose turnover for those samples collected in August, 1978 (Table 13). This inhibition in glucose turnover was the result of a virtual cessation of increased rates of glucose uptake beyond a glucose substrate concentration of 265 μg l^{-1}.

Table 13. Comparative effects of PCB on natural glucose turnover at elevated glucose levels.

Date	Site[a]	Turnover Time[b] (h)	
		Natural	PCB Stressed
2-77	4	13	8
8-78	7	130	36,720*

[a]See Table 4, page 57.

[b]Turnover times were estimated from linear glucose uptake kinetics over a glucose concentration range of 50 to 1000 μg l^{-1} (increments of approximately 100 μg l^{-1}).

*Values significantly different from unstressed natural samples ($p < 0.05$).

Effect of PCB on Glucose Mineralization

PCB effects of substrate mineralization were examined by partitioning $^{14}CO_2$ evolution from total (^{14}C)glucose uptake (Table 14). An average of 61.9% of the total glucose uptake was respired by natural samples. In comparison, 63.1% of the total glucose uptake was respired by PCB-containing samples. These averages include all ranges of glucose substrate concentrations. There were no significant differences ($\alpha = 0.05$) between the mean proportions of $^{14}CO_2$ evolved.

In an effort to explain the nature of the variation in glucose uptake and turnover in the presence and absence of PCB, an analysis of the effects of environmental variables was undertaken. A mixed-model, three-way analysis of variance examining the effects of sample type, sample treatments, and glucose substrate concentration was performed (Table 15). Statistically significant sample type, treatment, and substrate concentration effects were observed. The results indicated significant variation in terms of glucose uptake, in response to both treatment and glucose concentration effects. Because there were no significant interaction terms among any of the samples, treatments and glucose concentrations, the weight of each source of variation can be calculated from the mean squares (Table 15). Most of the variation in glucose uptake, 50.9%, was explained by the glucose concentration. The differences among samples explained 18.2% of the variation, while the presence or absence of PCB explained 31.0% of the variation. Preliminary correlation analysis indicated no apparent correlation between bacterial cell densities (TVC and PCB) and glucose uptake velocities, although uptake

Table 14. Comparative effects of PCB on glucose mineralization by Center Hill Reservoir samples.

Treatment	No. of Observations	% CO_2 Evolved	Standard Deviation
Natural	33	61.9	24.0
PCB	36	63.1	26.4

Table 15. Three-way analysis of variance of glucose uptake velocities of the Center Hill Reservoir microbial community.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F[a]
Main Effects				
Sample[b]	7	7.49×10^7	1.07×10^7	3.476*
Treatment[c]	1	1.83×10^7	1.83×10^7	5.927*
Substrate concn.[d]	3	9.00×10^7	3.00×10^7	9.736*
Interactions				
Sample X treatment	7	1.45×10^8	4.68×10^6	1.520
Sample X substrate concn.	21	9.00×10^8	4.28×10^6	1.390
Treatment X substrate concn.	3	9.78×10^8	3.26×10^6	1.058
Explained	41	3.28×10^8	7.82×10^6	2.537*
Residual	21	6.47×10^7	3.08×10^6	
Total	63	3.93×10^8	6.24×10^6	

[a]*Significant F statistic, $\alpha = 0.05$.

[b]Sample site and data.

[c]With or without PCB.

[d]$(U\text{-}^{14}C)$Glucose concentration range.

velocities themselves were significantly intercorrelated.

Regression analysis was performed to quantify the variations in glucose uptake velocities as a function of the independent variables. Initially, uptake velocities were regressed only on glucose substrate concentrations in model I linear regression. The regression lines were:

natural glucose uptake velocity $y = 0.01 + 0.0018\ x$

PCB glucose uptake velocity $y = 0.01 + 0.0038\ x$

X is the glucose concentration ($\mu g\ l^{-1}$) and y is the glucose uptake velocity ($\mu g\ l^{-1}\ hr^{-1}$). The r^2 values were 0.28 for the natural samples and 0.27 for PCB-dosed samples. Statistically significant linear regression relationships were found for each of three uptake velocity responses examined. However, as indicated by the r^2 values, only 28 and 27% of the variation of the independent variable, glucose uptake velocities for TVC and PCB, respectively, could be attributed to the linear regression of glucose uptake velocity on the glucose substrate concentration. Second- and third-order curvilinear models were also examined, and, although significant regressions were observed, there was no greater explained variation attributed to the regression model. These results indicated that variations in glucose uptake were attributable, in part, to other independent variables in addition to glucose substrate concentration.

To elucidate these effects more fully, a multiple regression analysis between glucose uptake velocities, substrate concentration, and field sampling variables was performed. For the purpose of this analysis the following variables were examined: glucose uptake

velocities for untreated samples (VTVC) and PCB-stressed samples (VPCB); glucose substrate concentrations; logarithmic transformation of TVC and PCB-degrading or PCB-resistant bacterial counts; water temperature; dissolved oxygen; Secchi disk transparency; conductivity; and pH (Table 16). As expected, the major contributing factor to variation in uptake velocity was the glucose substrate concentration. However, the added variance components explained 46 and 45% comparatively, for VTVC and VPCB of the total variation attributed to the multiple regression. Aside from the substrate concentration, the two greatest sources of variation were conductivity and pH. Increases in the multiple r^2 due to conductivity, relative to increased variation in uptake, were 0.8 for both VTVC and VPCB. Variation attributable to pH increased r^2 values by 0.2 and 0.0, respectively, for VTVC and VPCB. Although a significant portion of the total variation of glucose uptake velocity was accounted for by the individual regression equations, the overlapping 95% velocity confidence limits at the mean glucose substrate concentration (57 $\mu g\ l^{-1}$) indicated no significant difference between the natural uptake and the uptake of those samples dosed with PCB.

Table 16. Comparison of multiple regression coefficients of glucose uptake velocities for natural samples, PCB-stressed samples, and PHE-stressed samples.

Independent Variable[a]	Regression Coefficient		Multiple Coefficient of Determination (r^2)	
	VTVC	VPCB	VTVC	VPCB
SUBCON (X_1)	0.18	0.36	0.28	0.26
TEMP (X_2)	0.12	0.36	0.28	0.28
DO (X_3)	-0.24	0.33	0.30	0.30
SECC (X_4)	0.15	0.15	0.30	0.33
COND (X_5)	-0.26	-0.14	0.38	0.45
PH (X_6)	-0.11	0.75	0.46	0.45
TVC (X_7)	0.14	0.47	0.46	0.45
Y intercept (C)	0.01	-0.03		
Mean velocity ($\bar{Y}$)[c]	0.11	0.22		
95% confidence limits of $\bar{Y}$	±0.04	±0.09		

[a]SUBCON, glucose substrate concentrations; TEMP, water temperature; DO, dissolved oxygen; SECC, Secchi disk transparency; COND, conductivity; PH, pH.

[b]b X 10^2.

[c]$\mu g\ h^{-1}\ l^{-1}$.

VI. DISCUSSION

Polychlorinated biphenyls are complex mixtures containing well over 100 different compounds. Their chemical and physical properties, such as solubilities, partition coefficients, vapor pressures and densities, are actually wide-ranging averages dependent on the specific composition of the mixture. As a result, they present considerable analytical difficulties. Consequently, the choice of analytical methods for the extraction and quantitation of PCBs is crucial to the investigative outcome.

Hexane batch extraction of PCB was utilized for the majority of experimentation with adequate success. The relatively high recovery values achieved in this study (95 to 98%) were in close agreement with values reported by other investigators. Dawson and Riley (41) observed 95 to 96% PCB recoveries with a double hexane extraction and Paris et al. (130) reported 92 to 96% PCB recoveries with organic solvent extraction. In contrast, XAD-2 PCB extraction methods yield only 78 to 86% recovery (35) and exhaustive steam distillation results in only 80 to 90% recovery (46).

Even with conscientious analytical technique, a high degree of variability was associated within the hexane batch extraction method and more so with the Tenax method. Sources of error are inherent in several steps of the extraction procedure. According to Hutzinger (86), the quantitative precision of PCB extraction methods can be expected to approximate the commonly employed chlorinated hydrocarbon pesticide analysis techniques (standard

deviation ± 20%). In this study, triplicate control flask variation of PCB recovery ranged from 2 to 6% for hexane batch extraction (page 51) and 7 to 21% for Tenax-GC extraction (Table 2, page 54). The physical state of the PCB residue probably explains the discrepancy between observed and literature error values for batch extraction. The PCB in control flasks was of known composition and was not affected by biological activity while the reported samples were of environmental origin and of unknown PCB composition.

Concentration of initial hexane extracts to a small analysis volume was found to be the most critical step in PCB quantitation. Approximately a 50% loss occurred by reducing the extraction volume to 1 ml, whereas loss of PCB by volume reduction to 2 ml was decreased to insignificant levels. Loss by volatilization is a problem for a variety of organic pollutants (170) and places a lower limit on the sensitivity of the quantitative assay. In the present case, however, sufficiently high PCB concentrations in the biodegradation flasks and ample electron capture detector sensitivity alleviated the need for further extreme concentration of the extraction volume.

A comparison of the batch versus Tenax extraction methods confirmed previous studies (86, 170) that liquid-liquid batch extraction is a more efficient extraction technique. Several aspects of the Tenax method were inadequate for use with the biodegradation assays. First, only 69.7% of the added PCB was recovered and even with a hexane rinse of the glass vessel, total recovery is only 76.5%. The requirement for a hexane rinse tends to negate the Tenax advantage of simplicity and speed. Another inadequacy of the Tenax extraction

is the significant interference of extraction efficiency by low amounts of organic matter. It is suggested that organic matter solubilization of PCB as well as organic matter interaction with the Tenax, acts to reduce the number of PCB adsorption sites. This resulted in the passage of significant amounts of PCB through the column with the water sample.

The cumulative effect of these difficulties was a high degree of variability. This variability was observed to increase with increasing concentration of organic matter as well as increasing aqueous sample sizes (Table 2, page 54). Coefficients of variation ranging from 40 to 206% (calculated from data in Table 3, page 55) were unacceptable for use in biodegradation assays.

Use of hexane batch extraction with triplicate, experimental, biodegradation flasks also yielded considerable variation in PCB recovery (Table 5, page 60). Much of the variation was undoubtedly attributable to the establishment of separate "microenvironments" among the triplicate flasks. This well-known phenomenon is typically a nuisance with smaller experimental volumes. Perhaps an increase in biodegradation vessel size or replicate numbers would have decreased the standard deviation, however, due to the large size of the experimental design, this was unfeasible. Unfortunately, the experimental variation may have masked the importance of some environmental parameters on potential PCB biodegradation.

Of all the environmental parameters monitored during sampling, only aerobic, heterotrophic bacterial counts significantly correlated with the extent of laboratory PCB biodegradation, indicating that the

size of the heterotrophic population is critical to PCB biodegradation. It is not completely clear why population size was so important to a long-term incubation study since the bacteria seemingly have adequate time to proliferate (Fig. 16, page 102). It appears, therefore, that a long lag was required for PCB degrading organisms to adapt or become selected. In effect then, a larger initial bacterial population requires a shorter lag phase.

Dissolved oxygen, suspended sediments, and dissolved organic carbon also explained much of the variation in PCB biodegradation but experimental error masked possible significant correlation. Furthermore, some variables such as dissolved oxygen, dissolved organic carbon, phosphate concentration and conductivity exhibited little fluctuation during the study period, perhaps further masking significant effects. In this respect, it is noteworthy to mention that laboratory incubation contributed as much as experimental variation in the masking of important environmental parameters. Because of the resistance of the PCB molecule to biodegradation, it was necessary to employ long incubation times. As a result, some of the environmental characteristics such as dissolved oxygen and dissolved organic carbon may have changed during the incubation. Consequently, their true importance may have been masked. Certainly the effect of the ambient reservoir temperature was masked by incubating flasks at 25 C.

Surprisingly, bacterial counts on PCB agar did not correlate with PCB biodegradation. Sayler et al. (151) failed to observe a correlation between Aroclor 1254 agar counts and ambient estuarine PCB levels. It was initially assumed that PCB counts reflected the

numbers of PCB degrading organisms, since Aroclor 1254 was the only chemo-organotrophic carbon and energy source added to the medium. The PCB mixture, however, as purchased from the commercial producer, probably contained enough contaminants (86, 87) to support some bacterial growth. It was concluded, therefore, that PCB counts represented low nutrient-requiring bacteria that are resistant to high levels of PCB and may include PCB degraders.

The effects of pH on biodegradation were consistent with an expected pH effect on most biological activities; that is, PCB biodegradation exhibited a bell-shaped curve with varying pH (Fig. 5, page 65). Significant biodegradation was not observed at lower pH's while an apparent optimal pH coincided with the original reservoir pH. The later phenomenon indicated that the reservoir microbial population was acclimated to the ambient reservoir pH. The ability of the microflora to adapt to the changing reservoir pH may also explain the lack of correlation between reservoir pH and PCB biodegradation.

PCB concentration also played a major role in the rate and extent of PCB biodegradation (Table 9, page 67). It appears that the biodegradation of many organic pollutants is concentration dependent, particularly at very low concentrations. Boethling and Alexander (22) reported that 2,4-dichlorophenoxyacetic acid (2,4-D), naphthol, and 1-naphthyl-N-methylcarbonate biodegradation rates decreased with substrate concentrations. PCB biodegradation also conformed to this phenomenon. Some compounds, such as 2,4-D, displayed threshold concentrations in the part-per-billion concentration range (22) below which no significant biodegradation took place. This implies that

these pollutants may persist at lower environmental concentrations.

The use of high concentrations of PCB may also account for reports that bacteria were unable to degrade PCB. Tulp et al. (162) did not detect significant degradation of 4,4'-dichlorobiphenyl at substrate concentrations of 50 mg l^{-1} and Herbst et al. (75) could not detect activated sludge degradation of various pure (^{14}C)chlorinated biphenyl isomers at substrate concentrations of 400 µg l^{-1}. The latter investigators, however, conducted the experiments with only 6 hour incubations.

In situ biodegradation studies corroborated laboratory experimentation results which indicated that PCB biodegradation can occur in nature. It was interesting to note that biodegradation rates from both were comparable (Table 5, page 60; Table 8, page 64). Some caution, however, is warranted in the interpretation of in situ biodegradation. First, the in situ studies were performed in open systems, allowing the exchange of small particles and dissolved materials with the reservoir environment. Laboratory studies, on the other hand, were performed in closed systems allowing for an analytic budget of added PCB. Unaccountable loss of PCB during in situ incubations, therefore, cannot be wholly attributed to PCB biodegradation. Hypothetically, dissolved organic compound excretion by the biota in the biochambers can solubilize more PCB than in sterile control chambers although polyurethane foam pads should retard such loss. The net result is a biologically induced loss of PCB via molecular diffusion rather than by degradation. The second important distinction between the two systems was the nature of the environmental conditions.

The laboratory assays were conducted at a constant temperature in the absence of environmental perturbations. The in situ chambers, on the other hand, were subject to fluctuating environmental conditions. Furthermore, light, even at a 1 meter depth, may have played a photodecompositional role (143). The role of algae and cyanobacteria may also have contributed to PCB biodegradation in the light since algae and other phototrophic organisms possess limited heterotrophic potential (5, 25). The role of algae, however, was not experimentally tested.

In sharp contrast to the biodegradation studies which indicated active PCB biodegradation, the results of two independent experiments indicated a lack of terminal decomposition of PCB to CO_2. Even extensive partial oxidation of chlorinated biphenyls should theoretically result in CO_2 evolution. It was, therefore, concluded that mineralization of Aroclor 1254-like mixtures is not a major route of PCB-removal from the environment. This conclusion is in agreement with the general view that higher chlorinated biphenyls are virtually nondegradable in nature (14).

The possibility exists that the lack of mineralization was a result of unfavorable experimental conditions. Two different lines of evidence indicated, however, that the negative results were an accurate reflection of PCB fate in nature. First, parallel (^{14}C)-naphthalene studies demonstrated the utility of the mineralization assay which is in accord with previous work (74). In essence, these results represent a positive control for (^{14}C)PCB mineralization in lieu of an active PCB mineralizing bacterial culture.

Active naphthalene mineralization was also indirect evidence that (^{14}C)PCB was in an accessible physical state for microbial utilization. There was initial concern that sorption of PCB by the XAD resin might physically separate the bacteria from the PCB. However, since napthalene and PCB both have low water solubilities, high octanol-water partition coefficients, high water activities, and other similar sorption interactions with XAD, it was felt that they acted in a similar manner in the assay. In addition, the intimate association of slime-forming bacteria with surfaces in the presence of PCB also indicated that the bacteria were in contact with the PCB.

The stimulation by XAD and XAD-PCB of heterotrophic growth provided evidence that the bacteria were not inhibited in the mineralization bottles. The nature of the XAD stimulation was not clear, but it is suggested that the physical presence of the XAD surface area was responsible for the stimulation. The addition of PCB further stimulated bacterial growth (Fig. 8, page 73), as did the presence of PCB in Celite-containing biodegradation flasks (Fig. 10, page 76).

It was notable that PCB alone did not affect bacterial growth over the control mineralization bottles. Only in the presence of XAD was the PCB addition stimulatory. This observation coupled with the PCB stimulation of bacteria in the biodegradation flasks as well as the SEM studies suggested that PCB stimulated only the growth of surface-associated bacteria. This hypothesis will be discussed in more detail later.

Transformation of 2-chlorobiphenyl to chlorobenzoic acid and chlorobenzyol formic acid was conclusive evidence that Center Hill

microflora decomposed PCB. Accumulation of chlorobenzoic acid is commonly encountered in PCB biodegradation (1, 13, 56-58, 162). Ohmori et al. (128) identified gamma-benzoyl butyric acid from biphenyl in addition to 4-chlorobenzoic from chlorobiphenyl by isolated bacteria. It appears, therefore, that natural populations of bacteria utilize the established biphenyl degrading pathway in the process of PCB biodegradation (29, 110).

The tentative identification of chlorobenzoyl formic acid was based on a very distinctive ion fragmentation pattern. A molecular parent peak (M+) was expected at m/e 184, but was not detected in the mass spectrum (Fig. 12, page 80). However, predominant peaks of distinctive chlorine doublets were present at m/e 139 (M-45), corresponding to the loss of the formic acid moiety, and at m/e 111 (M-73), corresponding to the further loss of a carbonyl group. Because of the natural mass abundance ratio of approximately 75% ^{35}Cl and 25% ^{37}Cl, chlorine-containing compounds are readily identifiable in mass spectra as unmistakable doublets. Therefore, it was recognized that the metabolite contained one chlorine residue per molecule.

The authentic benzoyl formic acid standard displayed a diminutive molecular parent ion peak at m/e 150. The two major fragmentation ion peaks were found at m/e 105 (M-45), corresponding to the loss of the formic acid constituent, and at m/e 77 (M-73), corresponding to the further loss of a carbonyl group. It is obvious that the major fragmentation peaks of both the metabolite and the standard were identical except for the difference of a chlorine residue. In addition, there was considerable correspondence among the less dominant, lower

weight fragmentation peaks as well. In spite of the overwhelming evidence, a mass spectrum of a chlorobenzoyl formic acid standard must be compared to the metabolite spectrum for absolute confirmation. Unfortunately, chlorobenzoyl formic acid was not available commercially and the laboratory was not equipped to synthesize the molecule de novo.

Although the isolation of chlorobenzoyl formic acid as a metabolite was novel, chlorobenzoic acid production via chlorobenzoyl formic acid is in agreement with the established pathway for chlorobiphenyl degradation (29, 40, 60, 110). Gibson et al. (61) demonstrated the dihydroxylation of a biphenyl ring. Catelani et al. (29) in turn demonstrated the cleavage of the dihydroxylated metabolite to form 2-hydroxy-6-ozo-6-phenylhexa-2,4-dienoate, the meta cleavage product. If a sequence of oxidations proceeds to cleave carbon dioxide moieties off the latter metabolite, chlorobenzoyl formic acid would be an expected intermediate in the formation of chlorobenzoic acid.

Benzoyl formic acid (BFA), alternatively known as phenylglyoxilic acid, is a well established intermediate of the L-madelate catabolic pathway, which is possessed by several microbial genera (44). BFA is, in fact, the principal inducer of the mandelate pathway enzymes. Livingstone and Fewson (108) have reported that several BFA derivatives also function as potent inducers of pathway. Therefore, the possible accumulation of chlorobenzoyl formic acid in aquatic environments, albeit in extremely low concentrations, could have important implications in the biodegradation of aromatic pollutants in nature.

Benzoyl formic acid has also been identified as a metabolite in

styrene (vinyl benzene) biodegradation. Milvey and Garro (118) reported that benzoyl formic acid was a nonmutagenic styrene metabolite that can be detected in the urine of factory workers. In contrast, the chloro-derivative of benzoyl formic acid has been rarely mentioned in the scientific literature and only with reference to experiments in theoretical ∝-keto acid chemistry (50, 101).

The results of the 2,4'-dichlorobiphenyl metabolism were not as explicative as the 2-chlorobiphenyl studies. The unusual migratory pattern of the dichlorobiphenyl residual (R_f = 0.99) during thin layer chromatography suggested that the extracted PCB was in an altered physical or chemical state. In view of the relatively inert chemical properties of PCB, it is likely that the extracted biphenyl was complexed with other microbial compounds from the growth flask. Furthermore, it was not clear whether the dichlorobiphenyl molecule was actively decomposed. Although free dichlorobiphenyl was not detected in the growth flask, quantitative analysis of the complexed residual was not performed. Therefore, it was not possible to determine if all the added PCB was recovered as complexed PCB. The failure to detect known PCB metabolites did not necessarily signify that decomposition did not occur.

It was also not clear if the phthalate compound played a role in the complexing of PCB. It should be mentioned that a NIH library match with an unknown compound is not necessarily a definite identification. A phthalate derivative is merely the best match of spectra contained in the NIH library; therefore, identification of the compound as a phthalate is speculative. Besides, it is not likely

that a polar chelating molecule such as phthalate would alone bind PCB. The possibility also exists that the tentative phthalate was a dechlorinated metabolite of dichlorobiphenyl degradation. The 2-chlorobiphenyl metabolites were unequivocally derived from PCB as marked by the chlorine residues. The phthalate, however, was not conveniently marked. A thorough examination of the pertinent literature divulged that phthalate or phthalate derivatives have not been previously implicated as PCB metabolites.

Bioaccumulation studies also provided valuable information concerning the environmental fate of PCB. The phase equilibrium nature of PCB bioaccumulation by PP1 supports the findings of other investigators (34, 133, 152). The PP1 results, in addition, extend this observation to the bacterial constituents of the aquatic environment. Clayton et al. (31) reported that phase equilibrium was the dominant process in PCB bioaccumulation by zooplankton. Pavlou and Dexter (131) found similar effects with phytoplankton and shellfish as well as zooplankton, while Scura and Theilacker (152) confirmed the phenomenon in a laboratory, marine, food chain.

The initial variability in the bioaccumulation experiment was attributed to non-equilibrium dynamics during PCB partitioning among the various components of the bioaccumulation vials (i.e., cells, reservoir water, dissolved organic matter, glass walls, head space, and rubber stoppers). Once a partial equilibrium was established, the ensuing decline of radiolabeled PCB from the aqueous phase was ascribed to sorption of PCB by the rubber stoppers. Jordan et al. (92) previously described the substantial transfer of

hydrophobic compounds from the aqueous phase to rubber stoppers in similar experimental designs. Due to the high water activity of PCB (161, 180), a significant amount will volatilize to the head space and in turn partition to the rubber stoppers. The rate limiting step was, therefore, at the air-water interface and may account for the slow observed rate of PCB loss from the water phase.

The approximate 97% reduction of PCB accumulation in autoclaved cells as compared to live cells was unexpected because it appears to contradict the observation that PCB accumulation is governed by a phase equilibrium phenomenon. Superficially, the results indicate an active PCB uptake mechanism was responsible, however, it is more likely that a cellular PCB binding component was dissolved or denatured upon autoclaving. It is tempting to postulate that such a component was involved in the binding of 2,4'-dichlorobiphenyl. The observed PCB-induced slime could feasibly play this role. Such a mechanism could act to sequester PCB thereby effectively protecting the cells' PCB sensitive sites.

PCB appeared to stimulate the number of heterotrophic bacteria in reservoir water. Wong and Kaiser (174) observed a similar response by various Aroclors on natural populations of lake bacteria. It appears, therefore, that this is a general PCB effect on natural mixed populations. The stimulation of natural bacterial populations in mineralization vials also supports this conclusion. It is difficult, however, to make meaningful comparisons with other investigations because experimental conditions and PCB concentrations differed. Keil et al. (96) and Sayler et al. (150) observed PCB

stimulation of microbial activity of bacterial isolates. Borquin et al. (23), on the other hand, reported that 26% of estuarine isolates were inhibited by milligram levels of Aroclor 1242, but did not report stimulatory responses. Similarly, Blakemore and Carey (21) focused only on isolates that were inhibited by PCB and made no mention of possible stimulatory phenomena.

With respect to PCB effects on bacterial growth, it is reiterated that Aroclor 1254 is a complex mixture of compounds. The overall stimulation stemmed from the collective effects of individual PCB compounds. Two different PCB compounds do not necessarily elicit the same response from an organism. Experiments with chicks and mice (138) demonstrated that separate and distinct effects of isomer toxicity were possible. In this respect, Wyndham et al. (176) reported that 4-chlorobiphenyl was highly mutagenic to sensitive Salmonella strains but that mutagenicity decreased with increasing degree of chlorination.

The growth stimulated bacteria also displayed an adaptive response to PCB additions. Bacteria that were acclimated to Aroclor 1254 responded with a 10-fold higher increase in numbers over un-acclimated cultures upon inoculation into reservoir water containing PCB (Fig. 16, page 85). This adaption mechanism, however, did not necessarily confer PCB resistance (Fig. 18, page 87), but suggested that one adaptive mechanism to relatively low PCB concentrations was slime formation. This hypothesis is strongly supported by the scanning electron microscopy studies on the biodegradation flasks.

The microscopic observations also indicated a PCB-induced

alterations of the bacterial community structure in the biodegradation flasks. Consequently, if the species composition is altered, how is bacterial activity affected? The possible interference of PCB with basic activities such as organic decomposition and nutrient cycling may be of grave concern to the ecology of rivers and lakes.

Short-term glucose uptake studies demonstrated a significant yet unpredictable PCB effect (Table 16, page 102). Either no effect, stimulation, or inhibition on a sample to sample basis (Tables 12 and 13, pages 94 and 96, respectively) suggests that PCB affected glucose uptake in a complex manner. Overall, however, no significant specific PCB effect on heterotrophic activity was detectable.

It is likely that the variable effect of PCB on glucose uptake was due to PCB interaction with some of the environmental parameters governing glucose uptake. Aside from direct PCB-microbial effects on activity, such phenomena for example, as PCB-organic carbon complexation may decrease available nutrients sources which in turn may affect glucose metabolism. It is felt, therefore, that the natural variability of glucose uptake and unknown interaction of PCB with environmental variables together masked the direct PCB effect and its impact on the activity of individual components of the microbial community. Pure culture work with reservoir isolates is required to evaluate the specific effect of PCB on glucose uptake at the cellular level. Chronic PCB effects may be more severe than the short-term effects. As indicated by SEM observations, the entire community structure may be altered. In turn, an abrupt change in microbial carbon metabolism could result.

A general picture of PCB interaction with naturally-occurring, freshwater, bacterial populations emerged from this study. During the course of a year, every sample of Center Hill Reservoir was capable of degrading a commercial PCB mixture. Correspondingly, several laboratories have previously demonstrated PCB degradation by PCB-enriched mixed cultures at concentrations of PCB between 10 and 1000 μg l^{-1} (13, 32, 160). Carey and Harvey (28) reported 1 to 4% conversion of tri- and tetrachlorobiphenyl to polar metabolites by natural populations of marine microorganisms and Kaiser and Wong (93) observed biodegradative removal of two Aroclor mixtures by lake bacteria, although Aroclor 1254 was not degraded. It appears, therefore, that PCB is subject to biodegradation by a variety of bacteria from different aquatic habitats. In situ experimentation provided compelling evidence that PCB biodegradation is not a laboratory artifact, but takes place in aquatic ecosystems under natural conditions by the resident microflora. These studies, therefore, support the contention that significant microbial biodegradation of PCB can occur in the environment (93, 150).

The rate of Aroclor 1254 biodegradation, however, was exceedingly low, ranging from 6.8 to 81.2 μg $l^{-1}month^{-1}$ with a mean of 46.5 μg $l^{-1}month^{-1}$. The rates reflect stable laboratory conditions, although the in situ rate (21.0 μg $l^{-1}month^{-1}$) was within the range of the laboratory-derived rates. The biodegradation rates also compare favorably with other reported values. Sayler et al. (150) reported that a Pseudomonas sp. growing at 100 μg Aroclor 1254 l^{-1} utilized 37.2 μg l^{-1} in 22 days and 69.8 μg l^{-1} in 60 days.

Inasmuch as biodegradation rates were low, much lower rates can be expected under unfavorable environmental conditions. In addition to pH, oxygen availability, and other environmental parameters, the ambient PCB concentration is also critical to biodegradation rates (22; Table 9, page 67). At the typically lower PCB concentrations encountered in the water column (73, 165), biodegradation rates would be proportionately lower. Sewage plant effluents and heavily contaminated waters are important exceptions where significant biodegradative PCB removal at higher PCB concentrations may be attributable to microbial activity (160).

Bacterial decomposition of monochlorinated biphenyls, and to a lesser extent, di-, tri-, and tetrachlorobiphenyls results in hydroxylated and acidic metabolites (5, 56-58, 158, 162). Microbial metabolism, therefore, is the most likely explanation for the conspicuous absence of the lesser chlorinated biphenyl residues in environmental samples (14, 178). The 2-chlorobiphenyl decomposition studies strongly support this view by demonstrating that natural freshwater microorganisms can transform the PCB molecule in the same manner as in laboratory culture.

The possible accumulation of PCB metabolites such as chlorobenzoic acid and chlorobenzoyl formic acid raise toxicological concerns in addition to PCB itself. The toxicity and persistance of some of these compounds (e.g., chlorobenzoyl formic acid) is unknown. Chlorobenzoic acids are biorecalcitrant and accumulate in nature (43). It is important to recognize and identify these compounds as well as their environmental hazards (4).

While PCB decomposition is one route of biodegradation, the complete fate of PCB is not entirely understood. In particular, bacteria displayed an effective mechanism of PCB sequestration which prevented reextraction with organic solvents. Such an irreversible complexing of PCB is distinguished from PCB bioaccumulation which is governed by reversible physical equilibrium characteristics.

Since PCB decomposition is inversely proportional to the extent of chlorine substitution (15, 32, 56-58, 93, 150, 161), it is highly unlikely that Aroclor 1254 components were extensively decomposed. The lack of preferential PCB peak biodegradation (Fig. 4, page 58) and the 2,4'-dichlorobiphenyl results suggest that irreversible complex formation between cellular components and PCB may be the major route of Aroclor 1254 biodegradation in the environment. It is not known if further processing of the compounds, including decomposition, ensues after binding.

PCB bioaccumulation is of environmental importance as well. PCB bioaccumulation is an initial mechanism leading to both irreversible PCB complexing and PCB decomposition. The specific role of slime production with respect to PCB accumulation is unknown, however, PCB partitioning between bacterial cells and the aqueous phase was dramatically decreased upon autoclaving the cells. It is suggested, therefore, that slime production or similar activity by the bacteria, may be a major mechanism of bioaccumulation, as well as biodegradation and microbial adaption to PCB. The occurrence of slime-production in conjunction with polychlorinated biphenyls has been reported by others (150).

While active PCB biodegradation was clearly established, it is felt that the terminal mineralization of PCB is not an important mechanism in this process. This hypothesis is supported by the mineralization studies and the results of other investigators. Carey and Harvey (28) and Furukawa et al. (57) failed to detect radiolabeled CO_2 evolution from PCB. Similarly, Crawford et al. (37) did not detect (^{14}C)hexachlorobiphenyl mineralization with natural soil samples of different habitats. On the other hand, Sayler et al. (150) isolated a pseudomonad capable of mineralizing hexachlorobiphenyl.

Although these studies demonstrated the decompositional fate of PCB in natural systems, it is apparent that the interactive effect of PCB on microbial populations accounts for a significant portion of the environmental removal of the pollutant. Aroclor was found to significantly stimulate the growth of mixed bacterial populations under specific culture conditions. This stimulation can result in the alteration of community structure giving rise to populations for enhanced PCB accommodation. This effect is suggested by studies on the microbial response to PCB enrichment and the ability of bacterial cells to partition PCB from aqueous solution.

VII. SUMMARY AND CONCLUSIONS

Laboratory and field PCB biodegradation assays demonstrated the ability of natural microbial populations to biodegrade Aroclor 1254, a commercial PCB mixture. Two different PCB extraction methods, hexane batch extraction and Tenax-GC column extraction, were evaluated for efficiency, precision, and feasibility for use with biodegradation assays. Hexane batch extraction in conjunction with electron-capture, gas-liquid chromatography was found to be a superior method with respect to PCB recovery, precision and variability.

Each of 24 independent water samples taken over the course of one year from Center Hill Reservoir actively biodegraded PCB. The rate of PCB biodegradation was exceedingly low, ranging from 6.5 μg $l^{-1}month^{-1}$ to 81.2 μg $l^{-1}month^{-1}$, with a mean of 46.5 μg $l^{-1}month^{-1}$. Statistical analysis revealed that the extent of PCB biodegradation was significantly ($p < 0.05$) explained by both temporal and spatial effects. Approximately 43% of the variation in biodegradation was explained by the following environmental site characteristics: site depth, water temperature, dissolved oxygen, dissolved organic carbon, water transparency, water conductivity, phosphate concentration, pH, nitrate concentration, suspended sediments, total viable bacterial counts, and PCB-resistant bacterial counts. However, the computed multiple regression equation did not significantly describe the biodegradation variation. Of all the environmental variables monitored, only total viable counts significantly correlated with

PCB biodegradation, although dissolved oxygen, suspended sediments, and dissolved organic carbon accounted for the majority of the remaining explained variation. In situ experiments with semi-permeable membrane chambers corroborated laboratory results. PCB biodegradation was observed after 1 month but not 1 week incubation in Center Hill Reservoir.

Adjustment of the reservoir sample pH within 1 pH unit of the ambient reservoir pH did not significantly affect PCB biodegradation. Lowering the pH to 3.0 or 5.0, however, completely inhibited significant biodegradation. The concentration of PCB also affected PCB biodegradation. A 10-fold increase in PCB concentration (from 10 $\mu g\ l^{-1}$ to 100 $\mu g\ l^{-1}$) decreased the percent of total PCB biodegraded by approximately 50%, however, the biodegradation rate increased approximately 5-fold. With additional 10-fold increments in PCB concentration, the degradation rate was significantly low to be masked by analytical error.

A technique employing Amberlite XAD-2 to partition (^{14}C)PCB in the aqueous phase was developed in order to assess mineralization of PCB by aquatic microflora. In two series of experiments using water samples from an oligotrophic and a eutrophic reservoir, no (^{14}C)PCB mineralization (as $^{14}CO_2$ evolution) was detected up to 96 days' incubation. (^{14}C)Naphthalene, on the other hand, was readily mineralized in parallel experiments. Plate counts of bacteria during the course of the mineralization assay demonstrated a PCB-generated stimulation of bacterial growth.

Reservoir water containing either 2-chlorobiphenyl or

2,4'-dichlorobiphenyl yielded PCB metabolites. In the former case, chlorobenzoic acid and (tentatively) chlorobenzoyl formic acid were identified by thin layer chromatography (TLC) and mass spectrophotometry. In the case of the dichlorobiphenyl, initial TLC indicated the complete microbial removal of the added PCB. Upon examination of the suspected metabolite by mass spectrometry, however, dichlorobiphenyl and a suspected phthalate derivative were identified.

An unidentified, Gram negative, bacterial rod (PP1) was isolated from PCB-enriched reservoir water to examine PCB bioaccumulation. The ability of PP1 to accumulate over 60% of the PCB added was lost upon autoclaving the cell suspension. Observation of an autoclaved PP1 culture with aid of light microscopy revealed intact cells that were indistinguishable from live cell suspensions.

Natural bacterial populations were stimulated by 100 µg Aroclor 1254 l^{-1} as indicated by total viable counts. Furthermore, populations enriched with PCB demonstrated an adaptive response upon reinoculation into fresh, reservoir water containing PCB. Examination of flask components (previously added diatomaceous earth) by scanning electron microscopy revealed the growth of surface-associated, slime-producing bacteria in the presence but not in the absence of Aroclor 1254.

The overall, short-term effect of Aroclor 1254 on heterotrophic glucose uptake and mineralization by reservoir microorganisms was insignificant. On a sample to sample basis, however, significant stimulatory as well as inhibitory PCB effects were observed. The

environmental variables that were monitored contributed significantly to the glucose uptake kinetics.

This investigation contributed considerably to the understanding of the fate and impact of PCB in freshwater ecosystems. A significant PCB-microbial interaction was demonstrated under conditions of moderate PCB contamination. Aquatic microorganisms are capable of accumulating a large proportion of the ambient PCB and, under favorable conditions, are able to degrade the contaminant. However, degradation rates are very low. Consequently, the residence time of PCB is expected to be exceedingly long as judged from biorefractility and resistance to physico-chemical degradation. It is also noted that the accumulation of PCB in microbial cells and the potential accumulation of biorefractile PCB metabolites, such as chlorobenzoic acids, may be of toxicological concern with respect to PCB persistence, microbial ecology, and food web dynamics in aquatic systems.

In view of the persistence of PCB in aquatic environments, chronic interaction with microbial populations suggests subtle shifts of community composition, in particular the enhancement of slime-producing attached bacteria. While short-term PCB exposure does not appear to significantly affect microbial heterotrophic activity in nature, chronic effects and displacement of community composition may be of concern.

The general conclusions that can be drawn from this study and applied to oligotrophic freshwater environments are:

(1) Natural freshwater microbial populations are capable of biodegrading commercial PCB mixtures under favorable environmental conditions. Environmental biodegradation rates are expected to be exceedingly slow.

(2) The extent of environmental PCB biodegradation is governed by a variety of interacting environmental variables. Under generally favorable conditions, however, the heterotrophic, bacterial, population size and the ambient PCB concentration are two of the most dominant factors regulating PCB biodegradation.

(3) PCB biodegradation by natural microbial populations conforms to established biphenyl and PCB catabolic pathways. In addition, recalcitrant metabolites, such as chlorobenzoates, can be expected to accumulate in the environment at relatively low concentrations.

(4) Terminal mineralization of Aroclor 1254 is not a primary route of biodegradation. This mixture is bioaccumulated and potentially bound to cellular components.

(5) Heterotrophic bacteria of aquatic origin bioaccumulate ambient PCB from solution according to phase partitioning characteristics of the cells and the aquatic medium.

(6) Bacterial slime production is a general response of natural bacterial populations to the presence of PCB. Slime production appears to participate in PCB bioaccumulation and accommodation by the bacteria.

(7) Moderate Aroclor 1254 concentrations are stimulatory to the growth of heterotrophic bacteria in the presence of adequate surfaces. Attached, slime-producing bacteria are selected in the presence of PCB.

(8) PCB has an insignificant overall effect on heterotrophic uptake and mineralization activity. However, due to the interaction of PCB with complex environmental variables, both significant inhibitory or stimulatory effects on activity occur in different spatial and temporal aquatic situations.

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APPENDIXES

APPENDIX A

MEDIA AND BUFFER FORMULAS

1. Yeast extract peptone glucose (YEPG) medium.

dextrose	1.0 g
polypeptone	2.0 g
yeast extract	0.2 g
NH_4NO_3	0.2 g
purified agar	15.0 g
distilled water	1000 ml

pH was adjusted to 7.0 to 7.2 and the medium was autoclaved.

2. Basal salts medium

$NaNO_3$	2.0 g
KH_2PO_4	1.5 g
Na_2HPO_4	0.5 g
$FeCl_2$	0.5 mg
$MgSO_4 \cdot 7H_2$	0.2 g
yeast extract	1.0 mg
distilled water	1000 ml

pH was adjusted to 6.8 to 7.0 and the medium was autoclaved.

3. PCB medium

basal salts medium	1000 ml
Aroclor 1254	1.0 g
purified agar	15.0 g

After autoclaving basal salts medium and cooling to approximately 60 C, the Aroclor was added aseptically. The biphasic medium was emulsified by 15 minute sonification.

4. Ringer's stock solution

NaCl	8.0 g
KCl	0.2 g
$CaCl_2 \cdot 2\ H_2O$	0.2 g
Na_2CO_3	0.01 g
distilled water	1000 ml

pH was adjusted to 7.0 to 7.2 and the buffer was autoclaved.

For dilution blanks, the buffer was diluted 1:4 in distilled water and 4.5 ml was dispensed into capped test tubes prior to autoclaving.

5. Phosphate-buffered saline

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
distilled water	1000 ml

pH was adjusted to 7.0 to 7.2 and the buffer was autoclaved.

APPENDIX B

PHYSICAL AND BIOCHEMICAL CHARACTERISTICS OF BACTERIAL ISOLATE PP1, A GRAM NEGATIVE ROD

Table 17. Characteristics of PP1.

Test	Reaction
NaCl 2%	NG[a]
4 C	G
37 C	G
42 C	NG
pH 5	NG
pH 5.9	G
pH 8.2	G
Catelase	(+)
Oxidase	-
Casein degradation	-
DNA degradation	-
Starch degradation	-
Tween 20 degradation	+
Tween 40 degradation	+
Tween 60 degradation	+
Tween 80 degradation	+
Levan production	-
Growth in presence of:	
Bismuth Citrate	+
Potassium Tellurite	-
Sodium Azide	-

[a]G, growth; NG, no growth.

VITA

The author was born on November 3, 1950, in Paterson, New Jersey. After a secondary school education in Bergen County, New Jersey, and Montgomery County, Maryland, he graduated in 1968 from Northwood High School, Silver Spring, Maryland. He received his B.S. in microbiology from the University of Maryland in 1972 and his M.S. in microbiology from Colorado State University in 1976.

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