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IN MUNICIPAL SLUDGE AND SLUDGE-AMENDED SOILS

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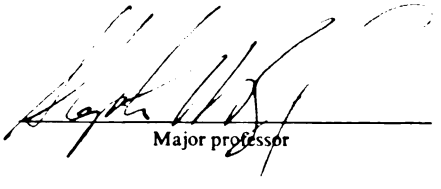

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ANAEROBIC BIODEGRADATION OF CHLOROPHENOLS IN MUNICIPAL SLUDGE AND SLUDGE-AMENDED SOILS

By

Mark Douglas Mikesell

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Sciences

1988

ABSTRACT

ANAEROBIC BIODEGRADATION OF CHLOROPHENOLS
IN MUNICIPAL SLUDGE AND SLUDGE-AMENDED SOILS

BY

Mark Douglas Mikesell

Chlorophenols are ubiquitous and toxic environmental contaminants. Because of their wide variety of applications they have been major industrial chemicals since the 1930s. Although they are known to be biodegradable and photochemically labile, chlorophenols, particularly pentachlorophenol, have been detected in soils, sediments, and natural waters throughout the world and found in foods and human urine. As a result several chlorophenols have been classified as priority pollutants by the U.S. Environmental Protection Agency.

Because anaerobic environments are significant habitats and are often found to be contaminated with chlorinated aromatic compounds, experiments were conducted to assess the biodegradability of pentachlorophenol, 2,4,6-trichlorophenol, three monochlorophenols, and the herbicides 2,4-D and 2,4,5-T in anaerobic sewage sludges from four Michigan communities. All the compounds were degraded in one or more of the sludges during the 70 day incubation. The

principal reactions were ortho and para dechlorination and, for the phenoxy compounds, ether cleavage. Sludge from Jackson, Michigan had the greatest biodegradative capabilities; the pathways of degradation were similar in three other sludges, which differed mainly in rates of degradation.

After acclimation of Jackson sludge to biodegradation of monochlorophenol isomers, the degradation of pentachlorophenol was examined. Monochlorophenol-acclimated sludge was able to dechlorinate pentachlorophenol at ring positions which were expected on the basis of earlier cross-acclimation studies (i.e., the positions for which dechlorination activity was enriched). When the monochlorophenol-acclimated sludges were mixed, pentachlorophenol was completely dechlorinated and the carbon mineralized to CH_4 and CO_2. Pentabromophenol was also degraded and the degradation of technical-grade pentachlorophenol proceeded more slowly after several additions, possibly due to accumulation of toxic impurities.

When Jackson sludge was added to soil contaminated with pentachlorophenol, the result was greatly enhanced degradation attributable to the dechlorinating activity of the sludge. The initial concentration of 10 - 30 mg Kg^{-1} was completely degraded within 28 to 35 days. The degradation was much slower in anaerobic soil without sludge and in aerobic soil with or without sludge. The apparent pathway was the same as observed in whole sludge and in the acclimated sludge mixture.

For

Mother,

and the rest of the family,
who never doubted

ACKNOWLEDGEMENTS

Without the diligent guidance and encouragement of my advisor Dr. Stephen Boyd, the work reported here would not have been possible. His ability to recognize what is important and what is not has been invaluable, helping me to focus on the right goals at the right times. Also, his exceptional insight into writing and publishing manuscripts has influenced me greatly.

Professor James Tiedje has been a wonderful inspiration to me. My participation in the frequent scientific (and social) meetings of his group has influenced my growth in microbiology in a way that nothing else could have done.

Dr. Lee Jacobs examined this dissertation very thoroughly, finding errors which otherwise would certainly have survived to the final copy. I sincerely appreciate his consciencious efforts. Dr. Matthew Zabik very kindly agreed to serve on my examining committee on extremely short notice and offered valuable insights on my work.

Finally, my association over the years with numerous professors, graduate students, and post-docs has truly been a pleasure. To acknowledge them all would require another chapter, so I'll trust that my gratitude has been appropriately revealed.

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CHLOROPHENOLS IN THE ENVIRONMENT

Chlorophenol formulations have been used on many applications for over fifty years. Used mainly as a fungicide and insecticide for wood preservation, pentachlorophenol (PCP), one of the most intensively used of all pesticides, has been applied for purposes ranging from pre-harvest herbicide in rice to molluscicide for the control of bilharzia (Crosby, 1981). This broad spectrum of availability attests to PCP's general biocidal nature, which results from its ability to uncouple oxidative phosphorylation (Weinbach, 1957). Because of their versatility and long history as major industrial chemicals, and their recalcitrance in the natural environment, chlorinated phenols have been detected in soils, sediments, ground and surface waters, as well as in food and human urine (Crosby, 1981; Paasivirta et al., 1985). The U.S. Environmental Protection Agency has designated five chlorinated phenols priority pollutants (Keith and Telliard, 1979).

In addition to their manufacture as industrial products and their use as biocides, several other sources of chlorophenols in the soil environment and in natural waters have been identified. They may occur as degradation products of a number of pesticidal compounds such as lindane (γ-hexachlorocyclohexane), pentachloronitrobenzene, hexachlorobenzene, and the phenoxy acetic acid herbicides (Ahlborg and Thunberg, 1980). Aquatic

environments have been severely contaminated by pulp mill effluents which contain chlorophenols resulting from the bleaching process (Crosby, 1981; Hakulinen and Salkinoja-Salonen, 1982). Chlorination of water supplies for the purposes of disinfection has also been shown to produce chlorophenols (Paasivirta et al., 1985).

Because of its toxicity and apparent ubiquity in the environment, PCP is the most thoroughly studied of the chlorophenols. It has been reported as a contaminant of soils and natural waters at concentrations as high as several thousand ppm, the highest levels being associated with dipping basins or storage areas at sawmills or wood-preserving facilities (Paasivirta et al., 1985; Valo et al., 1984).

PCP is known to be susceptible to photodegradation and microbial degradation and the products of these processes have been identified (Englehardt et al., 1986; Kaufman, 1978). However, the persistence of PCP and other chlorophenols in the environment suggests that under natural conditions these processes are slow. In addition, high concentrations such as may be present in soil are probably inhibitory to indigenous microorganisms. In animals and higher plants detoxification occurs by conjugation and dechlorination reactions. A summary of the potential fates of chlorophenols in the soil environment appears in Figure 1.

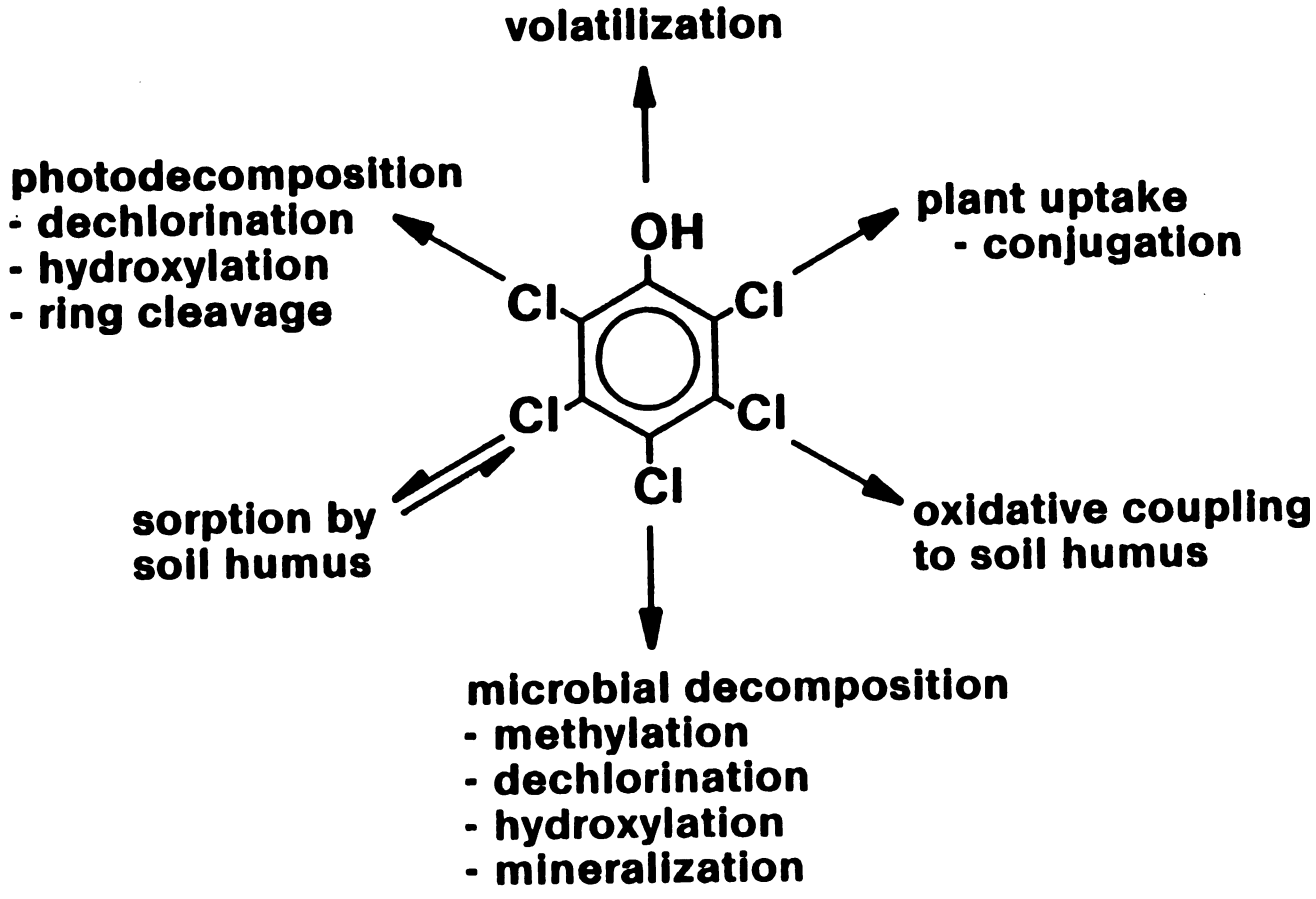

Figure 1. Potential fates of pentachlorophenol in the soil environment.

SORPTION AND BINDING

The mobility and biological availability of chlorophenols in soil are directly related to the degree of binding or sorption of the compounds by organic or inorganic soil components. Partitioning into the soil organic phase has been shown to control the uptake of non-ionic organic compounds (NOCs) in soil-water systems (Chiou et al., 1979, 1981, 1983). According to this mechanism, soil organic matter acts as a solubilizing medium for NOCs and is functionally and conceptually like a bulk-phase solvent such as hexane or octanol, in its uptake of NOCs from water. In soil-water systems, the mineral components do not function as sorbents for NOCs because of the strong dipole interactions between water and the polar mineral surfaces (Chiou et al., 1985).

The presence of ionizable functional groups confounds the sorptive behavior of organic solutes in soil-water systems which is particularly important for the chlorophenols whose pKa values range from roughly 5 to 9. In a recent study of the sorption of chlorophenols by sediments and aquifer materials, Schellenberg et al. (1984) found that the sorption of nonionized chlorinated phenols behaved in a manner which was consistent with the concept of solute partitioning. This includes isotherms which are highly linear over a wide range of relative solute concentrations, dependence of the sorption coefficient on organic

carbon content of the soil or sediment, and a linear relationship between the octanol-water partition coefficients (Kow) and the organic carbon-normalized sorption coefficients (Koc). Although some controversy still persists, these characteristics of solute partitioning have been clearly delineated by Chiou et al. (1979; 1983; 1985).

Schellenberg et al (1984) found that the sorption of both the non-ionized and the ionic forms of chlorophenols can occur. However, at low ionic strengths ($<10^{-3}$M) and at pH values not exceeding the pKa by more than one unit, sorption of the ionic form was insignificant. But for the tetrachlorophenols and pentachlorophenol, whose pKa values are less than 6, sorption of the phenolate ion was found to be important. They found that the sorption of the chlorophenolate ion was probably also a partitioning process since it was strongly dependent on the organic carbon content of the sediment or aquifer material. Materials containing very little organic carbon (< 0.10%) exhibited very low distribution ratios, a result consistent with the partitioning concept.

Westall et al. (1985) continued this work by investigating the octanol-water partitioning of chlorinated phenols and the effects of pH and ionic strength on distribution ratios. These experiments provided evidence for partitioning of the K^+ salt of tetra- and pentachlorophenols into the octanol phase. Solute partitioning between octanol and water is generally

presumed to be analogous to the partitioning between water and natural organic matter.

From a practical standpoint, these compounds are expected to be relatively mobile in soils due to the relatively high water solubilities of the chlorophenols, especially the lower chlorinate congeners, and the chlorophenolate anions. Evidence for such mobility has been provided recently in a study of chlorophenol-contaminated soil at wood-preserving facilities (Kitunen et al., 1987).

Another process which may significantly affect the fate of chlorophenols in soils is their incorporation through oxidative coupling into soil organic matter. Oxidative coupling of phenolic compounds is catalyzed by phenoloxidase and peroxidase enzymes produced by fungi, bacteria, and plants, and which are commonly found in surface soils (Sjoblad and Bollag, 1981). The reactions may also be catalyzed by a variety of soil minerals (Shindo and Huang, 1984; McBride, 1987) or be autooxidative in nature. The incorporation of chlorophenols into polymers in this manner is thought to be analogous to the process of humic substance synthesis from naturally-occurring phenolic compounds and thus it is possible that the xenobiotic phenols might become covalently bound to soil humic materials. Because of the electron-withdrawing properties of the Cl substituents, chlorophenols would be expected to be less reactive than most naturally occurring polyphenols (Berry and Boyd, 1984, 1985). As covalently bound residues, chlorophenols would be strongly immobilized and

stabilized against biodegradation. Stott et al.(1983) have shown that chlorocatechols added to soil are only partially mineralized, and that the remaining chlorocatechol carbon was biologically unavailable, presumably due to incorporation into soil humic substances.

The process of oxidative coupling of phenolic compounds has been investigated in considerable detail using model humus components such as vanillic and syringic acids. The initial step is the enzymatic formation of the aryloxy radical which can then polymerize with humus constituents. Bollag et al. (1977, 1980) have observed the formation of polymers after incubation of chlorophenols with a laccase from the soil fungus *Rhizoctonia praticola*. Dimers, trimers, tetramers and higher polymers were detected by mass spectrometry. Hybrid polymers between 2,4-dichlorophenol and model phenolic humus constituents have also been detected.

More recently the copolymerization of mono-, di-, tri-, tetra-, and pentachlorophenols has been demonstrated with syringic acid (Bollag and Liu, 1985). In this study two types of polymeric products were formed: quinoid oligomers and phenolic oligomers. In each case only a single chlorophenol moiety was present in the product and no dehalogenation products were detected.

In experiments which more closely simulated a natural soil environment, Weiss et al. (1982b) added ^{14}C-PCP to flooded rice soil in a growth chamber. The major pool of radioactivity recovered after one growing season (7-1/2 months) was 28.6%, in

the form of non-extractable soil residues, with humin, humic acid, and fulvic acid fractions each containing radioactivity.

Weiss et al. (1982a) also reported on their investigations of PCP uptake and metabolism by rice plants. Using ^{14}C-PCP they found that 12.9% of the added radioactivity was taken up by the plants in one growing season. They identified free and conjugated PCP as well as free and conjugated dechlorination products.

BIODEGRADATION

Although PCP is a highly chlorinated, exceptionally toxic compound, many reports of its biodegradation have appeared in the literature since the early 1970s. Because of its extensive use as a herbicide in Japanese rice paddy fields, many of the early reports focus on establishing a correlation between the persistence of PCP and soil properties. Kuwatsuka and Igarashi (1975) found that PCP was decomposed in both aerobic and anaerobic soils, but that it was degraded more rapidly anaerobically. The half-life of PCP was 30 days in flooded paddy soils and 50 days in upland soils, but almost no degradation occurred in a forest soil low in organic matter regardless of aeration. These results as well as those of other studies (Ide et al., 1972; Murthy et al., 1979) have shown reductive dechlroination to be the major degradative reaction in anaerobic (flooded) soil. In addition to the aeration status of soils, an important factor in PCP

degradation is the presence of an acclimated bacterial population. In mature paddy soils degradation occurred under both aerobic and anaerobic conditions, while in non-agricultural soils no PCP degradation was measured. Perhaps this difference can be attributed to an acclimated population of bacteria present in paddy soils because of previous PCP application (as a herbicide for rice crops). Also, accelerated decomposition of PCP was observed in fields which received repeated annual applications (Watanabe, 1977, 1978). Watanabe examined changes in soil bacterial populations using a most-probable-number technique. The PCP-degrading population increased early in the first year and the numbers remained high during the second and third years of application with no decrease in the intervening winters, implying that the population of PCP degraders was stable.

The list of PCP degradation products detected in soils treated with PCP include tetra-, tri-, and dichlorophenol isomers, as well as pentachloroanisole (Fig. 2). The methylation of PCP occurs in both aerobic and anaerobic soil (Murthy et al., 1979). Pentachloroanisole (PCP methyl ether) was a very minor product in anaerobic soil (5%), but 51.5% of the radioactivity added to aerobic soil could be recovered as the methylated product after 24 days. Also, pentachloroanisole added to soil was found to be reduced back to PCP much more readily under anaerobic conditions. In anaerobic soils the dechlorination productsdetected indicated that reductive dehalogenation had occurred at each of the ring positions relative to the hydroxyl, but a general feature of

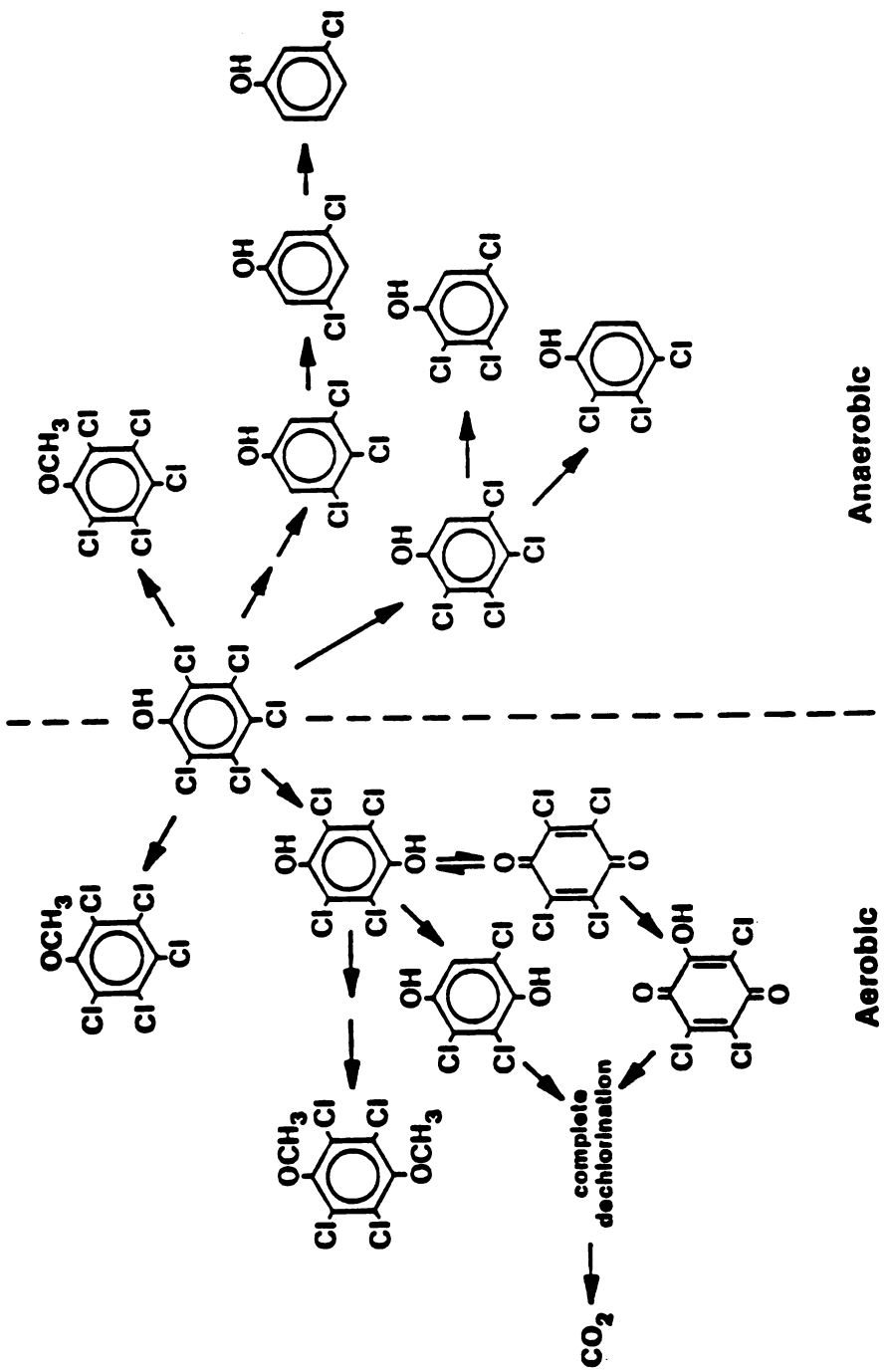

Figure 2. Comparison of the typical degradation pathways of pentachlorophenol in aerobic and anaerobic soils.

anaerobic PCP degradation is the preferential dechlorination at the ortho and para positions.

Methylated products and lower chlorinated phenols were also detected in the flooded rice soil used by Weiss et al. (1982b) in their growth chamber experiments. Several tetra- and trichlorophenols and tetra- and trichloroanisoles were detected but there was no further dechlorination. Dechlorination of the methylated compound was not demonstrated by Weiss et al. (1982b), nor has it been by other investigators.

Many organisms with the ability to degrade chlorophenols have been isolated from soils throughout the world. Cserjesi (1967) and Cserjesi and Johnson (1972) reported on fungal adaptation to PCP and its biotransformation, presumably because of concern that the fungal targets of the pesticide were becoming increasingly resistant to its presence in wood products. Tolerant strains of *Cephaloascus fragrans* were isolated, and in one case an increase in tolerance over time was demonstrated. Methylation of PCP by species of *Trichoderma* was shown to greatly reduce its toxicity to fungi and to fish in laboratory tests.

A phenol-utilizing yeast of the genus *Rhodotorula* was isolated from soil by Walker (1973) and was subsequently found to cometabolize monochlorophenols to the corresponding chlorocatechols. Spokes and Walker (1974) reported similar results with several diverse genera of soil bacteria which also produced chlorocatechols from chlorophenols.

Bacterial strains able to degrade pentachlorophenol under aerobic conditions have been isolated by several researchers. Chu and Kirsch (1972, 1973) reported the first such bacterium, which they designated KC-3, able to utilize PCP as its sole source of carbon and energy, releasing 75% of the PCP carbon as CO_2. They suggest that the organism belongs to the coryneform group of aerobic, saprophytic bacteria. Strain KC-3 is also able to grow on 2,3,4,6-tetrachlorophenol and 2,4,6-trichlorophenol, and does not appear to distinguish between bromo- and chlorophenols. Several other polychlorophenols were degraded by respiring cells but did not support growth of the organism, and the monochlorophenols and phenol itself were not incorporated or were highly toxic.

Using a soil perfusion apparatus, Watanabe (1973) isolated a *Pseudomonas* species from rice paddy soil capable of growth using PCP as its only source of carbon and energy. Growth of this organism was inhibited ot 100 ,ppm and stopped completely at 200 ppm. Suzuki (1977) also isolated a species of *Pseudomonas* from Japanese paddy soil, and in experiments with ^{14}C-PCP demonstrated that 50% of the PCP carbon was mineralized to $^{14}CO_2$. Other metabolites containing ^{14}C included pentachloroanisole, tetrachlorocatechol dimethyl ether, and tetrachlorohydroquinone dimethyl ether.

Using a new enrichment protocol which involved the use of a technical-grade PCP formulation, Stanlake and Finn (1982) isolated four strains of PCP-catabolizing bacteria of the genus

Arthrobacter from soil and aquatic habitats. One strain came from the base of a PCP-treated utility pole, but others came from environments with no known history of PCP exposure. The four strains were quite similar in their growth requirements and degradative capacity, and were also similar to the strain KC-3 described previously. The *Arthrobacter* strains were able to catabolize 2,4,6-trichlorophenol as well as PCP and appeared to be more sensitive to the decrease in pH of the medium which occurred with chlorophenol degradation than they were to the chlorophenols themselves.

Edgehill and Finn (1982, 1983), using a method resembling that of Stanlake and Finn (1982), isolated another *Arthrobacter* strain from contaminated soil and characterized its growth and substrate utilization kinetics. The organism displayed unusual inhibition characteristics as the PCP concentration was increased, in which zero-order growth kinetics were observed over a wide range of PCP concentrations (constant specific growth rate from 10 to 135 mg L^{-1}). This unusual behavior may reflect an ability of this PCP-adapted organism to regulate PCP uptake, thus reducing its inhibitory effect. An alternative explanation could be that the cell membrane of this bacterium may be somehow resistant to the uncoupling capability of PCP.

A group of researchers in Finland has published several papers on the chlorophenol-degrading activity of mixed populations and pure cultures of bacteria. Apajalahti and Salkinoja-Salonen (1984) reported on the importance of bark chips which absorbed the

PCP, permitting the PCP-degrading bacteria present in the mixed culture to tolerate more than a 10-fold higher concentration. Although this work was done in a bioreactor, the results should apply as well to soil containing organic material, in which the PCP would exist in equilibrium between aqueous and organic phases, reducing the aqueous PCP concentration and thereby protecting the soil organisms from its detrimental effects.

Valo et al. (1985) continued with physiological studies of the mixed culture, and Apajalahti and Salkinoja-Salonen (1986) studied chlorophenol metabolism by a pure culture of an actinomycete, *Rhodococcus chlorophenolicus*, isolated from the mixed culture described in the bioreactor research. *Rhodococcus chlorophenolicus* was able to degrade many polychlorinated phenol congeners completely to CO_2. It is distinguished by being the first chlorophenol-degrading isolate to be characterized sufficiently to be given a species designation.

The isolation and characterization of several strains of *Flavobacterium* able to degrade PCP has been described by Saber and Crawford (1985). The isolates all originted in Minnesota soils which had history of PCP contamination, having PCP conccentrations ranging from 12 to 800 ppm. The bacteria were able to mineralize 100 to 200 ppm PCP, releasing 73% to 83% of the PCP carbon as CO_2. Some differences were apparent in the ability of the strains to metabolize PCP; all strains contained an 80-100 kilobase plasmid. Steiert and Crawford (1986) elucidated the initial steps in the pathway of PCP metabolism in *Flavobacterium*. They performed ^{18}O

labelling experiments to show that the initial step was a hydrolytic dechlorination in which hydroxyl from H_2O replaced chlorine, yielding tetrachlorohydroquinone (Fig. 2). This was followed by two reductive dechlorination steps to form 2,6-dichlorohydroquinone.

Although most of the earlier studies on the metabolism of chlorophenols in soils indicated that anaerobic habitats fostered more active chlorophenol-degrading populations than aerobic, to date no anaerobic organisms capable of chlorophenol degradation have been characterized. However, strictly anaerobic degradation of chlorophenols in anaerobically digested municipal sewage sludges has been described recently in some detail. Boyd et al. (1983) showed the dechlorination and mineralization of 2-chlorophenol in 10% anaerobic sludge. In fresh, whole (undiluted) sludge all the monochlorophenols were degraded, the rates being 2-chlorophenol > 3-chlorophenol > 4-chlorophenol (Boyd and Shelton, 1984). Boyd and Shelton also experimented with anaerobic sludge which had been acclimated to chlorophenol degradation by weekly additions of 2-, 3-, or 4-chlorophenol over a period of about two years. Specific patterns of cross-acclimation were observed: sludge acclimated to 2-chlorophenol could degrade 4-chlorophenol and 2,4-dichlorophenol but not 3-chlorophenol; 3-chlorophenol acclimated sludge degraded 3-chlorophenol, 3,4- and 3,5-dichlorophenol but not 2-chlorophenol, 2,3- or 2,5-dichlorophenol; and sludge acclimated to 4-chlorophenol could degrade both 2- and

3-chlorophenol as well as 2,4- and 3,4-dichlorophenol (Boyd and Shelton, 1984).

BIOAUGMENTATION

Since a major environmental problem associated with chlorophenols is soil contamination in the vicinity of wood-preserving facilities (Kitunen et al., 1987; Paasivirta et al., 1985; Valo et al., 1985), efforts have been focused on utilization of biological methods for the remediation of contaminated sites.

Technical-grade chlorophenol formulations have been widely used as lumber preservatives against fungal and insect damage. The activities at wood-preserving plants have caused widespread contamination in the U.S. (Crawford and Mohn, 1985; Goerlitz et al., 1985) and elsewhere (Kitunen et al., 1987). At least 600 of these sites exist in the U.S. alone and pentachlorophenol is the principal contaminant. Other lower chlorinated phenols, creosote components, and highly toxic trace contaminants (polychlorinated dibenzodioxins, dibenzofurans, and phenoxyphenols) have been found in soil, surface water, and ground water in the vicinity of these operations (Goerlitz et al., 1985; Kitunen et al., 1987).

At least three groups have published reports of promising methods for the remediation of PCP contaminated soils using inoculation with aerobic bacteria. Crawford and Mohn (1985) studied the use of a *Flavobacterium* species to decontaminate soils in Minnesota. Their method was successful for a PCP concentration of up to about 300 ppm, but required repeated inoculations over a period of several months, and a residual PCP concentration of 10-

50 ppm was difficult to eliminate. Edgehill and Finn (1983) reported some similar results using their *Arthrobacter* strain with soil to which PCP was added in the laboratory. Most recently, Valo and Salkinoja-Salonen (1986) have employed composting and inoculation with *Rhodococcus chlorophenolicus* to achieve substantial decomposition of PCP in heavily contaminated soils.

In each of these examples of bioaugmentation, however, residual PCP at levels of 10 to 50 ppm was present, even after incubations as long as 500 days. This may reflect a common kinetic characteristic of the organisms involved or, perhaps more likely, be a manifestation of PCP's behavior in soil related to its tendency to partition into the soil organic phase, becoming only very slowly available for biological activity.

SUMMARY

Because of their widespread use as industrial chemicals and biocides, chlorophenols and especially pentachlorophenol are important environmental pollutants. Several different processes are significant in determining the impact of these compounds in the soil environment. Sorption of both chlorophenols and chlorophenolate anions by sediment and soil organic matter has been described as a partitioning process, analogous to solute distribution between octanol and water. The sorption coefficients are inversely related to the water solubility of the chlorophenol and directly related to the organic matter content of the soil or

sediment. Chlorophenols may also be incorporated into soil humics by formation of covalent bonds between the chlorophenol residue and the humic polymer. These bound residues are strongly immobilized and appear to be biologically unavailable.

Early studies on the biodegradation of chlorophenols focused on the fate of pentachlorophenol in rice paddy soils where it was widely used as a herbicide. Reductive dechlorination, primarily at the ortho and para positions, and methylation are the reactions most often observed in soil degradation studies. Anaerobic degradation is more rapid than aerobic, but critical to degradation regardless of aeration status is the presence of an adapted microbial population. Pure cultures of bacterial able to utilize PCP have been characterized. Species of *Pseudomonas*, *Flavobacterium*, *Rhodococcus*, and coryneform bacteria have been described, some of which have been used successfully in attempts to reclaim chlorophenol-contaminated soils. No anaerobic bacteria able to metabolize chlorophenols have been identified, but bacterial populations present in certain anaerobic sewage sludges are able to dechlorinate and mineralize chlorophenols.

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CHAPTER II

REDUCTIVE DECHLORINATION OF THE HERBICIDES 2,4-D, 2,4,5-T, AND PENTACHLOROPHENOL IN ANAEROBIC SLUDGES

ABSTRACT

The degradation of seven chlorinated aromatic compounds in anaerobic sewage sludge from three Michigan communities was examined. The compounds tested were 2,4-D (2,4-dichlorophenoxy acetic acid), 2,4,5-T (2,4,5-trichlorophenoxy acetic acid), PCP (pentachlorophenol), 2,4,6-trichlorophenol, and 2-, 3-, and 4-chlorophenol. All of the compounds tested were degraded in one or more of the sludges during a 70 day incubation period. Overall, 4-chlorophenol was the most persistent compound tested followed by 3-chlorophenol. The most rapid degradative reactions were cleavage of the ether linkage of the phenoxy acetic herbicides 2,4-D and 2,4,5-T, and the removal of Cl atoms *ortho* to the phenolic OH group of the chlorophenols. The initial products of 2,4-D and 2,4,5-T degradation were 4-chlorophenol and 2,4,5-trichlorophenol. Reductive dechlorination of 2,4,5-trichlorophenol (produced from 2,4,5-T) gave 3,4-dichlorophenol and 4-chlorophenol which were the final products of 2,4,5-T

degradation. Dechlorination of PCP gave 3,4,5-trichlorophenol as the initial transformation product, and further dechlorination gave 3,5-dichlorophenol as the final product of PCP degradation. The Jackson sludge had the greatest capacity to degrade the compounds tested. With the exception of 3- and 4-chlorophenol, all other compounds tested were completely degraded in 7 to 14 days in the Jackson sludge. Mason and Adrian sludges were similar in their degradative pathways overall, but significantly less active than the Jackson sludge.

INTRODUCTION

The manufacture and use of large quantities of chlorinated aromatic compounds, especially in pesticide formulations, have resulted in considerable concern over their environmental fate and toxicological effect. Because of their resistance to biological degradation in natural environments and their toxicity and potential mutagenicity, many chlorinated aromatics have been placed on the U.S. Environmental Protection Agency's list of organic priority pollutants (Keith and Telliard, 1979).

The great majority of studies on the degradation of chloroaromatic compound have examined their fate in aerobic environments. More recently, the anaerobic metabolism of chloroaromatic compounds has gained attention, particularly with the observation that Cl substituents can be removed directly from the aromatic ring in anaerobic habitats. Removal of the Cl atom from the aromatic ring, often referred to as reductive dechlorination, does not appear to occur in aerobic habitats. Reductive dechlorination is important because the dechlorinated products are usually less hazardous and more susceptible to further degradation by aerobic or anaerobic microorganisms.

The anaerobic degradation of chloroaromatic compounds has been investigated in a variety of biological systems including

municipal and industrial wastes (Hakulinen and Salkinoja-Salonen, 1982; Horowitz et al., 1982; Boyd et al., 1983; Boyd and Shelton, 1984; Guthrie et al., 1984), sediments (Horowitz et al., 1982; Attaway et al., 1982), soils (Murthy et al., 1979; Yamada and Suzuki, 1983), and bacterial cultures (Bollag and Russel, 1976; Suflita et al., 1982, 1984). Reductive dechlorination has been reported for several chloroaromatic compounds including the chlorobenzoates (Suflita et al., 1982) and chlorophenols (Murthy et al., 1979; Boyd et al., 1983; Boyd and Shelton, 1984) and for the pesticides diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea) (Attaway et al., 1982), chlornitrofen (4-nitrophenyl-2,4,6-trichlorophenyl ether) (Yamada and Suzuki, 1983), and 2,4,5-T (2,4,5-trichlorophenoxy acetic acid) (Suflita et al., 1984).

The fate of chloroaromatic pesticides in anaerobic sewage sludge is largely unknown. Since anaerobic digestion of sewage sludge is a common treatment method prior to sludge disposal, an understanding of the potential for anaerobic degradation of sludge is important. This is true from the standpoint of establishing reasonable guidelines for acceptable industrial discharges into wastewater treatment plants and for developing safe and economical methods of sludge disposal such as cropland application. Contamination of soil by sludge-borne organic pollutants may adversely affect plant growth. Additionally, these chemicals or their degradation products may be transported to ground or surface waters or be introduced into the food chain as a result of plant uptake.

In the present study, several chlorinated aromatic compounds including the pesticides 2,4-D (2,4-dichlorophenoxy acetic acid), 2,4,5-T, and PCP (pentachlorophenol) were studied with respect to their degradation in three anaerobically digested municipal sewage sludges. The objectives of this study were to (i) compare three municipal sludges for their ability to degrade the test compounds and (ii) determine the degradative pathways of those compounds subject to anaerobic biodegradation with particular emphasis on reductive dechlorination reactions.

MATERIALS AND METHODS

Materials. Anaerobically digested municipal sewage sludge was collected from primary digesters in three southern Michigan communities (Jackson, Mason, and Adrian). The Jackson Wastewater Treatment Plant receives about 40% of its wastewater from industrial sources and the remainder is residential. Retention time in the primary anaerobic digester is approximately 13 days. The Jackson sludge is typically about 40 g solids Kg^{-1} sludge and has a total N content of about 40 g N Kg^{-1} dry sludge (J. St. Andre, R.A. Greene Wastewater Treatment Plant, 1985, personal communication). The Adrian Wastewater Treatment Plant receives about 50% of its wastewater from industrial sources. Retention time of the primary anaerobic digester is approximately 35 days. The Adrian sludge is typically about 30 g solids Kg^{-1} sludge and has a total N content of about 350 mg N L^{-1} wet sludge (R. Deline, Adrian Wastewater Treatment Plant, 1985, personal communication). The Mason Wastewater Treatment Plant receives about 20% of its wastewater from industry. This is from a single source which produces infant formula. Retention time in the primary digester is approximately 20 days. The Mason sludge typically has 15 to 20 g solids Kg^{-1} sludge and a total N content of about 65 g N Kg^{-1}

dry sludge (J. Dean, Mason Wastewater Treatment Plant, 1985, personal communication).

The liquid sludge was transported to the laboratory in 4 L aspirator bottles which were tightly closed. In the laboratory the headspace of each bottle was flushed with and 80% N_2-20%CO_2 gas mixture which had passed through hot (350° C) copper filings to remove traces of O_2. The bottles were stoppered and stored for 48 hours at room temperature.

The three monochlorophenol isomers, 2,4,6-TCP (2,4,6-trichlorophenol), PCP, and 2,4-D were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI); 2,4,5-T was obtained from Sigma Chemical Co. (St. Louis, MO).

Methods. All incubations were carried out in 160 mL glass serum bottles which were thoroughly sparged with O_2-free 80% N_2-20% CO_2 gas mixture using a Hungate gassing apparatus (Hungate, 1969; Kaspar and Tiedje, 1982). While continuously sparging with the O_2-free gas, sludge was dispensed into the serum bottles in 75 mL aliquots using a glass pipette. The chlorinated aromatic compound were added from ethanol stock solutions using a Hamilton syringe to give the initial (extractable) concentrations shown in Table 1. The serum bottles were then capped with 1 cm thick butyl rubber stoppers and aluminum crimp seals. All compounds were tested individually in triplicate for each of the sludges. Sterile contrls were prepared for each sludge by autoclaving bottles of

sludge for 30 minutes on two consecutive days. The bottles were incubated in the dark at 37°C for 70 days. Two mL samples of liquid sludge were withdrawn with a syringe at 0, 7, 14, 21, 28, 35, 42, 56, and 70 days and stored frozen until analyzed.

At the end of the experiment, samples were analyzed by reverse-phase high pressure liquid chromatography to determine the disappearance of the added compounds and the appearance of degradation products. All compounds were identified by co-chromatography with authentic standards. Retention times were identical to the standard compounds +/- 0.01 min. The 2 mL samples were mixed with 1 mL of acetonitrile on a vortex mixer, centrifuged for 10 minutes at 12,000 x g, and filtered through 0.45 um membrane filters (Millipore, type HVLP). Recoveries from fresh sludge spiked with approximately 25 mg L^{-1} of the test compound all exceeded 80% of the initial amount added. A Waters liquid chromatograph was used, consisting of the following model number components: 6000A pump, M45 pump, 720 system controller, and 480 variable wavelength UV detector. All compounds were detected at their absorbance maxima: 280 nm for the lower chlorophenols and phenoxyacetic acids, 300.5 nm for PCP. The detection limit was 0.5 mg L^{-1}. The samples were injected using a Rheodyne 7125 sample injector fitted with a 20 uL loop. The analytical column was a Waters Radial-Pak C-18 cartridge held in an RCM-100 radial compression module. Peak area values were obtained with a Waters Data Module integrator. The mobile phase consisted of acetonitrile/5% aqueous acetic acid in proportions

which were varied to give retention times for the compounds tested of 4 to 10 minutes at a flow rate of 2.0 mL minute^{-1}. The mobile phase composition ranged from 60:40 to 45:55 CH_3CN:5% aqueous CH_3COOH.

RESULTS AND DISCUSSION

The capacity of the sludge-borne microbial populations to degrade the compounds tested varied considerably among the three sludges. None of the test compounds was degraded in the autoclaved controls. The times required for complete disappearance of the parent compounds (Table 1) indicated that the sludge from Jackson was most effective at degradation. For the Jackson sludge, 4-CP (4-chlorophenol) was the only compound of the seven tested that was not completely degraded during the 70 day incubation. All of the other compounds were degraded within 7 to 14 days by the Jackson sludge with the exception of 3-CP (3-chlorophenol) which required 56 days for complete disappearance. In general, the compounds tested were much more persistent in the sludges from Mason and Adrian, which were similar in their degradative capacities. The 2,4-D and 2-CP (2-chlorophenol) were the only compounds for which the Mason and Adrian sludges showed dissimilar times for complete disappearance of the parent compound.

In a previous study (Horowitz et al., 1982), the Jackson and Adrian sludges were incubated with the three monochlorophenol isomers and PCP, and CH_4 production was monitored as evidence of

Table 1. Initial concentration and time for complete disappearance of the parent compounds in three anaerobic sewage sludges.

Compound†	Initial concentration‡	Jackson	Mason	Adrian
	μM (ppm)		d	
2,4,5-T	62.7 (16.2)	7	42	35
2,4-D	75.2 (16.6)	7	(14.9)§	28
PCP	46.9 (12.5)	14	(28.5)	(14.6)
2,4,6-TCP	101 (19.9)	7	21	28
2-CP	158 (20.5)	14	42	70
3-CP	137 (17.7)	56	(127)	70
4-CP	177 (22.9)	(142)	(137)	(31.1)

† 2,4,5-T = 2,4,5-trichlorophenoxy acetic acid; 2,4-D = 2,4-dichlorophenoxy acetic acid; PCP = pentachlorophenol; 2,4,6-TCP = 2,4,6-trichlorophenol; 2-, 3-, and 4-CP = 2-, 3-, and 4-chlorophenol.

‡ Initial extractable concentration. Values given are averages of three samples.

§ Final concentration in μM after 70 d incubation.

degradation. All four compounds were observed to be persistent (i.e., no gas production over background was observed) over 56 days in both sludges. The reason for the disparity in results can be attributed to the fact that the monochlorophenol isomers inhibit CH_4 production even while undergoing primary degradation (Boyd et al., 1983). With respect to PCP, no gas production would be observed unless primary degradation (i.e., dechlorination--see results below) was followed by mineralization of the ring carbons.

Figures 1 through 4 show time courses for the degradation of 2,4-D, 2,4,5-T, PCP, and 2,4,6-TCP in Jackson sludge. The degradation pathways proposed seem plausible based on the sequential appearance of degradation products. Pathways indicated for the Jackson sludge were identical to those in the Mason and Adrian sludges when degradation occurred. Some similarities were apparent in the degradation of 2,4-D and 2,4,5-T (Fig. 1 and 2). The first product to be detected in both cases was the chlorinated phenol, which appeared after cleavage of the ether bond. Ether cleavage of the methoxy group of guaiacol has been previously observed in the Jackson sludge (Boyd et al., 1983). The 2,4-D was degraded within 7 days to 4-CP which disappeared gradually over the ensuing 49 days. No 2,4-dichlorophenol was detected, indicating that removal of the *ortho* chlorine was rapid, consistent with earlier studies on chlorophenol biodegradation in the Jackson sludge (Boyd and Shelton, 1984). Complete degradation of 2,4,5-T also occurred within 7 days giving 2,4,5-

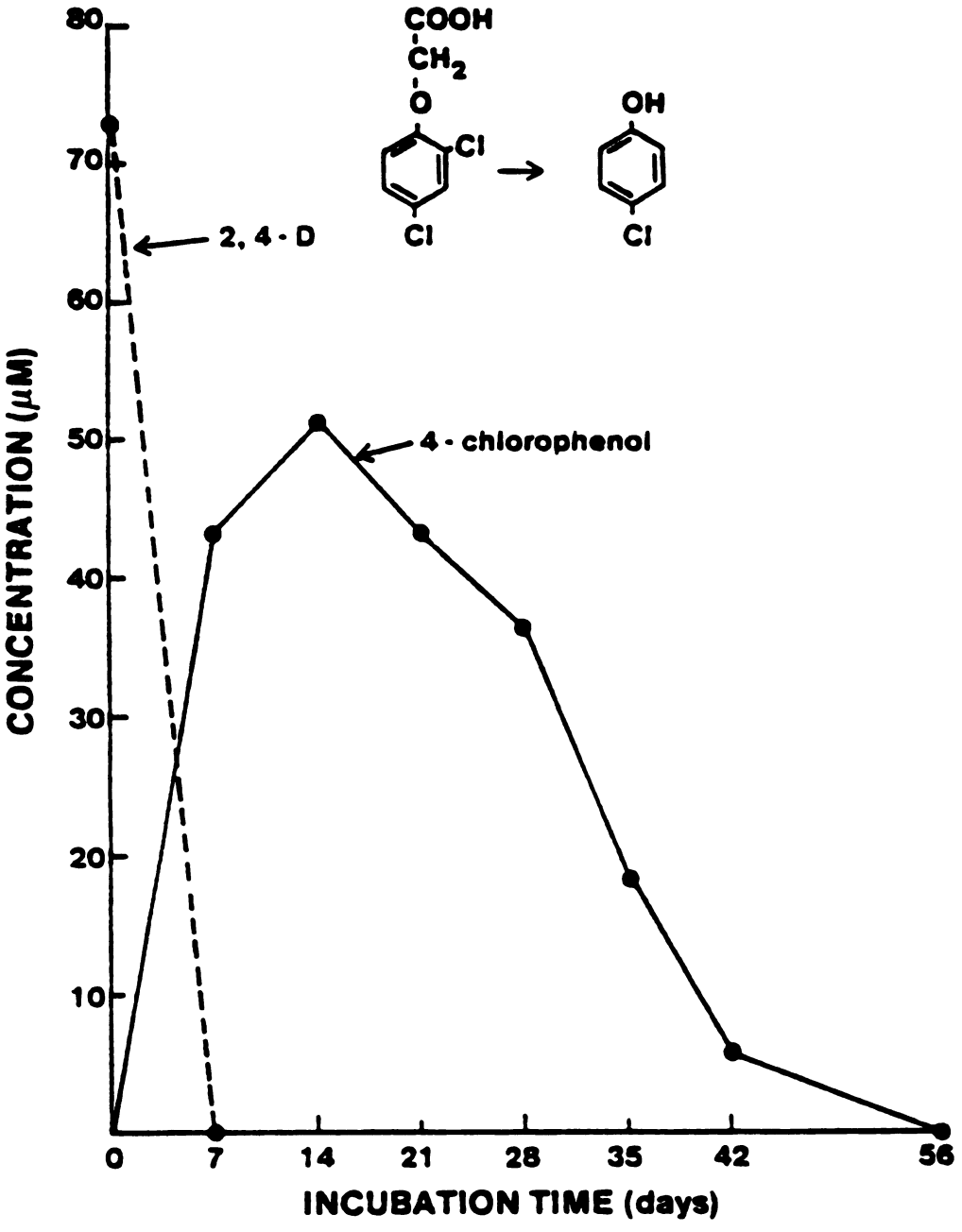

Figure 1. Degradation of 2,4-D in Jackson sludge.

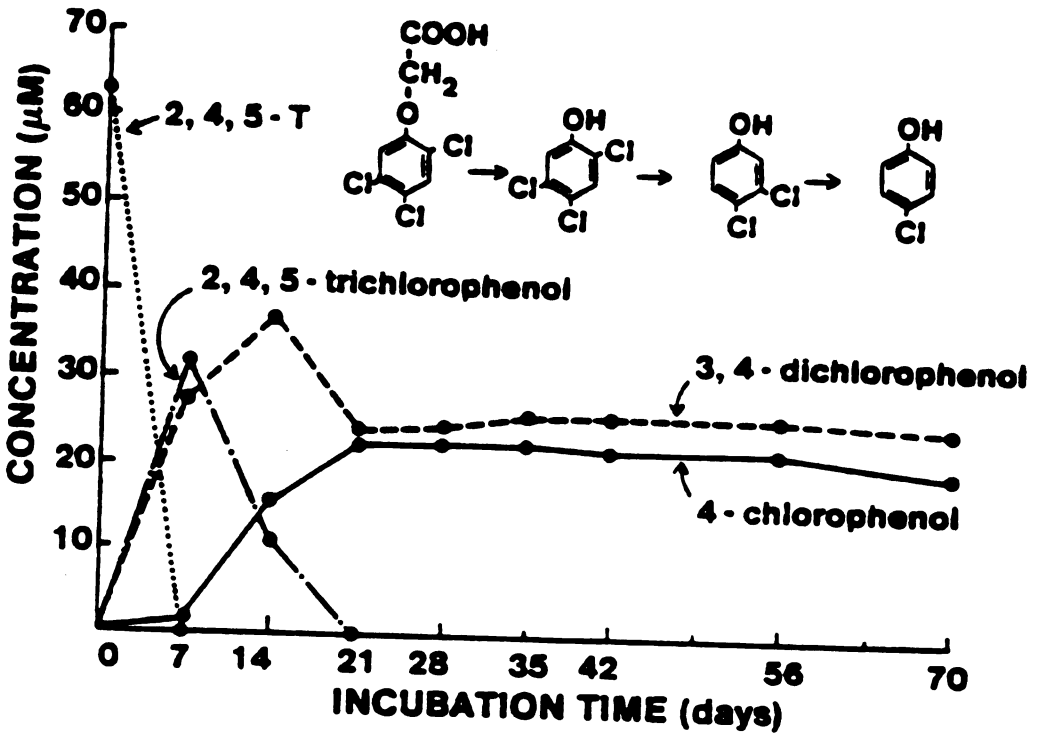

Figure 2. Degradation of 2,4,5-T in Jackson sludge.

trichlorophenol, which was subsequently dechlorinated to 3,4-dichlorophenol (Fig. 2). The 4-CP was also observed as a degradation product, presumably resulting from the dechlorination of 3,4-dichlorophenol. Dechlorination of the *meta* Cl atom of 3,4-dichlorophenol was interesting because in our previous studies dechlorination of chlorophenols in fresh sludge was limited to Cl substituents *ortho* to the phenolic OH (Boyd et al., 1983; Boyd and Shelton, 1984). Another feature distinguishing 2,4,5-T degradation from that of 2,4-D was the observation that 4-CP accumulated in the 2,4,5-T amended sludge. Depending on the original substrate added, 4-CP could persist or be slowly degraded, a result which cannot be readily explained. However, when 4-CP was added directly to sludge it was consistently the most persistent of the three monochlorophenol isomers (this work and Boyd and Shelton, 1984). The degradation pathway of 2,4,5-T reported in this study (Fig. 2) differs from that reported by Suflita et al. (1984) who observed 2,5-dichlorophenoxy acetic acid as the sole product of the reductive dechlorination of 2,4,5-T. However, in the latter study the active organisms had been selected by prior growth on 3-chlorobenzoate.

The two *ortho* Cl atoms were removed rapidly from PCP to give 3,4,5-trichlorophenol (Fig. 3), a result which is consistent with the rapid *ortho* dechlorination observed in the 2,4-D and 2,4,5-T experiments, and which is also consistent with the dechlorination observed by Boyd et al. (1983) and Boyd and Shelton (1984). Tetrachlorophenol was not detected. Removal of Cl from the 4

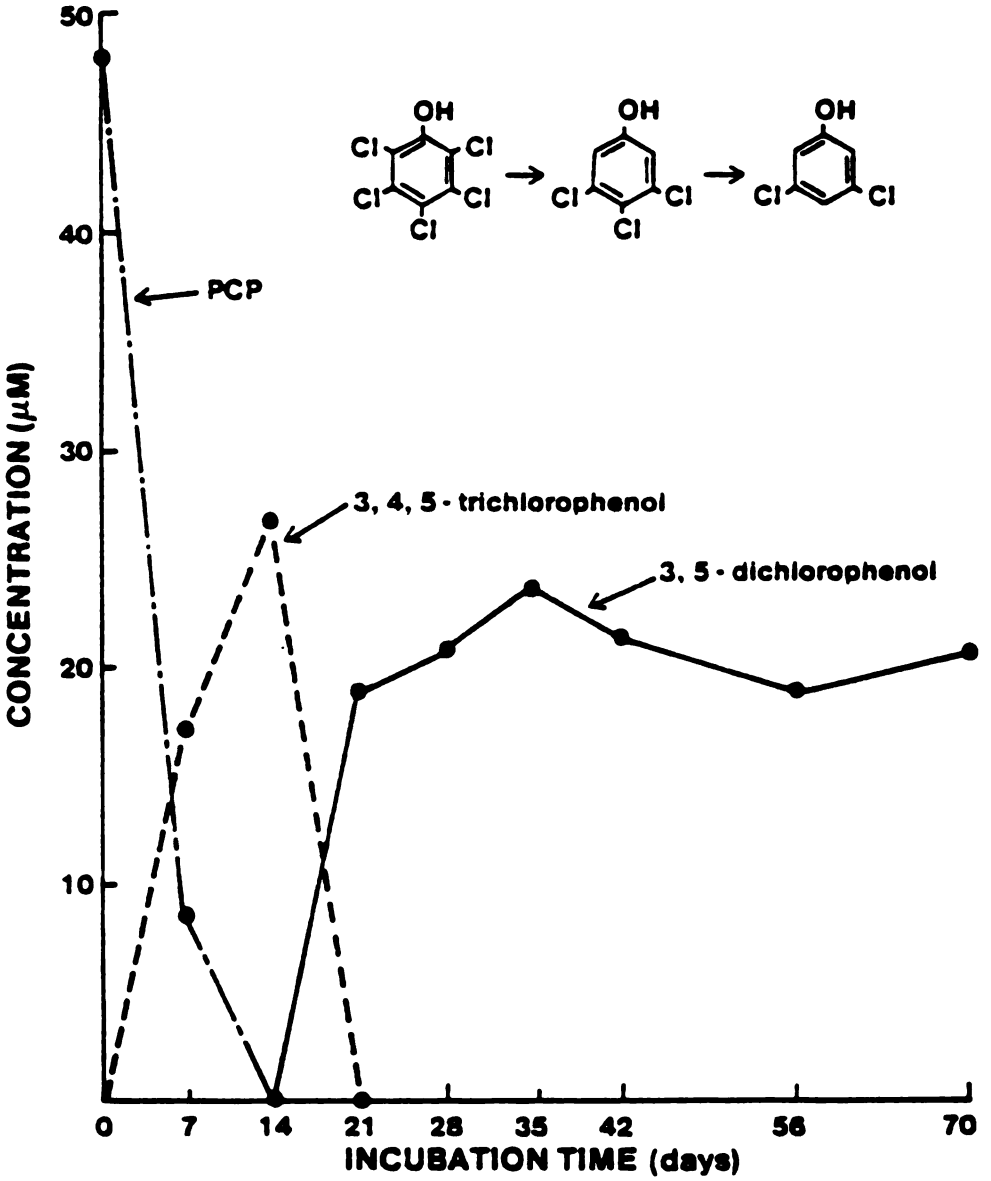

Figure 3. Degradation of PCP in Jackson sludge.

(*para*) position followed, giving 3,5-dichlorophenol as the final degradation product of PCP. These results appear to be consistent with those of Murthy et al. (1979), who observed removal of Cl atoms *ortho* and *para* to the phenolic OH of PCP in anaerobic soils. Based on the accumulation of 3,5-dichlorophenol, mineralization of PCP was not indicated in the present study of anaerobic sludges nor demonstrated previously in anaerobic soil (Murthy et al., 1979) or anaerobic sludge (Guthrie et al., 1984).

The ortho Cl atoms of 2,4,6-TCP were rapidly removed during degradation and resulted in accumulation of 4-CP (Fig. 4). The concentration of 4-CP appears to decrease very slowly from 28 to 70 days. The degradation of 2,4,6-TCP in anaerobic sludge has not previously been observed.

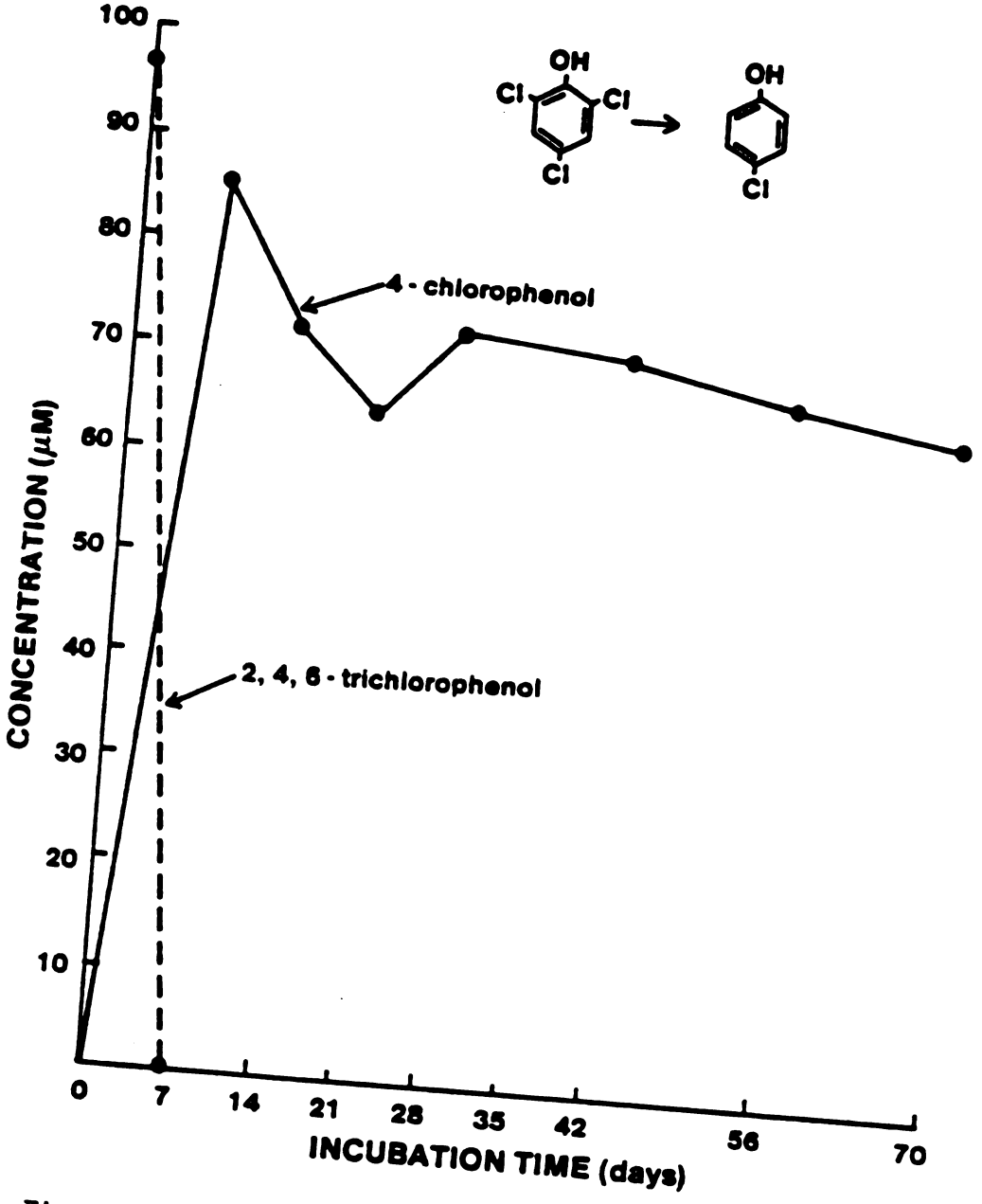

Figure 4. Degradation of 2,4,6-TCP in Jackson sludge.

CONCLUSIONS

The results of this study both confirm and expand previous results. The most consistent result was the rapid removal of Cl atoms *ortho* to the phenolic OH. With the exception of 2-CP added to Adrian sludge, *ortho* Cl atoms were very labile. The ether linkages of 2,4-D and 2,4,5-T were also cleaved rapidly, resulting the release of chlorophenols as the initial degradation products. Although 3- and 4-CP were the most persistent of the compounds tested, Cl atoms in the 3 position of 3,4-dichlorophenol (released from 2,4,5-T) and the 4 position of 3,4,5-TCP (released from PCP) were removed. Thus, Cl atoms located *ortho*, *meta*, and *para* to the phenolic OH were subject to reductive dechlorination. Removal of Cl atoms from the *meta* and *para* positions appeared to occur more readily in the higher chlorinated phenols.

The rapid ether cleavage and *ortho* dechlorination observed in the Jackson sludge suggests that lower chlorinated phenols, especially those with Cl atoms in the 3 and 4 positions, would tend to accumulate and pass through the wastewater treatment system. As a result, these compound would be introduced into the soil during land application of sludge. The reductive dechlorinations of the parent compounds should render the lower chlorinated products more susceptible to further degradation under

aerobic conditions by soil microorganisms. The Mason and Adrian sludges were less active in degrading the compounds tested. For these sludges, degradation did not appear to occur at a significant rate relative to the retention times of most municipal sludge digesters. However, given a sufficient incubation period, degradation did occur according to the pathways observed in the Jackson sludge.

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CHAPTER III

COMPLETE REDUCTIVE DECHLORINATION AND MINERALIZATION OF PENTACHLOROPHENOL BY ANAEROBIC MICROORGANISMS

ABSTRACT

Anaerobically digested municipal sewage sludge which had been acclimated to monochlorophenol degradation for more than two years was shown to degrade pentachlorophenol (PCP). Di-, tri-, and tetrachlorophenols accumulated when PCP was added to the individual acclimated sludges. When the 2-chlorophenol- (2-CP), 3-CP-, and 4-CP-acclimated sludges were mixed in equal volumes, PCP was completely dechlorinated. The same results were obtained in sludge acclimated to the three monochlorophenol isomers simultaneously. With repeated PCP additions, 3,4,5-trichlorophenol, 3,5-dichlorophenol, and 3-CP accumulated in less than stoichiometric amounts. All chlorinated compounds disappeared after the PCP additions were stopped. Incubations with [^{14}C]PCP resulted in 66% of the added ^{14}C being mineralized to $^{14}CO_2$ and $^{14}CH_4$. Technical-grade PCP was found to be degraded initially at a rate very similar to that of reagent-grade PCP, but after repeated additions the technical PCP was degraded more slowly.

Pentabromophenol was also rapidly degraded by the mixture of acclimated sludges. These results clearly show the complete reductive dechlorination of PCP by the combined activities of three chlorophenol-degrading populations.

INTRODUCTION

Pentachlorophenol (PCP) is a broad-spectrum biocide that is widely used as a wood preservative, as a pre-harvest herbicide, as a molluscicide, and in a variety of other applications. As a potent inhibitor of oxidative phosphorylation, it is toxic to a variety of organisms. In 1978 about 80% of the 23 x 10^6 Kg of PCP used in the United States was used for commercial lumber treatment (Crosby, 1981). Along with other chlorinated phenols, PCP has been placed on the U.S. Environmental Protection Agency list of priority pollutants (Keith and Telliard, 1979).

The impact and distribution of PCP in the environment has been extensively documented. For example, it has been used for many years as a herbicide in Japanese paddy fields. However, owing to some serious fish kills, its use was limited in 1971 to upland fields only (Watanabe, 1977). PCP has been identified as a contaminant in the vicinity of lumber mills and pulp and paper factories. The behavior of PCP in paddy soil has been an important research topic, especially with regard to bacterial degradation in relation to soil physical and chemical characteristics (Ide et al.,1972; Kuwatsuka and Igarashi, 1975; Sato, 1983; Watanabe, 1977).

In Finland, environmental samples (soil, ground water, fauna) have been found to contain compounds originating from the technical-grade PCP formulation used for lumber treatment. The contaminants identified include polychlorophenoxyphenols, polychlorophenoxyanisoles, polychlorodibenzodioxins and furans, and polychlorinated phenols, catechols, and guaiacols (Humppi, 1985; Humppi et al., 1984; Paasivirta et al., 1985; Valo et al., 1984, 1985).

Pure bacterial cultures able to metabolize PCP under aerobic conditions have been isolated by a number of research groups. Among these are several strains of a *Flavobacterium* sp. (Saber and Crawford, 1985), a pseudomonad (Watanabe, 1973), and a coryneform bacterium (Chu and Kirsch, 1972). A strain of *Arthrobacter* which utilizes PCP has been added to soil in an attempt to decontaminate polluted areas (Edgehill and Finn, 1983). Such prophylactic measures are effective in reducing the half-life of PCP in soil. No anaerobic bacteria with the ability to degrade PCP have been isolated or identified.

Despite the voluminous literature on PCP in the environment and its metabolism by various organisms, very few reports have adressed the fate of PCP in anaerobic environments. A few studies have presented data on PCP transformations in flooded soils, but generally the anaerobic status of the samples was not confirmed. Murthy et al. (1979) studied PCP degradation in aerobic and anaerobic soil and identified the products as pentachloroanisole, tetrachlorophenols (TeCPs), and trichlorophenols (TCPs). The

principal mode of degradation in anaerobic soil was described as dehyrodehalogenation. This is presumably the same process as the reductive dehalogenation which has been reported for PCP in anaerobic sewage sludge, in which the principal product was 3,5-dichlorophenol (3,5-DCP) (Mikesell and Boyd, 1985).

Hakulinen and Salkinoja-Salonen (1982) used an anaerobic fluidized bed reactor to treat pulp and paper industry wastewaters with considerable success. They accomplished complete mineralization of PCP to CO_2 in a system composed of both aerobic and anaerobic phases. Guthrie et al. (1984) examined the fate of PCP during anaerobic digestion of anaerobic sludge solids. They found that an acclimation period was necessary, after which primary degradation of PCP occurred, although the extent of degradation was not determined.

PCP has been shown to be strongly inhibitory to methanogenesis both in pure cultures (Guthrie et al., 1984; Roberton and Wolfe, 1970) and in anaerobic sludge and sediment (Horowitz et al., 1982). Thus, the presence of PCP in wastewater is a potentially serious problem if anaerobic digestion is to be successful. Knowledge of its fate in and effect on wastewater treatment systems is important if satisfactory guidelines for industrial discharges are to be established.

Reductive dechlorination, or removal of Cl atoms directly from the ring of aromatic compounds in a single step, does not appear to occur under aerobic conditions and is a significant process because the dechlorinated products are usually less toxic

and more readily degraded either anaerobically or aerobically. Reductive dechlorination has been reported for a variety of compounds, including chlorobenzoates (Suflita et al., 1982), chlorophenols (Boyd et al., 1983; Boyd and Shelton, 1984; Mikesell and Boyd, 1985; Murthy et al., 1979), and the pesticides diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] (Attaway et al., 1982), chlornitrofen (4-nitrophenyl-2,4,5-trichlorophenyl ether) (Yamada and Suzuki, 1983), and 2,4,5-T [(2,4,5-trichlorophenoxy)acetic acid] (Suflita et al., 1984; Mikesell and Boyd, 1985), in soils, sediments, and anaerobic sewage sludges.

Our objective in this research has been to use acclimation strategies to obtain complete dechlorination of PCP and eventual mineralization of the ring carbon to CH_4 and CO_2. The process of acclimation has been used successfully to obtain new or improved biodegradation activities. Boyd and Shelton (1984), for example, increased both the rate of degradation of CPs and the substrate range of degradative activity by using acclimated anaerobic sewage sludges.

In the present study, anaerobic sludges acclimated over a long period to the degradation of monochlorophenols (MCPs) were used for PCP degradation, in the hope that the acclimation process would improve both the rate and extent of PCP transformation.

MATERIALS AND METHODS

Anaerobically digested municipal sewage sludge was taken from a primary digester at the wastewater treatment plant in Jackson, Mich. The plant receives about 40% of its wastewater from industrial sources and 60% from residential sources. The retention time in the primary anaerobic digester is approximately 13 days. Typically the sludge contains about 40 g of solids per kg of sludge, with a total N content of 40 g of N per kg of dry sludge (J. St. Andre, personal communication).

The sludge was collected from the digester, placed in 4-liter glass bottles, and transported to the laboratory where the headspace of each bottle was thoroughly flushed with an O_2-free gas mixture (80% N_2-20% CO_2) by using a Hungate apparatus (Hungate, 1969; Kaspar and Tiedje, 1982). Separate bottles of sludge have been maintained for more than 2 years with CP as the only added carbon or energy source. CP was added weekly from 6000 ug/mL aqueous stock solutions to give CP concentrations of approximately 25 ug/mL. Batches of sludge have also been maintained with simultaneous feedings of all three MCP isomers. This sludge received approximately 10 ug/mL of each MCP isomer weekly from aqueous stock solutions. CP concentrations in the acclimated

sludges were checked each week to monitor against accumulation of CP as a result of excess feeding.

The studies with PCP and pentabromophenol (PBP) were conducted with the following three sludges: (i) the individual MCP-acclimated sludges; (ii) a mixture of equal volumes of the three MCP-acclimated sludges; and (iii) sludge acclimated simultaneously to the three MCP isomers. In each case the sludge was dispensed with a glass pipette in duplicate into 160 ml serum bottles which had been thoroughly flushed with an O_2-free gas mixture. For (i) and (iii), 75 mL of sludge was transferred; for (ii), 25 mL of each MCP-acclimated sludge was transferred to the serum bottle. PCP (40uM, 11 ug/mL) was added to the bottles from a concentrated (15%) ethanol stock solution with a microliter syringe. After the headspace was flushed for an additional 2 to 3 min., the bottles were sealed with butyl rubber stoppers and aluminum crimp seals. Incubation was in the dark at 37°C without shaking, and samples (1 to 2 mL) were withdrawn daily with a disposable syringe and frozen until the time of analysis. Every 4 days duplicate samples were taken so that an immediate analysis could be made to determine whether re-feeding was necessary. Sterile controls were provided by autoclaving sludge twice on successive days. The individual acclimated sludges received four additions of PCP over 30 days. The acclimated sludge mixture and the simultaneously acclimated sludge received five and three PCP additions, respectively, in 18 days.

For extraction of the CPs, the 1.0 mL sample was mixed with 0.5 mL of acetonitrile on a vortex mixer, centrifuged for 10 min. at 12,000 x g, and filtered through a 0.45 um pore-size membrane filter (type HVLP, Millipore Corp., Bedford, Mass.). This procedure resulted in recoveries of 85% or higher when sludge was spiked with CP at 25 ug/mL. The concentration of CPs was determined by using a high performance liquid chromatography system (Waters Associates, Inc., Milford, Mass.) consisting of the following components: model 6000A and M45 pumps, model 720 system controller, and model 480 variable-wavelength UV detector. The CPs were detected at their absorbance maxima, which ranged from 280 nm for the lower chlorinated phenols to 300.5 nm for tetra- and pentachlorophenol. The detection limit was 0.5 to 0.1 ug/mL. The samples were injected onto a C_{18} column (4.6 x 250 mm) by using a Rheodyne 7125 sample injector (Rheodyne Corp., Cotati, Calif.) with a 20 uL loop. Peak area values were obtained with a Waters data module integrator. The mobile phase consisted of acetonitrile and 5% aqueous acetic acid in proportions which were adjusted to give retention times of 4 to 8 min. at a flow rate of 2.0 mL/min. The mobile phase composition ranged from 70:30 to 45:55 acetonitrile: 5% acetic acid.

The experiments with [^{14}C]PCP were conducted in the same serum bottle incubation system as described above. Approximately 4 uCi of [^{14}C]PCP was added from an ethanol solution to each bottle with a microliter syringe. The experiment was performed with the mixture of acclimated sludges, with half the bottles being fed

MCPs (approximately 2 ug of each isomer per ml per week) and the other half containing only [^{14}C]PCP. At the end of the incubation the headspace of each bottle was checked for $^{14}CO_2$ after acidification of the aqueous phase with H_3PO_4. The headspace CO_2 was trapped by being flushed through a series of five scintillation vials, each containing 10 ml of 0.1 N NaOH. A sample of trapping solution was removed from each vial and added to scintillation cocktail for the determination of radioactivity. That CO_2 was the source of radioactivity in the trapping solution was verified by precipitation of CO_2 with $BaCl_2$ and counting of a sample of the remaining liquid. In all cases the radioactivity remaining after precipitation of the CO_2 was at the background level. The recovery of $^{14}CO_2$ from sludge spiked with [^{14}C]bicarbonate was 78%. The values reported have been adjusted for this recovery.

After the headspace was flushed from the serum bottles the sludge was centrifuged at 26,000 x g for 20 min. to separate the solids from the acidified aqueous phase. The solids were the extracted twice with methanol and twice with ethyl acetate and the ^{14}C in these extracts was determined. The ^{14}C remaining in the air-dried sludge solids was collected as $^{14}CO_2$ after the solids were combusted in a model OX200 Biological Materials Oxidizer (R.J. Harvey Instrument Corp., Hillsdale, N.J.). The ^{14}C radioactivity was determined with a model LS 8100 (Beckman Instruments, Inc., Fullerton, Calif.) using external standard quench correction.

All CPs were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., and were used without further purification; [^{14}C]PCP (10.57 mCi/mmol), uniformly ring labeled, was purchased from Pathfinder Laboratories, St. Louis, Mo., and had a radiochemical purity of >98%. PBP was purchased from Ultra Scientific, Hope, R.I.

RESULTS AND DISCUSSION

PCP (10 ug/mL) was degraded in each of the individual MCP-acclimated sludges (Fig.1). Degradation occurred most rapidly in the 2-CP-acclimated sludge (Fig. 1a), in which the PCP was degraded within 3 days. PCP degradation in 3- and 4-CP-acclimated sludges (Fig.1b and c, respectively) was considerably slower, requiring 12 and 19 days, respectively, for complete disappearance. No PCP degradation occurred in the autoclaved controls. The time-course for MCP degradation is also given in Fig. 1, which shows that 2-CP was degraded more rapidly than the other MCP isomers. These data, together with our previous results on the dechlorination of CPs (2, 3, 18), suggest that Cl substituents ortho to the phenolic OH group are removed more rapidly than Cl in the meta and para positions. Thus, removal of the ortho-Cl substituents of PCP by the 2-CP-acclimated sludge probably occurred faster than removal of the removal of the meta or para-Cl substituents of PCP by the 3- or 4-CP-acclimated sludges. Differences in cell numbers between the three acclimated sludges may also have contributed to the different rates.

The individual CP acclimations dechlorinated PCP principally but not exclusively at the position corresponding to the MCP to which they were acclimated. Figure 2 indicates the products of

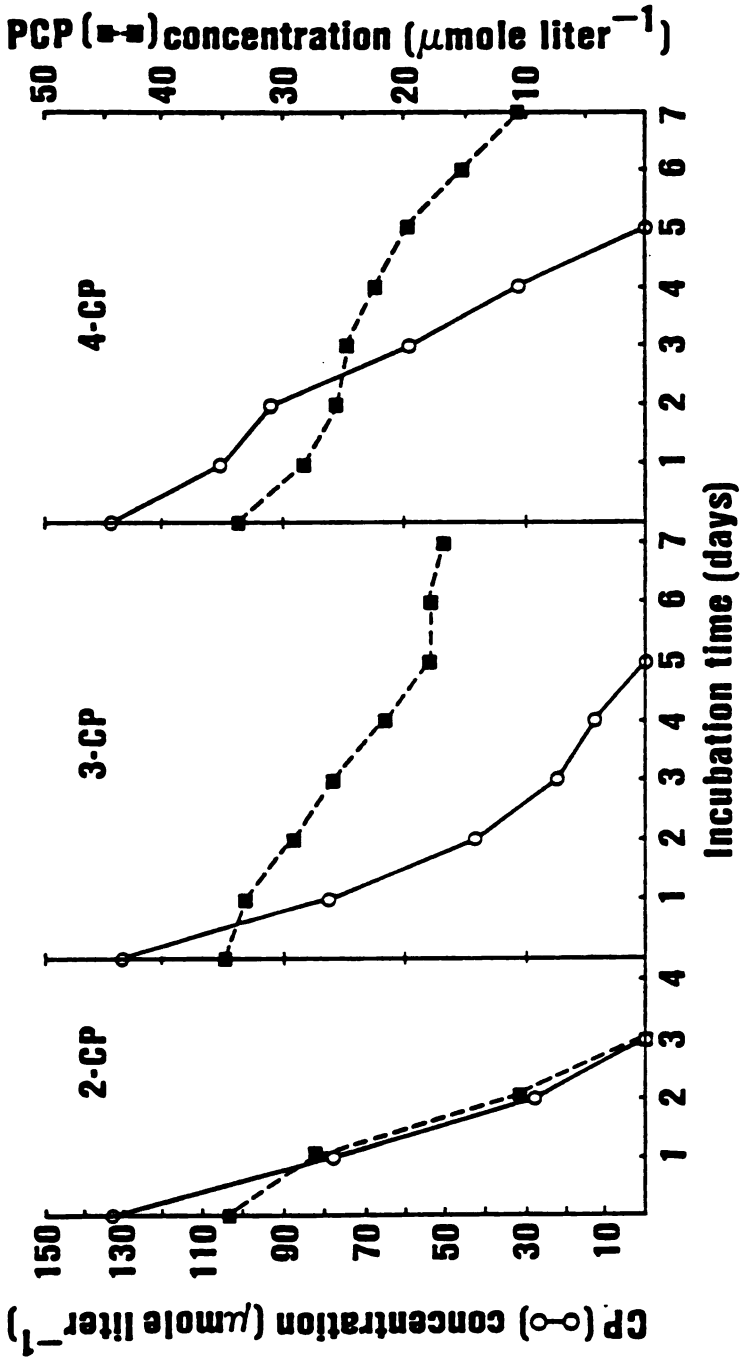

Figure 1. Degradation of MCPs and PCP in sludge acclimated to (a) 2-CP, (b) 3-CP, and (c) 4-CP.

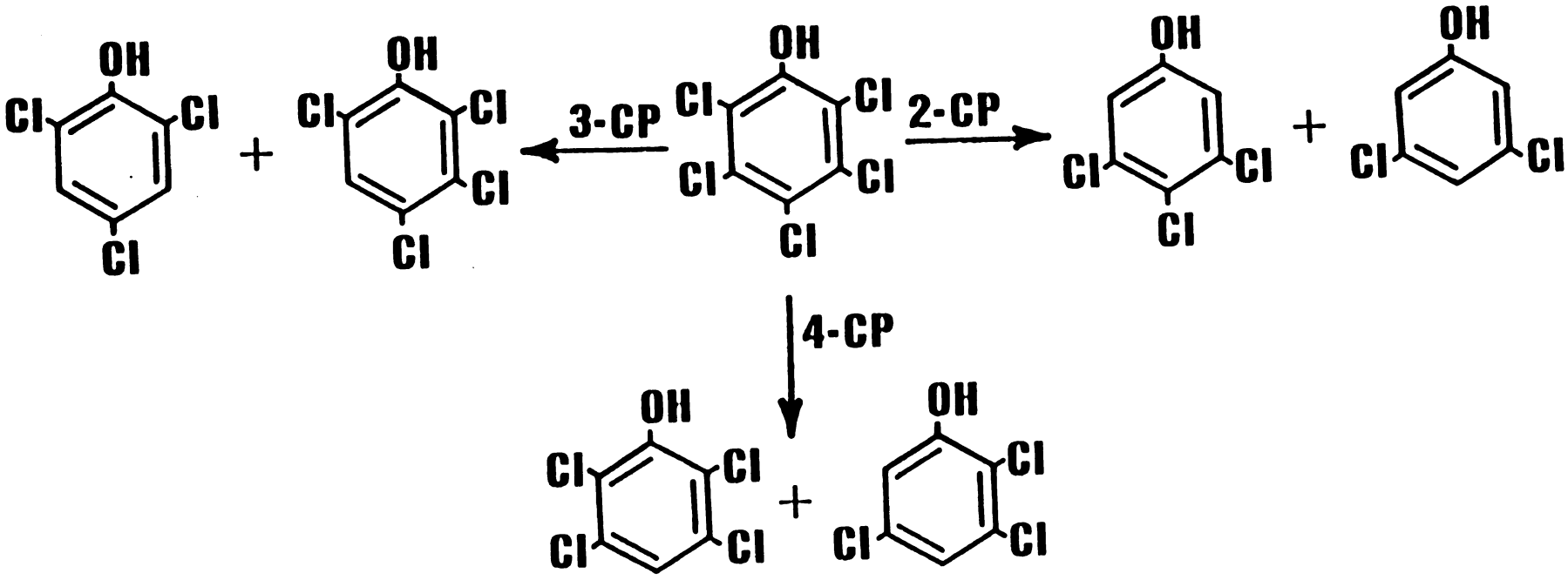

Figure 2. Products resulting from the dechlorination of PCP in sludges acclimated to 2-, 3-, or 4-CP.

PCP dechlorination which accumulated in the acclimated sludges. In 2-CP-acclimated sludge 3,4,5-TCP and 3,5-DCP accumulated in a ratio of 2:1; in 3-CP-acclimated sludge 2,3,4,6-TeCP and 2,4,6-TCP accumulated in a 1:2 ratio; and in 4-CP-acclimated sludge 2,3,5,6-TeCP and 2,3,5-TCP accumulated in a ratio of 3:1. These data show that the less extensively chlorinated phenols (TeCPs and TCPs) were less subject to dechlorination reactions than PCP and thus tended to accumulate. It was also apparent that the dechlorinating populations present in the three acclimated sludges were qualitatively different. The pattern of dechlorination in the acclimated sludges was similar to that obtained in an earlier study (Boyd and Shelton, 1984) in which MCP-acclimated sludges were tested for activity on different CP isomers. In the earlier study the 2-CP-acclimated sludges were able to dechlorinate 4-CP. The specificity of the populations with regard to the site of dechlorination argues strongly for an enzymatic event.

The three MCP-acclimated sludges, if considered together, were able to dechlorinate at all positions on the aromatic ring. The next step toward our objective of complete PCP degradation was to combine the three dechlorinating activities and determine whether PCP was completely dechlorinated. Figure 3 shows the results of the experiment in which equal volumes of 2-, 3-, and 4-CP-acclimated sludges were mixed and given five PCP additions over a period of 18 days. Each time PCP was added (40 uM, 11ug/mL), it was degraded within 3 to 6 days, with the gradual accumulation of 3,4,5-TCP, 3,5-DCP, and 3-CP. The maximum accumulation of

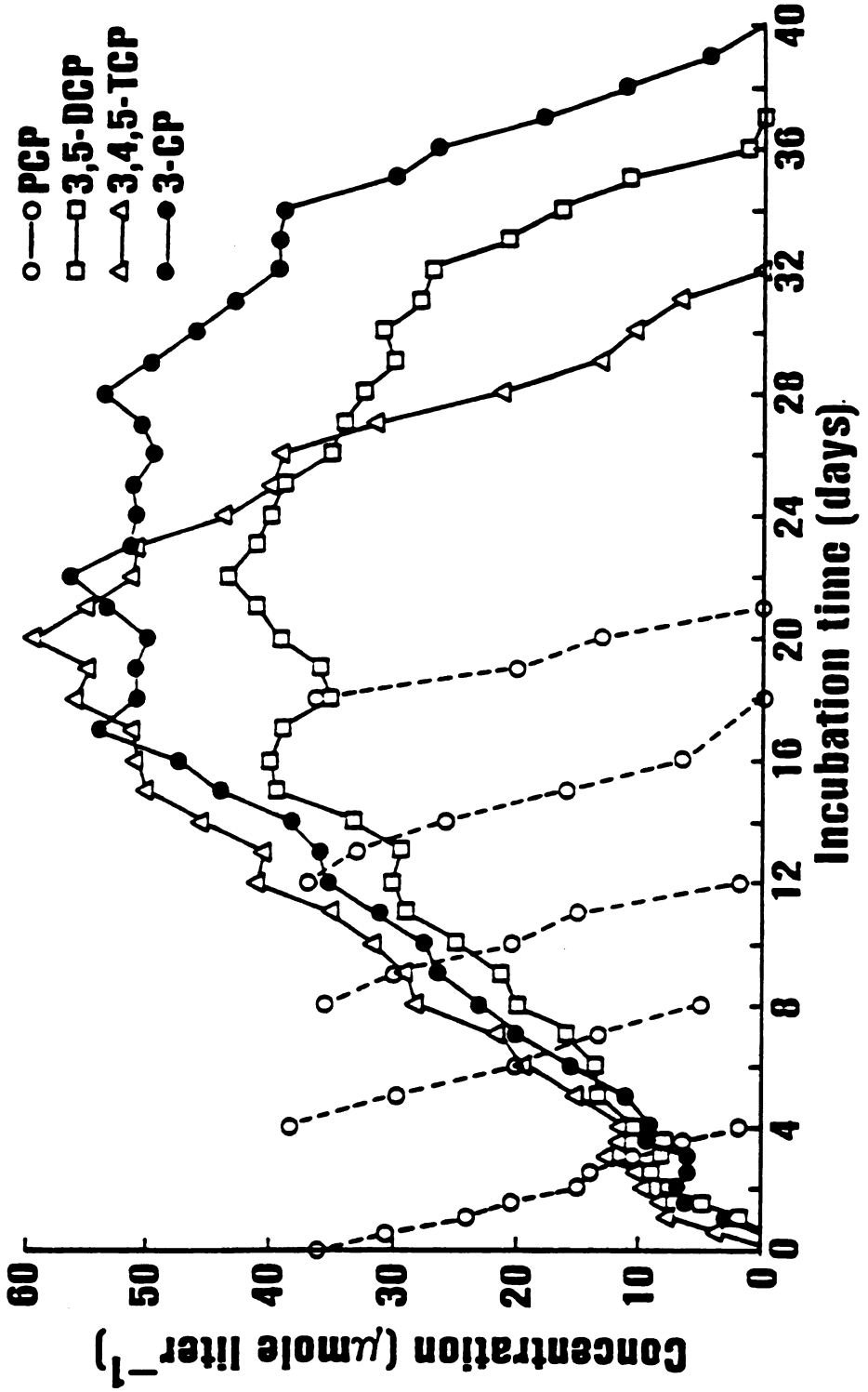

Figure 3. PCP degradation in a mixture of 2-, 3-, and 4-CP-acclimated sludges, showing the sequential appearance and disappearance of dechlorination products.

dechlorinated products (day 21) was 75% of the PCP added. No PCP was present after day 21, and the concentration of accumulated products gradually declined until, on day 40, no CPs could be detected. The products disappeared in the expected sequence, with 3,4,5-TCP giving way to 3,5-DCP, and finally 3-CP. In general, accumulation of phenol was not observed in the acclimated sludges or enrichments; phenol was apparently consumed as rapidly as it was produced from the final dechlorination step. No PCP dechlorination occurred in the autoclaved controls.

The complete dechlorination of PCP with this type of accumulation and disappearance of lower chlorinated phenols was also observed with sludge which had been acclimated over a long period to all three MCP isomers simultaneously (results not reported). The same product accumulation and disappearance pattern occurred in the simultaneous acclimation system as in the mixed system; the only significant difference was that the rate of PCP degradation was slower in the former case.

These results clearly show the complete reductive dechlorination of PCP through the combined activities of two or three anaerobic CP-degrading populations. We have proposed a PCP degradation pathway based on the sequential appearance and disappearance of 3,4,5-TCP, 3,5-DCP, and 3-CP (Fig. 4). This pathway appears to result from the relatively higher rate of PCP dechlorination by the 2-CP-acclimated sludge (Fig. 1). This sludge rapidly removes Cl from the 2 and 6 positions of PCP to

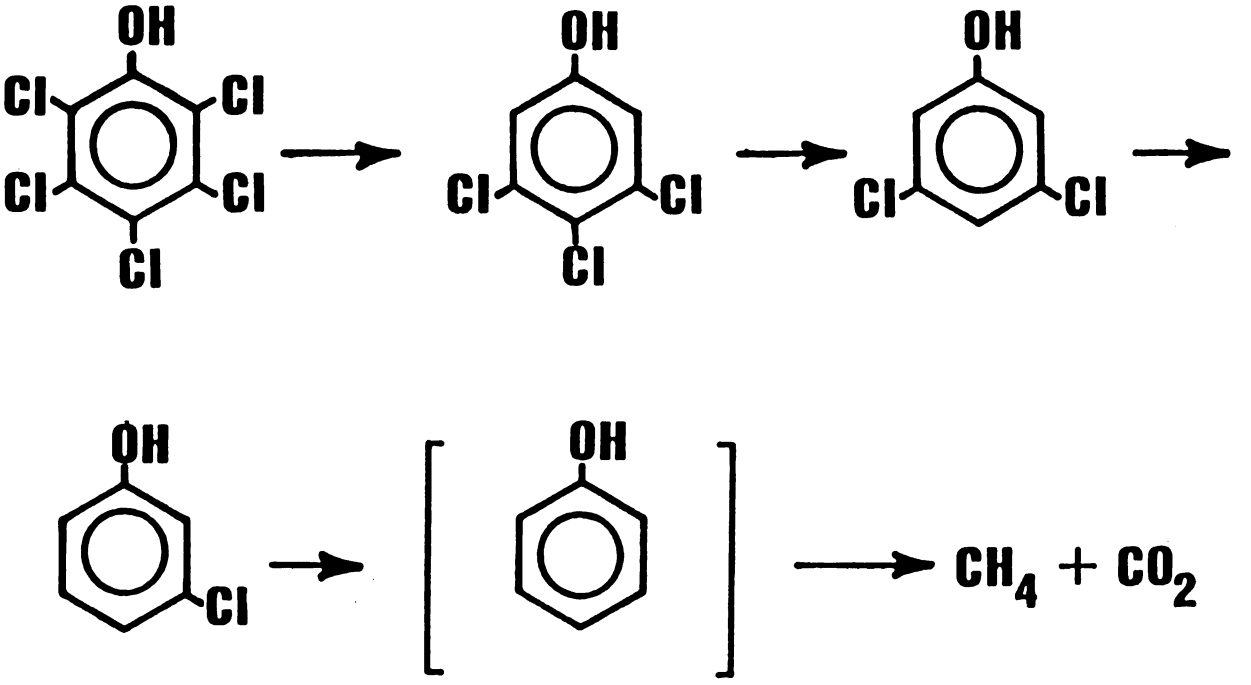

Figure 4. Proposed pathway for PCP degradation in a mixture of CP-acclimated sludges.

give 3,4,5-TCP (Fig. 2). The para-Cl was then removed by populations present in the 2- or the 4-CP-acclimated sludge, or both, which have been shown to dechlorinate at this position (Boyd and Shelton, 1984) (Fig.2). 3,5-DCP and 3-CP were probably dechlorinated by the 3-CP-acclimated sludge. Previous results showed that the 3-CP-acclimated sludge was cross-acclimated to the degradation of 3,5-DCP.

The ultimate fate of the PCP carbon was investigated by adding [^{14}C]PCP to the CP-acclimated sludge mixture. After a 2 month incubation, duplicate bottles were analyzed for ^{14}C in gas, aqueous, and solid phases, and in extracts of the solids. The results are summarized in Table 1. The data in the two columns are for a sludge mixture which was given weekly additions of the three MCPs and a mixture which was incubated without additions. The reason for comparing the two treatments was the determination of whether the presence of CPs affects the dechlorination and mineralization of PCP. The data show that the presence of CPs results in less mineralization of PCP, as indicated by lower amounts of gaseous ^{14}C and higher aqueous and extractable levels of ^{14}C. When the amounts of ^{14}C trapped as $^{14}CO_2$ were combined with the calculated amount of $^{14}CH_4$ produced (according to the stoichiometry of phenol mineralization in methanogenic consortia [Healy and Young, 1979]), the total carbon mineralized was substantial: 54.9% and 65.7% for the fed and not-fed bottles. This difference is quite small and may be due in part to a small isotopic dilution effect resulting from the CP additions. The

TABLE 1. Mineralization of [^{14}C]PCP

Product	% of added ^{14}C in fraction:	
	Amended with 2-, 3-, and 4-CP	Not amended
CO_2	22.9	27.4
CH_4[a]	32.0	38.3
Total ^{14}C in gas phase	54.9	65.7
Aqueous phase	13.7	10.9
Methanol extract of solids	16.9	12.3
Ethyl acetate extract of solids	5.5	4.8
Extracted solids	4.3	4.1
Total ^{14}C	95.3	97.8

[a] Calculated from the measured CO_2 according to the equation phenol$\rightarrow$2.5CO_2 + 3.5CH_4

total recovery of the added ^{14}C was greater than 95% for all bottles analyzed.

The degradation of technical-grade PCP and reagent-grade PCP were compared (Fig. 5). No significant difference in the rate of disappearance of the two forms was apparent until the third PCP addition, after which the technical-grade PCP was degraded more slowly. Technical-grade PCP formulations are known to contain a number of highly toxic contaminants (Humppi, 1985; Humppi et al.,1984) not present in reagent-grade PCP. The potential contaminants include chlorinated dibenzo-p-dioxins, dibenzofurans, phenoxyphenols and anisoles and could be responsible for the retarded disappearance of the technical-grade material.

The degradation of PBP by the mixed acclimated sludges was also observed (Fig. 5). The rate of disappearance of PBP was greater than that of PCP through all three additions (over 28 days). Thus it appears that dehalogenation occurs for both chloro- and bromoaromatic compounds, and that removal of Br may be more facile.

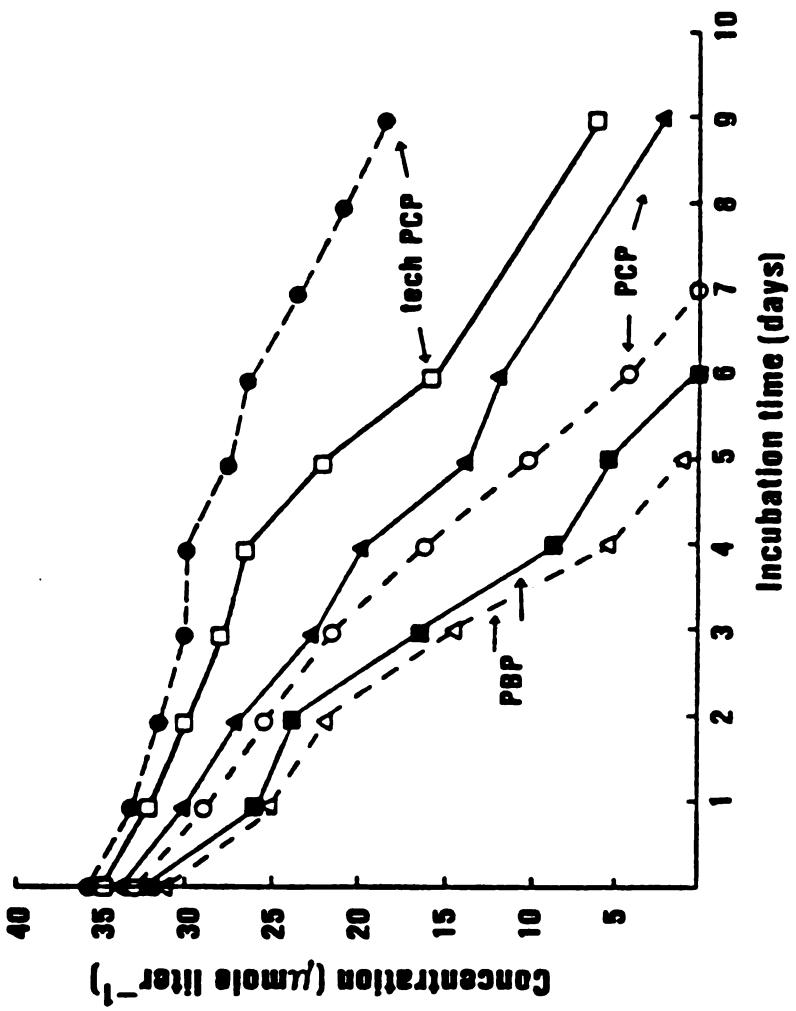

Figure 5. Degradation of technical-grade PCP, reagent-grade PCP, and PBP in the mixture of CP-acclimated sludge; solid lines represent the first addition, dashed lines represent the third addition.

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CHAPTER IV

ENHANCEMENT OF PENTACHLOROPHENOL DEGRADATION IN SOIL THROUGH INDUCED ANAEROBIOSIS AND BIOAUGMENTATION WITH ANAEROBIC SEWAGE SLUDGE

ABSTRACT

The addition of anaerobic sewage sludge, previously shown to dechlorinate chlorophenols, to soil contaminated with pentachlorophenol (PCP) resulted in greatly enhanced rates of PCP degradation. The sludge was added to soil at a rate of 5 g Kg^{-1} (dry weight basis) and the mixture incubated anaerobically. Initial PCP concentrations of 10-30 mg Kg^{-1} (ppm) were completely (to < 0.5 ppm) degraded within 28 to 35 days. In anaerobic soil without sludge or aerobic with or without sludge, PCP persisted, with 65% and 90%, respectively, remaining after 56 days. Higher rates of sludge addition (10 and 25 g Kg^{-1}) gave only small differences in the rate of PCP degradation. PCP was degraded by sequential dechlorination and the products of PCP degradation in sludge-soil mixtures were the same as those observed in sludge alone. The sequence of products was: PCP--> 2,3,4,5-

tetrachlorophenol-->3,4,5-trichlorophenol-->3,5-dichlorophenol-->3-chlorophenol. Small amounts of 3,4-dichlorophenol were observed as well. These results clearly demonstrate that the dechlorinating activity present in sludge could be transferred to soil to achieve PCP degradation. This suggests a simple, effective and economical treatment of soil through inducing anaerobiosis and bioaugmentation with anaerobic sewage sludge.

INTRODUCTION

The contamination of soils with wood preserving chemicals has been well documented in the U.S. and elsewhere (Valo et al., 1985; Paasivirta et al., 1985; Crawford and Mohn, 1985; Kitunen et al., 1987). The contaminants may include chlorinated phenols, their impurities, and creosote components, as well as oils and sometimes chromium, copper, and arsenic. The highly chlorinated compounds, principally pentachlorophenol (PCP), are of concern because they are sometimes present in surface soils at very high concentrations (thousands of ppm) and are generally resistant to degradation even at lower levels. As a result, PCP can be found in ground water and surface water where it undergoes biomagnification (Lu et al., 1978).

Despite its recalcitrance, a number of bacterial strains that degrade PCP have been isolated and characterized (Chu and Kirsch, 1972; Stanlake and Finn, 1982; Edgehill and Finn, 1982; Pignatello et al., 1983; Saber and Crawford, 1985; Apajalahti and Salkinoja-Salonen, 1986). Some of these strains have been considered potentially useful for the decontamination of PCP-laden soils (Crawford and Mohn, 1985; Edgehill and Finn, 1982; Valo and Salkinoja-Salonen, 1986). The successful use of a strain of *Flavobacterium* on a soil containing about 300 ppm PCP has been

reported by Crawford and Mohn (1985). Several inoculations and maintenance of favorable environmental conditions were required. Edgehill and Finn (1982) published similar results with what they reported to be an *Arthrobacter* strain. The soil used in this case was spiked in the laboratory with PCP (120-150 mg L^{-1}) and immediately inoculated. Experiments with soil inoculations on a larger scale in an outdoor shed were also successful at reducing the half-life of PCP (85% disappearance in 12 days compared with 30% in uninoculated soil).

Composting of soil contaminated with chlorophenols has also been explored as a potential method of soil decontamination (Valo and Salkinoja-Salonen, 1986). In this case the degradation of PCP (measured by evolution of $^{14}CO_2$ from ^{14}C-PCP as well as by disappearance of PCP) in sterilized compost soil was accelerated by the addition of a chlorophenol degrading actinomycete, *Rhodococcus chlorophenolicus*. In each of these studies the treated soil contained a residual chlorophenol concentration of 10-30 ppm.

We have recently demonstrated the reductive dechlorination of PCP by anaerobic microorganisms found in municipal sewage sludge (Mikesell and Boyd, 1985). In fresh sludge from Jackson, Michigan, PCP was rapidly dechlorinated first at the *ortho* positions and then at the *para* position, resulting in the formation of 3,5-dichlorophenol. The reductive dechlorination of PCP in sludges from two other locations also occurred but at slower rate than in the Jackson sludge. When the same sludge was

acclimated to the degradation of the monochlorophenol isomers (2-, 3-, and 4-chlorophenol), PCP degradation proceeded rapidly through the same tri- and dichlorophenols (Mikesell and Boyd, 1986). However, in this case dechlorination continued through 3-chlorophenol until no chlorophenol could be detected. These results clearly showed the complete reductive dechlorination of PCP by chlorophenol-degrading anaerobic microorganisms. Incubations with ^{14}C-PCP resulted in 66% of the added ^{14}C being mineralized to $^{14}CH_4$ and $^{14}CO_2$.

The reductive dechlorination of PCP is important because the dechlorinated products are less toxic, less likely to bioaccumulate, and more susceptible to further degradation either aerobically or anaerobically. One potential application of this reductive dechlorination reaction is in the remediation of contaminated soils at wood preserving sites. These sites are numerous (over 600 in the U.S.), and PCP is the primary contaminant. Thus, it was of interest to determine if the PCP-degrading activity present in anaerobic sewage sludge would manifest itself when sludge was added to soil containing PCP. This may offer an effective *in-situ* treatment of PCP contaminated soils through inducing anaerobiosis (flooding) and bioaugmentation with anaerobic sewage sludge.

Our objective in these experiments was to attempt to transfer anaerobic dechlorinating activity from whole sludge (Jackson, MI) to PCP-containing soil by adding the sludge at rates that would be reasonable in agricultural settings. Initially the experiments

were performed with contrived (spiked) soil, then with a partially treated contaminated soil from the site of a wood-preserving facility.

MATERIALS AND METHODS

Materials. The soil used throughout these experiments was the Spinks loamy fine sand (Psammentic Hapludalfs) which was collected from the top 15 cm of a plowed field near the Michigan State University Agronomy Farm, East Lansing. The soil was stored at 4°C until it was sieved (<2mm) and the moisture content determined. Anaerobic sewage sludge was collected from the R.A. Greene Wastewater Treatment Plant, Jackson, Michigan. The influent at this plant is 40% from industrial sources, 60% residential. The sludge typically contains 40 g Kg^{-1} nitrogen (dry weight) and 30-40 g solids L^{-1}; the retention time in the primary digestor is approximately 13 days. The soil used in the first part of this study was the Spinks loamy fine sand (Typic mesic Psammentic Hapludalfs; 81% sand, 14% silt, 5% clay, 1.1% organic carbon; pH 5.8). A contaminated soil was collected by representatives of BioTrol, Inc., a Minnesota-based company involved in remediation of contaminated soils and ground water . Details of the physical and chemical properties of this soil are not available; the soil was partially treated by physical extraction to remove all but approximately 30 mg Kg^{-1} PCP. The result was an extremely sandy material very low in organic material.

The chlorophenols were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, and used without further purification.

Methods. Soil and sludge-soil mixtures were incubated in 125 mL Erlenmeyer flasks. To insure anaerobiosis the flasks were thoroughly flushed with O_2-free N_2 gas using a Hungate gassing apparatus (Hungate, 1969) before the soil was added and for an additional 10 min prior to the addition of the anaerobic sludge. The sludge was dispensed with a disposable syringe fitted with a length of Tygon tubing. The sludge amendment rates are reported in the units g sludge (dry solids) per Kg dry soil. The flasks were sealed with rubber stoppers and air-tight plastic tape. Anaerobiosis was monitored throughout the experiments by periodic measurement of CH_4 in headspace samples. Methane was measured by injecting headspace gas into a Carle 8515 gas chromatograph equipped with a microthermistor detector and a 2 meter steel column packed with Poropak QS. Aerobic flasks were flushed with air and closed with foam plugs.

When PCP was added to the soil it was as a concentrated aqueous solution of the sodium salt. For each experiment the NaPCP solution was added slowly with a syringe to the bulk soil with thorough mixing to insure a uniform mixing of the water and dissolved NaPCP. Depending on the experiment, the resulting soil contained 10, 20, or 30 mg PCP Kg^{-1} (37, 75, and 113 umole PCP Kg^{-1}), or with the already contaminated soil, the PCP concentration was increased from 30 to 45 and 60 mg Kg^{-1}; in all cases the

moisture content was adjusted to 18% (60% of field capacity) prior to the addition of the sludge. Sterile materials were obtained by autoclaving twice on successive days.

The experiments were set up in duplicate with two flasks for each treatment and for each pre-arranged sampling time. At the prescribed time the flasks were immediately frozen until the time of extraction and analysis. The extraction was performed on the entire contents of each flask, i.e., approximately 50g of soil or sludge-soil mixture, which was first dried in a Soxhlet extraction thimble at 75°C. The spiked material was extracted in a Soxhlet apparatus for 6 hours at 6-8 cycles per hour using 250 mL dichloromethane; the contaminated BioTrol soil required a 24 hour extraction. The extract was then shaken with two 25 mL volumes of 0.1 M K_2CO_3 (pH 12). After filtering through a 0.45 um membrane filter (type HA, Millipore, Inc.) the extract was ready for analysis. The extraction efficiency was 95% or greater for pentachlorophenol and all other chlorophenol congeners as determined with freshly spiked samples.

The chlorophenols in the aqueous K_2CO_3 extract were analyzed by reverse phase HPLC with detection by UV absorbance. The details of the chromatographic procedure have been reported previously (Mikesell and Boyd, 1986).

RESULTS AND DISCUSSION

The addition of anaerobic sludge (5 g Kg^{-1}) to anaerobic soil resulted in significant enhancement of PCP degradation, reducing the concentration from the initial 70 umol Kg^{-1} to below the detection limit (less than 0.5 umol Kg^{-1}) in 28 days (Fig.1). By comparison, anaerobic incubation of the soil without sludge addition resulted in a loss of 45% of the PCP in 56 days. In soil incubated aerobically with or without sludge addition the decrease in PCP concentration was 10% at the end of the 56 day incubation. Autoclaved sludge-soil mixtures showed no decrease in PCP concentration. Alternative carbon sources were also added to PCP-containing soil (no sludge addition) and in a 28 day incubation the results with glucose and ground soybean residues (data not shown) were not significantly different from the result in unamended anaerobic soil.

The results of this initial experiment indicated that the reductive dechlorinating activity present in sludge (Mikesell and Boyd, 1985, 1986) was successfully transferred to soil with the addition of sludge . The slow and partial loss of PCP in the unamended anaerobic soil indicates that the indigenous microflora have some capability to degrade PCP. PCP degradation in anaerobic soil has been reported previously (Ide, et al., 1972; Kuwatsuka

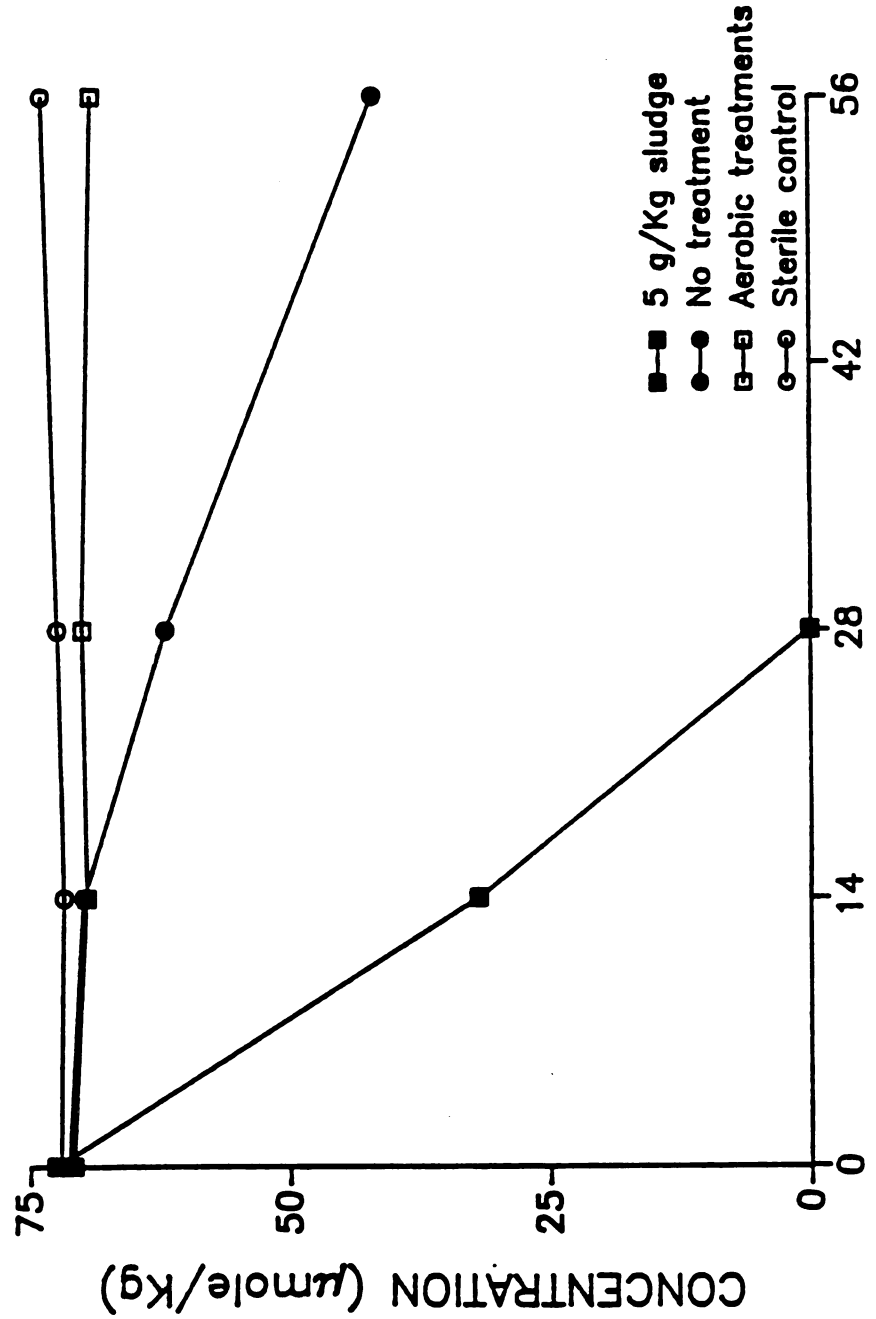

Figure 1. Disappearance of PCP in Spinks soil under a variety of incubation condition. Closed symbols: anaerobic incubation. Open squares: aerobic incubation (average of all treatments). Open circles: sterile, anaerobic incubation.

and Igarashi, 1975; Murthy et al., 1979). The absence of stimulated degradation with the addition of alternative organic amendments demonstrates that the great bulk of the dechlorinating activity originates in the sludge; no abiotic losses of PCP were detected.

In an attempt to more closely mimic actual field conditions prior to treatment using the proposed bioaugmentation process, we incubated the soil aerobically for 28 days before splitting the experiment, imposing anaerobiosis and adding sludge to a portion of the flasks. After a second 28 day period the anaerobic portion of the experiment was split again, with half of the flasks being returned to the aerobic state. The results of the complete 84 day experiment are summarized in Figure 2. During the continuous aerobic incubation (84 days) the initial PCP concentration decreased by only 20%. In contrast, within 32 days of the onset of anaerobiosis and addition of sludge (at 28 days, closed arrow in Fig. 2) the PCP was completely degraded and lower chlorophenols accumulated in nearly stoichiometric amounts. In the third 28 day period (open arrow in Fig. 2) the concentration of the dechlorination products began to decrease under anaerobic conditions while remaining roughly constant in aerobic flasks. At the end of the anaerobic incubation 63% of the initial PCP concentration was detected as lower chlorinated phenols, principally tri-, di-, and monochlorophenols. These results clearly show the importance of maintaining anaerobic conditions for the degradation of PCP as well as the dechlorination products.

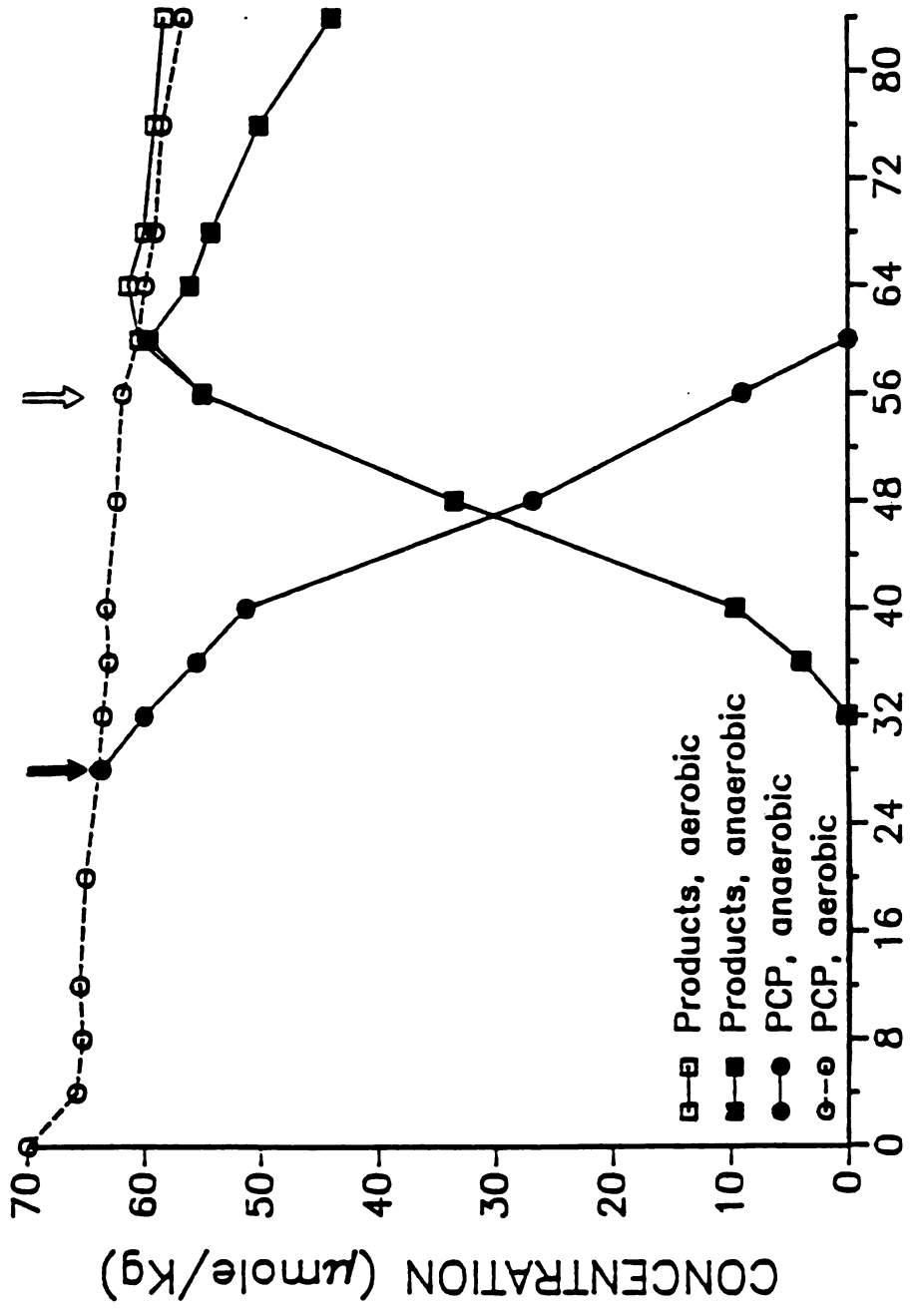

Figure 2. PCP degradation and varying aeration status in soil and soil-sludge mixtures (5 g Kg^{-1}).

To fully demonstrate the necessary role of biologically active anaerobic sludge, we conducted 28 day incubations with combinations of active and sterile soil and sludge (Fig. 3). When autoclaved sludge was added to either active or autoclaved soil the PCP concentration remained high and no dechlorination products were detected. The slightly lower PCP concentration in the active soil/sterile sludge mixture may indicate a stimulation of the indigenous soil organisms by the addition of the sludge but the products of this metabolism are unknown, and in any event the small decrease was not significant. When active sludge was added to active or sterilized soil the PCP degradation was substantial and the typical series of dechlorination products was detected.

The dechlorination process was more extensive when the sludge was added to sterile soil: 3,4,5-trichlorophenol (TCP) was the main product, accounting for 64% of the total chlorophenols present, and some dichlorophenol (DCP) was found. When sludge was added to active soil 2,3,4,5-tetrachlorophenol was the major product (61% of the total CPs) and comparatively little 3,4,5-TCP was detected. The comparison of active and sterile soil indicate that the indigenous microbes in the soil are competing with the added sludge organisms. Another possibility is that the autoclaving of the soil released nutrients which subsequently stimulated the activity of the sludge population. Most importantly, this experiment shows that active sludge is required for PCP degradation, thus substantiating the results shown in Figure 1.

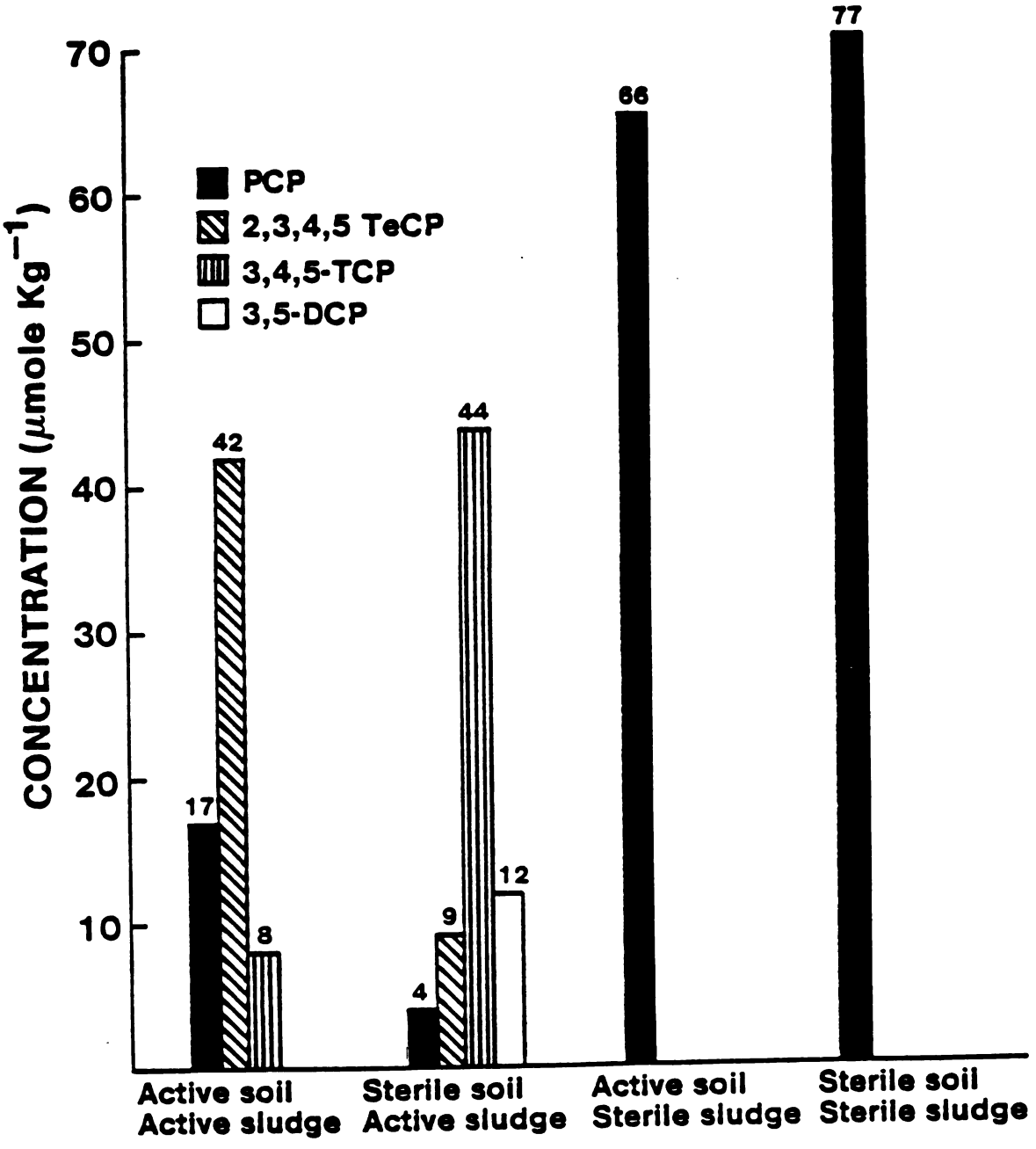

Figure 3. PCP degradation in combinations of active and sterile soil-sludge mixtures.

In addition, identical degradation products are observed in both sludge (Mikesell and Boyd, 1985, 1986) and in sludge-amended soil, confirming that the dechlorinating activity present in the sludge was expressed in the sludge-amended soil. Taken together, these results strongly suggest that sludge application to soil may be an effective and practical means of bioaugmentation for the purpose of reclaiming PCP-contaminated soils.

We next attempted to define the influence of sludge application rate and initial PCP concentration on the rate of PCP degradation. These results are summarized in Figure 4, where the data are presented as percentages of the PCP originally present. At the higher sludge application rate of 25 g Kg^{-1} (Fig.4c) the rate of PCP degradation and product accumulation was somewhat greater than at the 10g Kg^{-1} rate (Fig. 4a). At the higher sludge rate the product level had begun to decrease at the 40 day sampling after reaching its maximum at 28 days. The accumulation of products had begun to level off at the final sampling of the lower rate flasks, but longer incubation would have been required to observe a decrease in product concentrations. At the higher initial PCP concentration of 30 ppm (Fig. 4b), the initial rate of degradation was slightly greater than at 10 ppm, although an additional week was required for complete PCP disappearance. Clearly the sludge bioaugmentation was effective up to a PCP concentration of 30 ppm.

The disappearance of PCP and sequential appearance of individual dechlorination products is illustrated in Figures 5 and

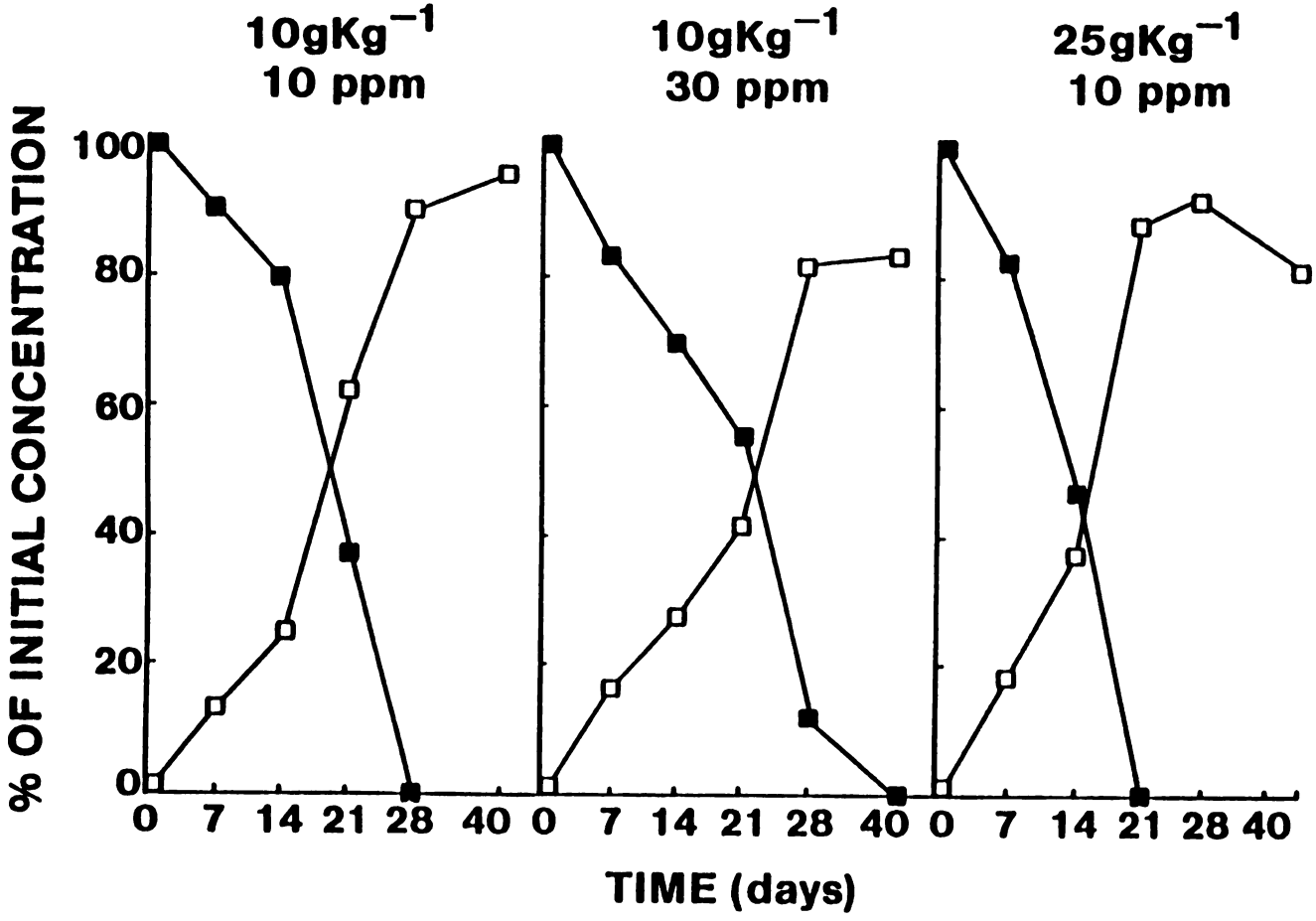

Figure 4. PCP degradation and product accumulation at two PCP concentrations (10 and 30 mg Kg^{-1}) and two sludge application rates (10 and 25 g Kg^{-1}). Closed circles: PCP. Open circles: degradation products.

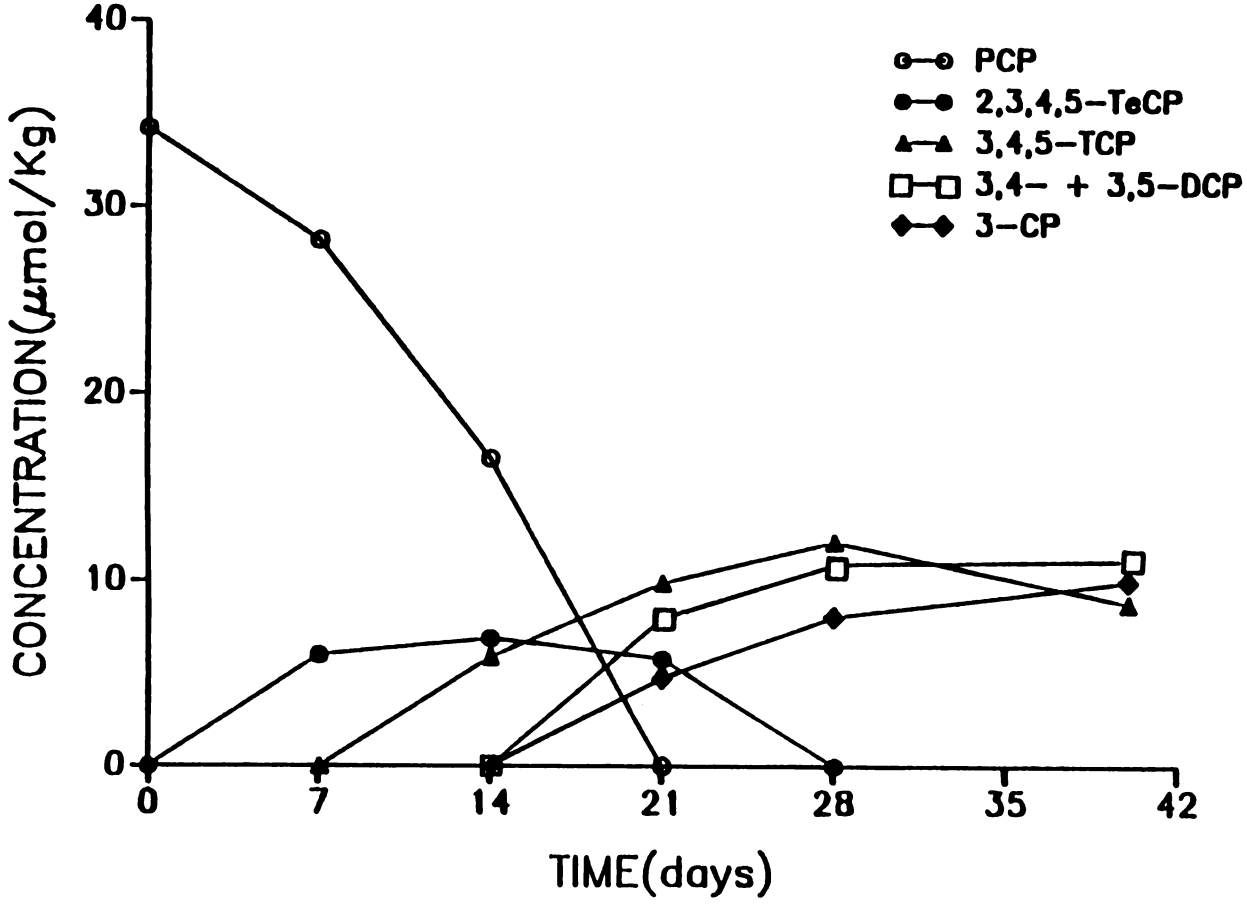

Figure 5. PCP disappearance and sequential product appearance in 25 g Kg^{-1} treatment.

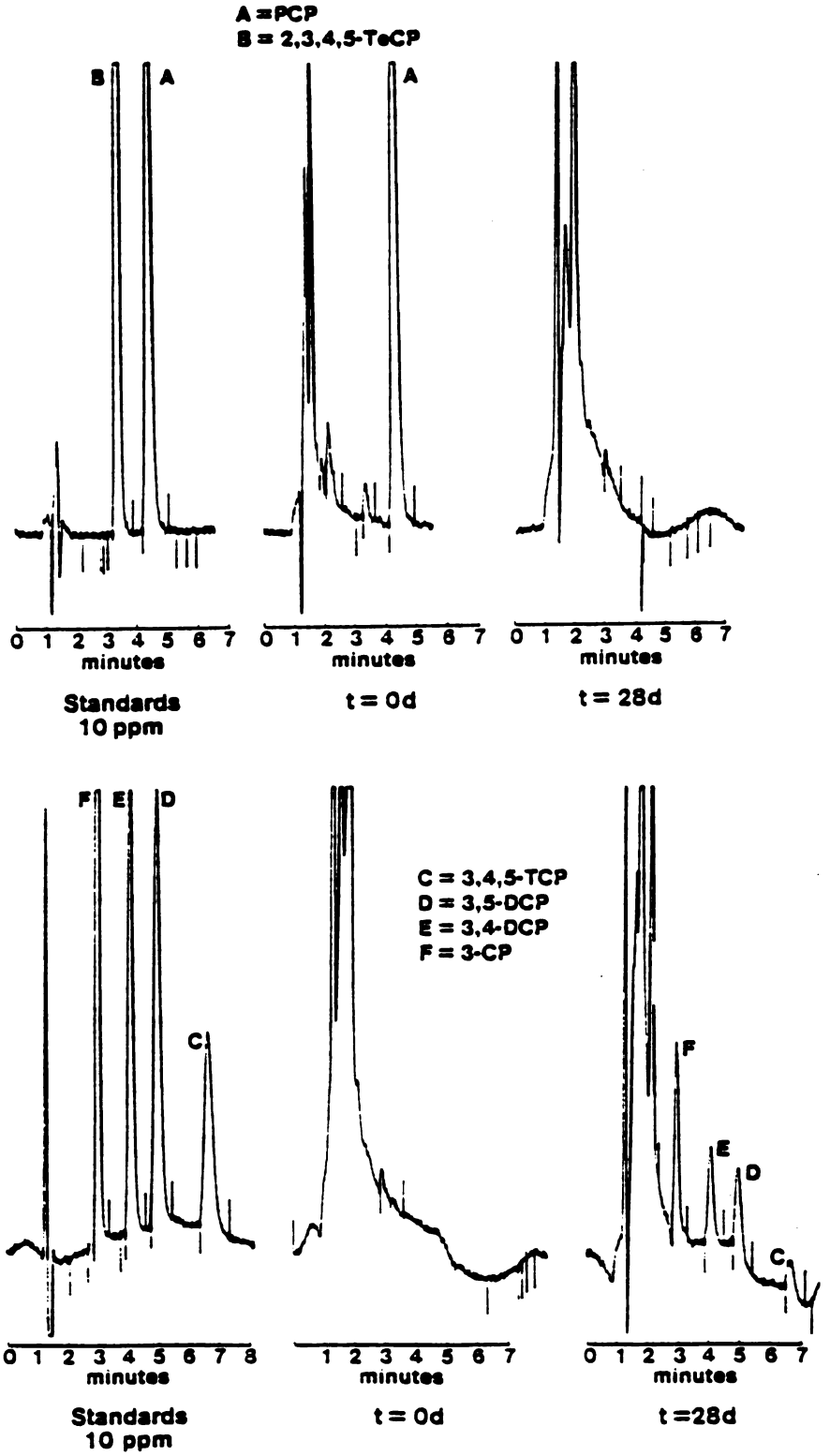

Figure 6. HPLC chromatograms for PCP and lower chlorophenols; t=0 and 28 day chromatograms are for the 25 g Kg^{-1} treatment.

6. At the end of the incubation approximately 80% of the PCP could be recovered as tri-, di-, and monochlorophenols. The pathway of dechlorination is the same as that we reported earlier (Mikesell and Boyd, 1986) for the dechlorination of PCP in chlorophenol-acclimated anaerobic sludge. The difference is that the present pathway includes 2,3,4,5-TeCP which was not observed in our previous studies. Figures 6a and 6b are HPLC chromatograms which show the disappearance of PCP and the appearance of dechlorination products in the 28 day incubation of the 25 g Kg^{-1} treatment.

We have demonstrated that the addition of anaerobic sewage sludge to soil containing 10 to 30 ppm of PCP resulted in the removal of PCP within approximately one month. Our next objective involved the use of the partially extracted BioTrol soil which contained nearly 30 mg Kg^{-1} PCP. Figure 7 summarizes the results of our initial experiments.

Sludge was added at three rates: 5 g-Kg^{-1}, 10 g $Kg^{-1}$with a second addition at 40 days, and 25 g Kg^{-1}. The rate of removal of PCP and accumulation of dechlorination products was slower than that observed in the experiments with spiked soil. The effect of sludge application rate was not dramatic and was not very apparent until the last 20 days of the 90 day incubation. The extent of degradation was 45%, 77%, and 71%, respectively for the three application rates. Lower chlorinated phenols accounted for 30%, 38%, and 35% of the PCP initially present in the three treatments. It thus appears that in these incubations both PCP and the

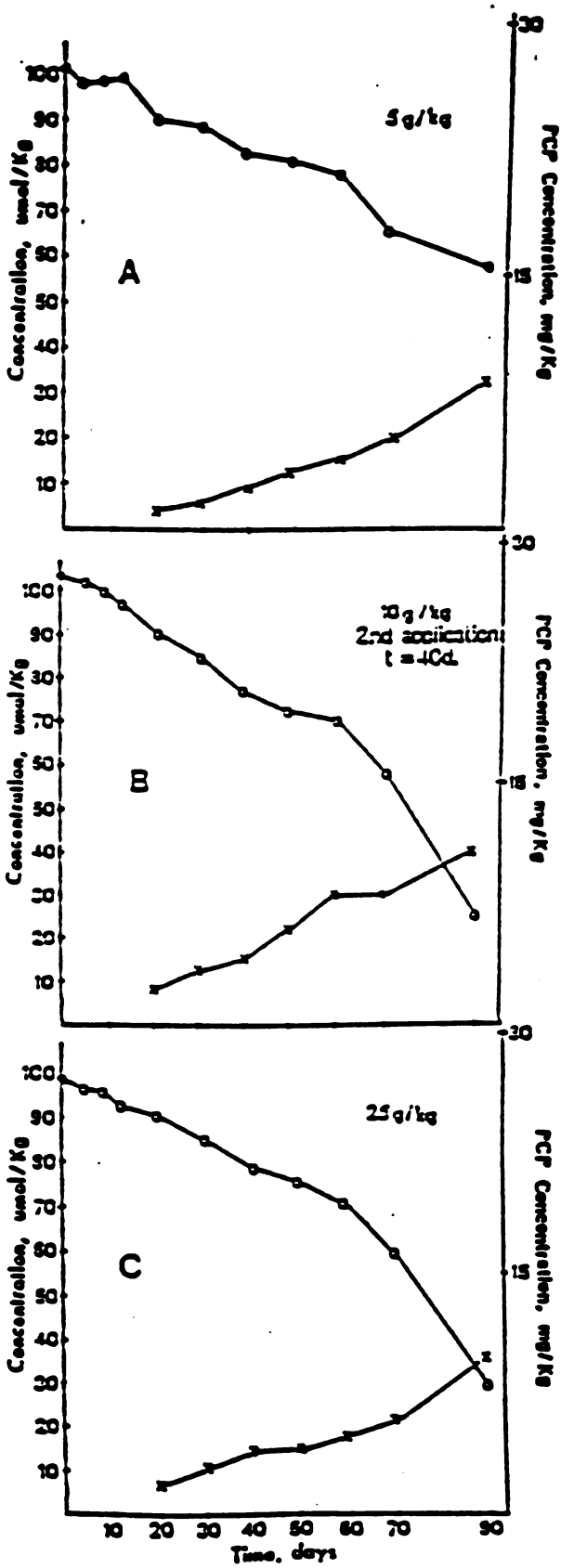

Figure 7. PCP degradation in contaminated soil provided by BioTrol, Inc., with three rates of sludge addition.

Table 1. Degradation of PCP at three initial concentrations in sludge-amended BioTrol soil (Jackson sludge, 10 g Kg^{-1}).

	Initial PCP Concentration					
	30 mg/kg		45 mg/kg		60 mg/kg	
Days	Concen.	% Remaining	Concen.	% Remaining	Concen.	% Remaining
0	27.5	-	43.1	-	56.1	-
10	24.9	90.6	37.2	86.3	47.9	85.2
20	21.9	79.6	35.5	82.4	47.0	83.8
30	20.3	73.8	32.0	74.3	44.1	78.7
50	20.7	75.3	30.3	70.3	37.2	66.3

products of its dechlorination are being significantly degraded. In each treatment the rate of PCP degradation was highest between 60 and 90 days. Incubation for an additional period (e.g. 30 days) would likely have resulted in further significant reduction in the concentration of PCP, and another addition of sludge may have enhanced the degradation rate.

In another experiment PCP was added to the contaminated soil to give final concentrations of 45 and 60 mg Kg^{-1} in addition to the original 30 mg Kg^{-1} soil. Table 2 summarizes the results of a 50 day incubation of these soils amended with 10 g Kg^{-1} Jackson sludge. Rather than observing an inhibition of PCP degradation activity at higher PCP concentrations, these data indicate a more rapid loss of PCP in the soils with the higher PCP levels. This result probably reflects a difference in the bioavailability of the freshly added chemical compared with that present as a result of the original contamination. In any case, this experiment shows that the PCP dechlorinating activity is not inhibited at PCP concentrations as high as 60 mg Kg^{-1}.

These data clearly show that induced anaerobiosis along with bioaugmentation with anaerobic sewage sludge having dechlorinating activity is a potentially useful stategy for the remediation of chlorophenol-contaminated soils. The amount of sludge required to achieve decontamination is comparable to that which is routinely used as a soil amendment for agricultural purposes, and anaerobiosis can be imposed over limited areas of soils by flooding with water. Thus this potential environmental technology

for remediating PCP-contaminated soils appears to have the essential elements for actual field practice.

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