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Sandra L. Woods

Knowledge of a compound's degradation pathway is crucial to the assessment of risk at contaminated sites. Pentachlorophenol and its metabolic products can serve as model compounds to study the fate and transport of halogenated aromatic compounds in the environment. Acclimation of anaerobic sewage sludge to pentachlorophenol produced a microbial consortium with the ability to remove chlorines from all three positions on the chlorophenol molecule. A series of split degradation pathways were observed in batch experiments of the reductive dechlorination of pentachlorophenol and its metabolites. Pentachlorophenol was rapidly degraded to 2,3,4,5-tetrachlorophenol, 2,3,4,6-tetrachlorophenol, and 2,3,5,6-tetrachlorophenol. Experiments conducted with each metabolite revealed the complete pathways for the degradation of pentachlorophenol. Dechlorination of 2,3,4,5-tetrachlorophenol produced 3,4,5-trichlorophenol which was subsequently degraded to both 3,4-dichlorophenol and 3,5-dichlorophenol. 2,3,4,6-tetrachlorophenol was dechlorinated at both ortho and meta positions to produce 2,4,6-trichlorophenol and 2,4,5-trichlorophenol. 2,3,5,6-tetrachlorophenol was degraded to 2,3,5-trichlorophenol. Reductive dechlorination of 2,3,5-trichlorophenol produced 3,5-dichlorophenol. 2,4,6-trichlorophenol was degraded to 2,4-dichlorophenol and 2,4,5-trichlorophenol was dechlorinated at two positions to form 2,4-dichlorophenol and 3,4-dichlorophenol. Of the three

dichlorophenols produced, only 2,4-dichlorophenol was degraded significantly within the time of the study (3 weeks), producing 4-chlorophenol.

The sequence of metabolic products produced by pentachlorophenol-acclimated anaerobic sewage sludge during this study is identical to that observed during a previous study of reductive dechlorination of pentachlorophenol by a mixture of three sludges acclimated to 2-chlorophenol, 3-chlorophenol, and 4-chlorophenol respectively. These results suggest that separate groups of organisms or enzyme systems are responsible for the removal of ortho, meta, and para chlorines.

MEASUREMENT OF THE RATES OF REDUCTIVE DECHLORINATION OF CHLORINATED PHENOLS

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PREFACE

This thesis is a compilation of an introduction and a manuscript submitted for publication concerning the pathways of anaerobic biodegradation of pentachlorophenol.

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April 20, 1990

MEASUREMENT OF THE RATES OF REDUCTIVE DECHLORINATION OF CHLORINATED PHENOLS

1.0 INTRODUCTION

Chlorinated phenols are manufactured in the United States and Canada, among other places, and have been used for a variety of purposes. Pentachlorophenol (PCP) has been widely used as a wood preservative. Because of its strong ability to decouple oxidative phosphorylation (Weinbach, 1957), PCP has also been used as a fungicide/bactericide in a variety of materials, including paints, adhesives, textile and paper products, leather, and cooling tower water (Guthrie et al., 1984), and as herbicides and molluscicides (Crosby, 1981). Chlorinated phenols are also used as precursor chemicals in the production of pesticide formulations, especially 2,4-D and 2,4,5-T. Like other halogenated aromatic compounds, chlorinated phenols are persistent in the environment and accumulate in sediments and organisms. As a group, they are highly toxic and some, such as pentachlorophenol and 2,4,6-trichlorophenol, are known or suspected animal carcinogens (Boyd and Shelton, 1984; Mikesell and Boyd, 1986; and references cited within).

Reports of environmental contamination by PCP have been extensively documented in the literature. Until recently, PCP was widely used for the treatment of lumber. This usage accounted for about 80% of 1979 U.S. production of PCP (Crosby, 1981). Contamination of groundwater and surface waters by PCP and other chlorophenols has been reported at wood-treatment plants and sawmills in Oregon (Johnson et al., 1985), Minnesota (Ehrlich et al., 1982), Florida (Goerlitz et al., 1985), and Finland (Valo et al., 1984; Valo et al., 1985). An estimated 500 sites in the U.S. are contaminated with PCP as a result of wood-treating activities (Cirelli, 1978). Additionally, contaminants present in technical-grade PCP used as a wood preservative have also been identified in the environment. Samples of soil, groundwater, and organisms near one lumber mill

in Finland that used a technical-grade PCP formulation contained polychlorinated catechols, guaiacols, anisoles, furans, and dioxins (Valo et al., 1984; Valo et al., 1985; Humppi, 1985; Paasivirta et al., 1985).

PCP has also been used for many years as a herbicide, notably on rice paddy fields in Japan where it accounted for 25% of herbicide usage from the early 1950's to 1971 (Kuwatsuka and Igarashi, 1975). Its use for this application was restricted in 1971 following some large fish kills.

Other important sources of environmental contamination by chlorophenols include: degradation of several pesticides by soil microbes, including 2,4-D and 2,4,5-T (Mikesell and Boyd, 1985), lindane, pentachloronitrobenzene, and hexachlorobenzene (Ahlborg and Thunberg, 1980); the effluents from pulp mills, which contain chlorophenols as a byproduct of the bleaching process (Lander et al., 1977); and chlorination of municipal water and wastewater (Paasivirta et al., 1985). Additionally, many congeners of chlorophenol are contaminated with dioxins during the manufacturing process which may subsequently be released into the environment. The combustion of wood products treated with chlorophenols or municipal sewage sludges containing them may also lead to the formation of polychlorinated dioxins and furans (Boyd and Shelton, 1984).

Anaerobic biodegradation of chlorophenols is accomplished by the replacement of chlorines by hydrogen in a process called reductive dechlorination. The process is of considerable importance because lesser chlorinated phenols are generally less toxic and degraded more easily by aerobic organisms than the more highly chlorinated congeners. An acclimation period has generally been found to be necessary before degradation occurs. Initially, for unacclimated cultures, chlorines in the position ortho to the hydroxyl group are removed (Boyd et al., 1983; Boyd and Shelton, 1984; Mikesell and Boyd, 1985; Woods et al., 1989; Cozza and Woods, 19xx). With further acclimation,

meta and para chlorines can also be removed, eventually leading to the formation of phenol. Complete mineralization of chlorinated phenols to carbon dioxide and water has also been observed in anaerobic environments (Mikesell and Boyd, 1986).

Differences in the biodegradation potential for several sewage sludges (Mikesell and Boyd, 1985), and between sewage sludges, sediments, and aquifer material (Gibson and Suflita, 1986), suggest that separate groups of microorganisms are responsible for the removal of chlorines from different sites on the aromatic ring. Boyd and Shelton (1984) observed that sludges acclimated to 2-chlorophenol could degrade 4-chlorophenol and 2,4-dichlorophenol but not 3-chlorophenol, and those acclimated to 3-chlorophenol were incapable of degrading 2-chlorophenol. Additionally, sludges acclimated to individual monochlorophenols produced differing initial degradation products (Mikesell and Boyd, 1986).

The kinetics of anaerobic biodegredation of phenol has been reported by Neufeld et al. (1980) and Dwyer et al. (1986). Qualitative descriptions of the kinetic rates of reductive dechlorination for different chlorophenols have also been given (Boyd, et al., 1983; Boyd and Shelton, 1984; Mikesell and Boyd, 1985; Woods et al., 1989), however, these studies did not measure the rate constants directly.

The objectives of this thesis are to 1) develop a conceptual and mathematical model of sequential reductive dechlorination reactions of pentachlorophenol, 2) determine the anaerobic degradation pathways of pentachlorophenol utilized by a mixed culture of anaerobic bacteria, and 3) measure the relative rates of biotransformation of pentachlorophenol and its metabolic products.

The results of this thesis have potential applications for the formulation of discharge limits, the design of wastewater treatment facilities, and the understanding of the fate and transport of halogenated compounds in groundwater. Determination of the kinetic constants for the biodegradation of PCP will provide a better understanding of the rate at

which certain populations of anaerobic bacteria dechlorinate aromatic compounds. PCP has been shown to inhibit methanogenesis in concentrations in excess of 200 ug/L in unacclimated primary digester sludge (Roberton and Wolfe, 1970; Guthrie et al., 1984). Failure of anaerobic digesters may therefore occur in areas where pentachlorophenol is intermittently discharged in high amounts to the sewer system. Accordingly, the kinetics of anaerobic biodegradation of PCP has potential importance for the design of better wastewater treatment facilities, the formulation of discharge limits of pentachlorophenol to municpal sewage systems by industry, and the land disposal of digester sludges.

Hazardous compounds are often transformed into more mobile forms as they degrade, and therefore a knowledge of the degradation pathway is crucial to the assessment of risk at contaminated sites. Transport of halogenated organic compounds through groundwater is dependent on the structure of the molecule, degree of sorption onto organic particles, solubility, and other factors. Chlorophenols can serve as model compounds to study the fate and transport of halogenated aromatic compounds in the environment.

1.1 PREVIOUS INVESTIGATIONS

The earliest information on the degradation of chlorinated phenols is from studies of the toxicity of PCP in soils after its application as a herbicide (Alban and McCombs, 1949; Taylor, 1950; Loustalot and Ferrer, 1950; Young and Carroll, 1951), in which the toxicity of PCP was found to decrease with time. Young and Carroll (1951) also observed that PCP was removed more quickly from soils with a higher organic content.

Studies of the distribution and fate of PCP in Japanese rice paddy fields has been a source of early information on the degradation of PCP and other chlorinated phenols

(Ide et al., 1972; Kuwatsuka and Igarashi, 1975; Watanabe, 1977; Watanabe, 1978; Sato, 1983). These authors reported that PCP was degraded more rapidly in established fields than new fields. Ide et al. (1972) were the first to identify the degradation products of PCP as tetra-and tri-chlorophenols and pentachloroanisole, and attributed the degradation to a microbially-mediated reductive dechlorination process. They also reported that chlorines in positions ortho to the hydroxyl group were more easily removed that those in the meta and para positions, a phenomena that has been verified by later studies (Boyd and Shelton, 1984; Mikesell and Boyd, 1985; Mikesell and Boyd, 1986; Woods et al., 1989). Kaufman (1978), in a review paper of earlier PCP degradation studies, recognized that the more rapid degradation in established rice paddy fields was the result of the development of an acclimated microbial population of reductive dechlorinators after repeated applications of PCP.

Biodegradation of PCP by aerobic organisms has been extensively documented in soils (Ide et al., 1972; Kuwatsuka and Igarashi, 1975; Watanabe, 1977; Murthy et al., 1979; Edgehill and Finn, 1983; Sato, 1983; and others). Several strains of bacteria that aerobically degrade pentachlorophenol have been isolated, including *Flavobacterium sp.* (Saber and Crawford, 1985), *Arthobacter sp.* (Stanlake and Finn, 1982; Edgehill and Finn, 1983), and *Psuedomonos sp.* (Suzuki and Nose, 1971; Watanabe, 1973; Kamisako, 1975; Suzuki, 1975; Suzuki, 1977).

Biodegradation of chlorinated phenols by anaerobic microorganisms has been studied in anaerobic sewage sludges (Boyd et al., 1983; Boyd and Shelton, 1984; Guthrie et al., 1984; Mikesell and Boyd, 1985; Mikesell and Boyd, 1986; Boyd et al., 1989), sediments (Steep et al., 1985; Gibson and Suflita, 1986), soils (Murthy et at., 1979; Boyd et al., 1989), soils amended with sewage sludge (Mikesell and Boyd, 1988), aquifers (Ehrlich et al., 1982; Suflita and Miller, 1985; Suflita, et al., 1988), and bacterial cultures (Suflita et al., 1982; Woods, et al., 1989). The metabolic

products of the anaerobic degradation of PCP are a series of lesser chlorinated phenols that are created in a sequential manner. The sequence of degradation products observed varies with the characteristics of the microbial consortia present. However, in contrast to the aerobic degradation of PCP, individual species of anaerobic bacteria that degrade PCP or other chlorophenols have not been isolated.

2.0 PATHWAYS OF THE REDUCTIVE DECHLORINATION OF PENTACHLOROPHENOL

2.1 ABSTRACT

Knowledge of a compound's degradation pathway is crucial to the assessment of risk at contaminated sites. Although the reductive dechlorination of chlorinated phenols has been demonstrated, the complete degradation pathways for pentachlorophenol have not been previously described. Pentachlorophenol and its metabolic products can serve as model compounds to study the fate and transport of halogenated aromatic compounds in the environment.

The objectives of this research were to 1) characterize the changes in dechlorination potential that occur as the result of acclimation of anaerobic sewage sludge to PCP, and 2) describe the complete degradation pathways for pentachlorophenol in the PCP-acclimated sludge. Our approach was to utilize a continuous-flow reactor to serve as a reservoir of organisms for individual batch experiments conducted in smaller reactors.

Acclimation of anaerobic sewage sludge to pentachlorophenol produced a microbial consortia with the ability to remove chlorines from all three positions on the chlorophenol molecule. A series of split degradation pathways were observed in batch experiments of the degradation of pentachlorophenol and its metabolites. Pentachlorophenol was rapidly degraded to 2,3,4,5-tetrachlorophenol, 2,3,4,6-tetrachlorophenol, and 2,3,5,6-tetrachlorophenol. Experiments conducted with each metabolite revealed the complete pathways for the degradation of pentachlorophenol. Dechlorination of 2,3,4,5-tetrachlorophenol produced 3,4,5-trichlorophenol which was subsequently degraded to both 3,4-dichlorophenol and 3,5-dichlorophenol. 2,3,4,6-tetrachlorophenol was dechlorinated at both ortho and meta positions to produce 2,4,6-

trichlorophenol and 2,4,5-trichlorophenol. 2,3,5,6-tetrachlorophenol was degraded to 2,3,5-trichlorophenol. Reductive dechlorination of 2,3,5-trichlorophenol produced 3,5-dichlorophenol. 2,4,6-trichlorophenol was degraded to 2,4-dichlorophenol, and 2,4,5-trichlorophenol was dechlorinated at two positions to form 2,4-dichlorophenol and 3,4-dichlorophenol. Of the three dichlorophenols produced (2,4-dichlorophenol, 3,4-dichlorophenol, and 3,5-dichlorophenol), only 2,4-dichlorophenol was degraded significantly within the time of the study (3 weeks) to produce 4-chlorophenol.

The sequence of metabolic products produced by pentachlorophenol-acclimated anaerobic sewage sludge during this study is identical to that observed during a previous study of reductive dechlorination of pentachlorophenol by a mixture of three sludges acclimated to 2-chlorophenol, 3-chlorophenol, and 4-chlorophenol respectively. These results suggest that separate groups of organisms or enzyme systems are responsible for the removal of ortho, meta, and para chlorines.

2.2 INTRODUCTION

Hazardous compounds are often transformed into more mobile or toxic forms as they degrade, and therefore a knowledge of degradation pathways is crucial to the assessment of risk at contaminated sites. Like other halogenated aromatic compounds, pentachlorophenol (PCP) is persistent in the environment and accumulates in sediments and organisms. PCP and its metabolic products can therefore serve as model compounds to study the fate and transport of halogenated aromatic compounds in the environment.

PCP has been used as a wood preservative, as a fungicide/bactericide in a variety of materials, including paints, adhesives, textile and paper products, leather, and cooling tower water (Guthrie et al., 1984), and as herbicides and molluscicides (Crosby, 1981). The treatment of lumber accounted for about 80% of the 1979 U.S. use of PCP (Crosby, 1981). PCP is highly toxic to a wide variety of organisms (Crosby, 1981).

Environmental contamination by PCP is common. Contamination of groundwater and surface waters by PCP, other chlorophenols, and contaminants of technical-grade PCP formulations has been reported at wood-treatment plants and sawmills in Oregon (Johnson et al., 1985), Minnesota (Ehrlich et al., 1982), Florida (Goerlitz et al., 1985), and Finland (Valo et al., 1984; Valo et al., 1985). An estimated 500 sites in the U.S. are contaminated with PCP as a result of wood-treating activities (Cirelli, 1978).

Anaerobic biodegradation of PCP is accomplished by the replacement of chlorines by hydrogen in a process called reductive dechlorination. The process is of considerable importance because lesser chlorinated phenols are generally less toxic and degraded more easily by aerobic bacteria than the more highly chlorinated congeners. Biodegradation of chlorophenols by anaerobic microorganisms has been previously

studied in anaerobic sewage sludges, sediments, soils amended with sewage sludge, aquifers, and bacterial cultures (Table 2.1).

The degradation products of the reductive dechlorination of PCP are a series of lesser chlorinated phenols that are created in a sequential manner. Metabolic products of the various chlorophenols that have been observed to date are reported in Table 2.1. Metabolites created vary with the characteristics of the microbial consortia present. Initially, for unacclimated cultures, chlorines in the position ortho to the hydroxyl group are removed to form 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP) and 3,4,5-trichlorophenol (3,4,5-TCP) (Boyd et al., 1983; Boyd and Shelton, 1984; Mikesell and Boyd, 1985; Woods et al., 1989). With acclimation, meta and para chlorines can also be removed (Boyd and Shelton, 1984; Mikesell and Boyd, 1985; Mikesell and Boyd, 1986; Woods et al., 1989). Complete mineralization of monochlorophenols and phenol to carbon dioxide and water has also been observed in anaerobic environments (Boyd et al., 1983; Boyd and Shelton, 1984; Mikesell and Boyd, 1986).

In contrast to the aerobic degradation of PCP, individual species of anaerobic bacteria that degrade PCP or other chlorophenols have not been isolated, suggesting that a consortium of microorganisms is responsible. Additionally, variations in the biodegradation potential of several anaerobic sewage sludges and between sewage sludges, sediments, and aquifer material suggest that separate groups of microorganisms are responsible for the removal of chlorines from different sites on the aromatic ring. Boyd and Shelton (1984) observed that sludges acclimated to 2-chlorophenol (2-CP) could degrade 4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP) but not 3-chlorophenol (3-CP), and those acclimated to 3-CP were incapable of degrading 2-CP. Additionally, sludges acclimated to individual monochlorophenols produced differing initial degradation products (Mikesell and Boyd, 1986). Sludge acclimated to 2-CP produced 2,3,4,5-TeCP as the initial degradation

Table 2.1 Summary of pathways of the reductive dechlorination of pentachlorophenol

Parent Compound	Metabolite	Environment	Reference
PCP	2,3,4,5-TeCP	soil	Ide et al., 1972
		soil	Kuwatsuka and Igarashi, 1975
		soil	Murthy et al., 1979
		sewage sludge	Woods et al., 1989
	2,3,4,6-TeCP	soil	Ide et al., 1972
		soil	Kuwatsuka and Igarashi, 1975
		sewage sludge	Mikesell and Boyd, 1986
	2,3,5,6-TeCP	soil	Ide et al., 1972
		soil	Kuwatsuka and Igarashi, 1975
		sewage sludge	Mikesell and Boyd, 1986
2,3,4,5-TeCP	3,4,5-TCP	sewage sludge	Woods et al., 1989
2,3,4,6-TeCP	2,4,6-TCP	sewage sludge	Mikesell and Boyd, 1986
2,3,5,6-TeCP	2,3,6-TCP	soil	Murthy et al., 1979
	2,3,5-TCP	sewage sludge	Mikesell and Boyd, 1986
2,4,5-TCP	3,4-DCP	sewage sludge	Mikesell and Boyd, 1985
2,4,6-TCP	2,4-DCP	sewage sludge	Mikesell and Boyd, 1985
		sewage sludge	Woods et al., 1989
3,4,5-TCP	3,5-DCP	sewage sludge	Mikesell and Boyd, 1985
		sewage sludge	Mikesell and Boyd, 1986
	3,4-DCP	sewage sludge	Woods et al., 1989
		amended soil	Mikesell and Boyd, 1988
2,3-DCP	3-CP	sewage sludge	Boyd and Shelton, 1984
		sewage sludge	Woods et al., 1989
2,4-DCP	4-CP	sewage sludge	Boyd and Shelton, 1984
		sewage sludge	Mikesell and Boyd, 1985
		sewage sludge	Gibson and Suflita, 1986
		aquifer sediment	Suflita et al., 1988
		sewage sludge	Woods et al., 1989
2,5-DCP	3-CP	sewage sludge	Boyd and Shelton, 1984
		sewage sludge	Gibson and Suflita, 1986
		aquifer sediment	Suflita et al., 1988
2,6-DCP	2-CP	sewage sludge	Boyd and Shelton, 1984
		sewage sludge	Woods et al., 1989
3,4-DCP	4-CP	sewage sludge	Woods et al., 1989
3,5-DCP	3-CP	sewage sludge	Mikesell and Boyd, 1986
		sewage sludge	Woods et al., 1989

product of PCP, sludge acclimated to 3-CP produced 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP), and sludge acclimated to 4-CP gave 2,3,5,6-tetrachlorophenol (2,3,5,6-TeCP).

The objectives of this research were to 1) characterize the changes in the pathways and rates of dechlorination that occur as the result of acclimation of anaerobic sewage sludge to PCP, and 2) to completely describe the anaerobic degradation pathways for pentachlorophenol in PCP-acclimated sludge. Our approach was to utilize a continuous-flow reactor to serve as a reservoir of organisms for individual batch experiments conducted in smaller reactors. The "mother" reactor was operated at steady-state conditions with regards to retention time, substrate removal and gas production rates, and Eh/pH conditions to provide nearly identical initial conditions and species distribution for each batch experiment.

Complete degradation pathways for PCP have not been verified in a single set of experiments, and the reaction kinetics have only been described qualitatively. This research provides this information which is crucial to the assessment of the fate and transport of PCP at contaminated sites. This paper describes the effects of acclimation on the rates of degradation and the observed pathways for the reductive dechlorination of PCP. A companion paper (Woods et al., 19xx) reports the kinetics of biotransformation determined for these pathways.

2.3 METHODS AND MATERIALS

Anaerobically digested sewage sludge was collected from the primary anaerobic digester at the municipal wastewater treatment plant in Corvallis, Oregon on July 25, 1989. The digester is operated at a temperature of 35° C and has a solids retention time of about 30 days. Influent to the plant is dominantly domestic sewage. This sludge was transported to the laboratory in a 20-liter carboy and transferred to the "mother reactor" described below. The sludge was fed a solution that was formulated using a modification of the anaerobic media described in Owen et al. (1978), at a rate of one liter/day (Appendix A). Concentrations of inorganic species and vitamins (in mg/liter) in the feed solution were as follows: $CaCl_2 \cdot 2H_2O$, 139.2, NH_4Cl, 221.6, $MgCl_2 \cdot 6H_2O$, 1000.7, KCl, 722.6, $MnCl_2 \cdot 4H_2O$, 11.2, $CoCl_2 \cdot 6H_2O$, 16.7, H_3BO_3, 3.23, $CuCl_2 \cdot 2H_2O$, 1.51, $Na_2MoO_4 \cdot 2H_2O$, 1.44, $ZnCl_2$, 1.18, $NiCl_2 \cdot 6H_2O$, 8.42, $(NH_4)_2HPO_4$, 73.4, Biotin, 0.015, Folic acid, 0.015, Pyridoxine hydrochloride, 0.075, Riboflavin, 0.038, Thiamin, 0.038, Nicotinic acid, 0.038, Panthothenic acid, 0.038, B_{12}, 0.0009, p-aminobenzoic acid, 0.038, Thioctic acid, 0.038. 135 mg $Na_2S \cdot 9H_2O$ and 100 mg $FeCl_2 \cdot 4H_2O$ were also injected directly into the reactor each day. These compounds were not contained in the feed solution because they react to precipitate FeS.

Acetate was provided as the primary carbon source to give an influent chemical oxygen demand of 5600 mg/L. PCP was added to the feed solution at a concentration of 0.9 mg/L to acclimate the organisms to the conditions of the experiments. The sludge was acclimated for six weeks. Before experiments were conducted, the reactor was operated until steady-state conditions had been acheived to provide a microbial consortium with the same initial characteristics and species distribution for each experiment. Steady-state conditions were assumed to exist when the hydraulic

retention time, pH, and substrate removal and gas production rates were constant with time.

2,3,4,5-TeCP (98% purity) was obtained from Pfaltz and Bauer Co., Waterbury, CT. 2,3,5,6-TeCP (99%) and 3,4,5-TCP (98%) were obtained from Ultra Scientific Co., Hope, R.I. All other chlorophenols, 2,6-dibromophenol, and 2,4,6-dibromophenol were obtained from Aldrich Chemical Company, Inc., Milwaukie, WN, in purities of at least 98%. 2,3,4,6-TeCP was obtained through the Aldrich Chemical Co. rare chemical library. All chemicals were used without further purfication.

Reactor System

Experiments were conducted in the laboratory using a novel two-part reactor system, as depicted schematically in Figures 2.1 and 2.2. The experimental protocol is listed in Appendix B. A 9.5 liter airtight "mother reactor" (Figure 2.1) served as a reservoir of acclimated organisms. This reactor consisted of a cylinder of Kimax beaded-process glass with stainless steel and Teflon-lined flange fittings (Ace Glass Company, Vineland, N.J.). Brass valves on the top plate provided access for liquid sampling, feed influent, and gas effluent. These materials were chosen to minimize sorption of chlorophenols to the reactor surfaces. The sludge was kept in suspension by continuous stirring with a Teflon paddle driven by an electric motor. The reactor was housed in an environmental chamber maintained at 31° C. Feed solution was kept under nitrogen in a glass container at 4° C to minimize microbial growth and was delivered to the reactor through Teflon tubing by a FMI RP-G6 laboratory pump (Fluid Metering Inc., Oyster Bay, N. Y.). A constant flow rate of 0.6 ml/min was maintained to provide a hydraulic retention time of 10 days.

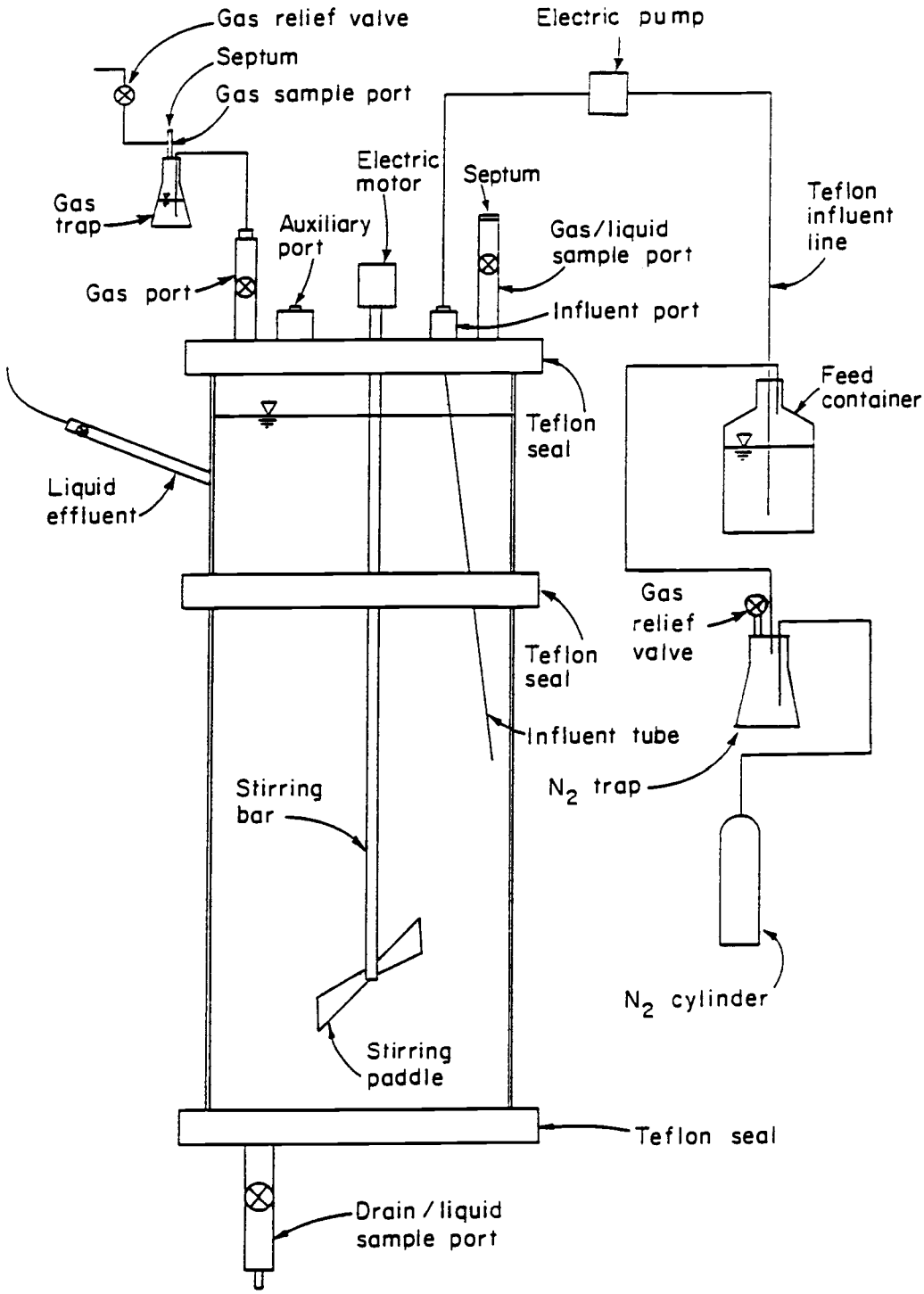

Figure 2.1. Schematic diagram of continuous-flow mother reactor.

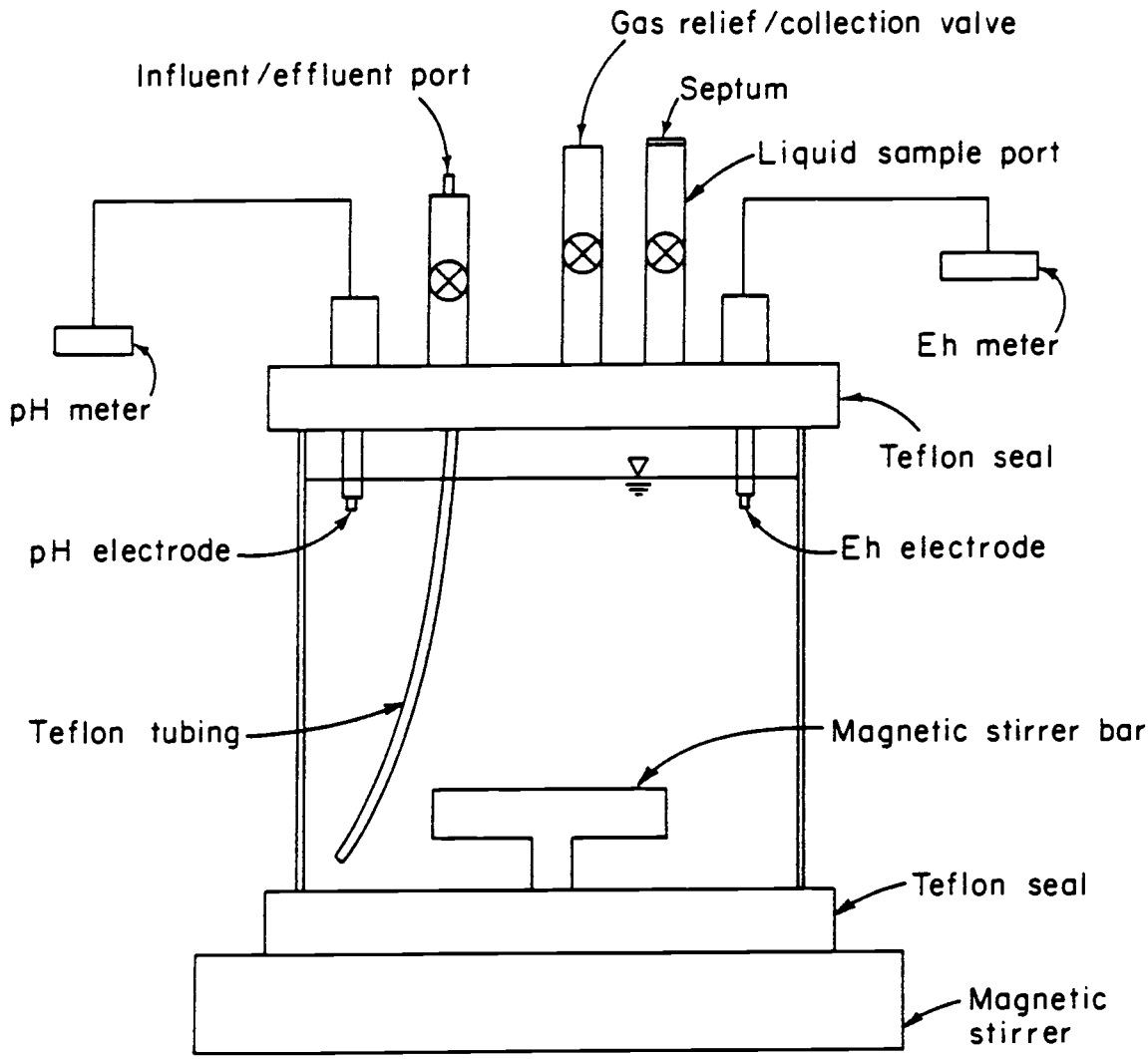

Figure 2.2. Schematic diagram of batch reactor.

Experiments were conducted in a 2.5-liter reactor of similar construction (Figure 2.2) that was operated in the batch mode. The batch reactor had a gas relief valve in addition to sampling and influent valves and was also stirred by a magnetic stir bar. The batch reactor also contained electrodes for measuring pH and Eh. Gas production was measured by a wet test gas meter connected to the gas relief valve. Transfer of the sludge from the mother reactor to the batch reactor was conducted under anaerobic conditions. The headspace of the batch reactor was purged with nitrogen until oxygen was not detectable by injection of 100 ml of headspace gas into a gas partitioner. Nitrogen was also flushed through the headspace of the mother reactor while the sludge was transferred to the batch reactor via the bottom drain of the mother reactor. For each experiment, the batch reactor was filled with sludge and then allowed to equilibrate until the Eh dropped below -330 millivolts to ensure conditions necessary for methanogenesis. A water solution containing the chlorophenol (s) of interest and sodium acetate was adjusted to pH 7.5 with concentrated HCl. The solution was then injected through the influent valve using a 50-ml glass syringe. The intitial acetate concentration was 1500 mg/L for all batch experiments.

Batch experiments for the degradation of dichlorophenols were conducted in 60-ml amber serum bottles. Chlorophenol solution was pipetted in and sludge added to fill the bottle. The entire bottle was filtered and 50 ml extracted for each sample taken. Hexane extracts were prepared in the same manner as the batch reactor samples.

Sampling Procedure

Liquid samples (20 ml) were taken immediately after injection of the chlorophenol/acetate solution and at 2-to 6-hour intervals during the course of the experiments using a 30-ml glass syringe. Samples were filtered using a Millipore filtration apparatus and Gelman type A/E glass fiber filters with a 1 micron pore size.

The first three ml of filtrate were discarded and the remaining sample split for separate analyses for concentrations of acetate and chlorophenols.

Analytical Procedures

10-ml aliquots were used for analysis of chlorophenols. These samples were acetylated and extracted into hexane using a modification of the method described by Voss et al. (1981) and NCASI (1981). The procedural protocol is listed in Appendix C. Each sample was added to 50 ml of glass-distilled water in a 150-ml separatory funnel using a 10-ml volumetric pipette. The internal standard (either 2,6-dibromophenol or 2,4,6-tribromophenol, depending on the identity of the metabolic products expected) was added using a 50 ul syringe. One ml of a 0.7 gram/ml solution of potassium carbonate and one ml of acetic anhydride were added and the funnel shaken for exactly two minutes. After 20 minutes (to ensure completion of the acetylation reaction), exactly 5 ml of hexane was added using a dedicated 5-ml volumetric pipette and the funnel again agitated for two minutes. After drawing off the water, the hexane layer was collected using a new pasteur pipette and stored in a 2-ml amber glass vial with Teflon-lined cap in the refrigerator until analysis by capillary gas chromatography.

Injections of hexane (1 ul) were made by autosampler into a Hewlett-Packard Model 5890A Gas Chromatograph (Appendix D) equipped with a ^{63}Ni electron capture detector (ECD) and a 30 m by 0.323 mm i.d. DB-5 fused-silica capillary column (J + W Scientific, Orangeville, CA). Helium (5 psi) was used as the carrier gas and 95% argon/5% methane was used as the ECD auxiliary gas. The detector was held at 320° C and the injector temperature was 250° C. The temperature program was as follows: an initial oven temperature of 45° C was held for 2 minutes, increased by 15° C/min to 105° C, and then by 5° C/min to a final temperature of 215° C that was held for 5

minutes. A special temperature program was necessary to separate 2,3,4,6-TeCP from 2,3,5,6-TeCP and 2,3,5-trichlorophenol (2,3,5-TCP) from 2,4,5-trichlorophenol (2,4,5-TCP). This program held an initial temperature of 45° C for 2 minutes, followed by temperature increases of 15° C/min to 105° C, 1° C/min to 150° C, and 5° C/min to a final temperature of 215° C that was held for 5 minutes. Peak areas were determined by a Hewlett-Packard Model 3392A Integrator. The identity of unknown metabolic products was determined by comparison of observed retention times to those of analytical standards (Appendix E).

Samples for acetate analysis were spiked with a few drops of 1 N sodium hydroxide solution, to minimize degradation of acetate during storage, and frozen in 8-ml amber glass vials. These samples were later analyzed using a Dionex Series 4000i Liquid Ion Chromatograph after filtration through Bond-Elute C-18 solid phase filter cartridges (Analytichem International, Harbor City, CA). The ion chromatograph was operated using a temperature compensation of 1.7, output range of 30 microsiemens, and flow rate of 2 ml/min. An eluant consisting of 0.191 g/L $NaCO_3$ and 0.143 g/L $NaHCO_3$ was used.

Total suspended solids and volatile suspended solids were determined for several samples during each experiment using procedures 2540 D and 2540 E, Standard Methods, 17th edition (1989). Solids determinations for each experiment are listed in Appendix F.

2.4 RESULTS AND DISCUSSION

An initial experiment (Figure 2.3) to evaluate the degradation of PCP and test the analytical procedures was performed on the unacclimated sludge during the first week of exposure to PCP. Degradation of PCP began after a lag period of five days and complete removal occurred in 10 days. 2,3,4,5-TeCP began to appear after five days and increased in concentration until day seven, then began to decline with a concomitant increase in the concentration of 3,4,5-TCP. By the tenth day, all PCP and 2,3,4,5-TeCP had been removed and a mass of 3,4,5-TCP equivalent to that of the initial PCP mass had accumulated. Therefore, PCP was sequentially dechlorinated at the ortho positions to form first 2,3,4,5-TeCP and then 3,4,5-TCP. Metabolites of 3,4,5-TCP were not detected during this experiment. An initial concentration of 3,4,5-TCP was present in the experiment, presumably as the result of the degradation of PCP in the mother reactor. These results are consistent with those of Mikesell and Boyd (1985), who observed dechlorination of PCP and the production of 3,4,5-TCP in fresh sewage sludge. These authors did not detect 2,3,4,5-TeCP, presumably because their sample frequency of one week did not detect the transient tetrachlorophenol concentration.

Acclimation of the sludge to PCP over a period of six weeks resulted in a shortened lag period, greatly enhanced degradation rates, and the development of split biodegradation pathways. The progress curve for the degradation of PCP is shown in Figure 2.4. PCP was rapidly degraded and completely removed from the reactor in one day. Degradation of PCP was accompanied by the production of nearly stoichiometric amounts of the three tetrachlorophenols. Thus, ortho dechlorination of PCP continued in sludge acclimated to PCP, but was accompanied by dechlorination at the meta and para positions.

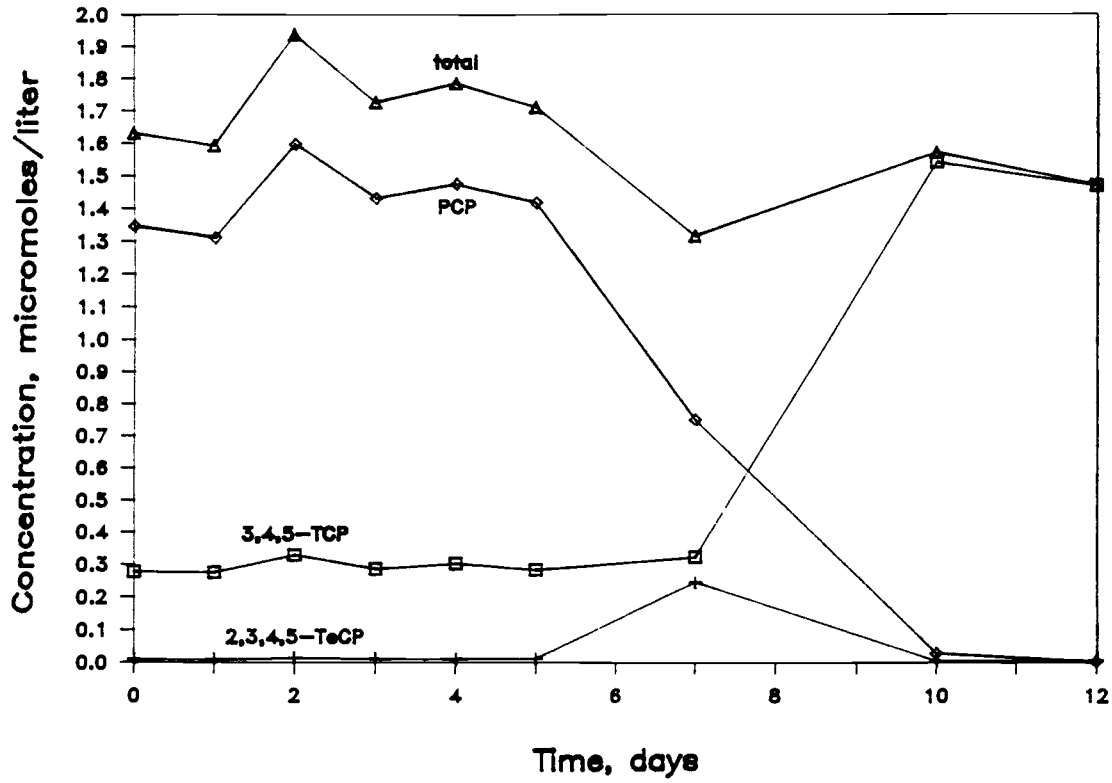

Figure 2.3. Progress curve for an initial PCP degradation experiment. (August 4-18, 1989, VSS=200 mg/L)

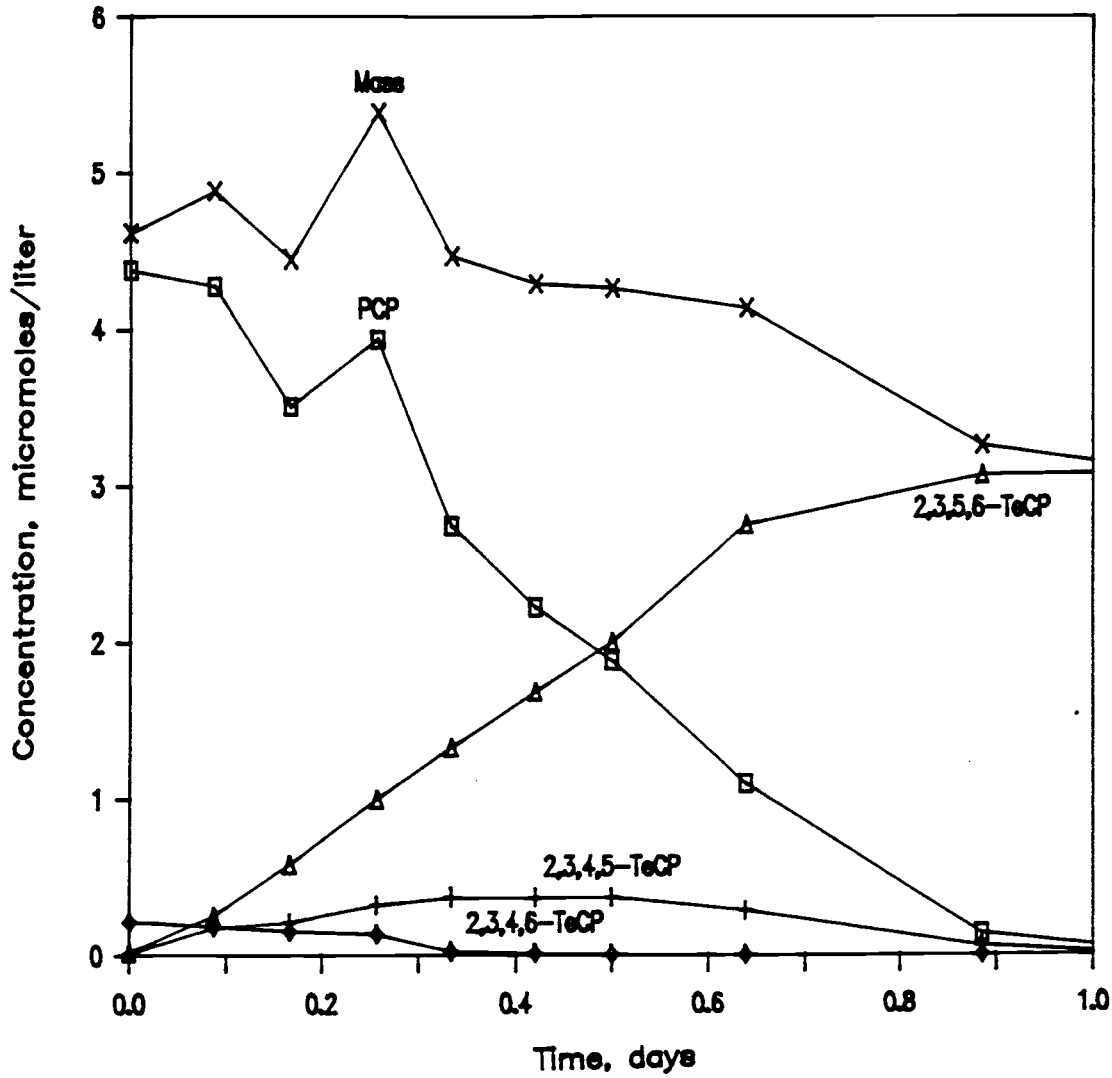

Figure 2.4. Progress curve for the degradation of pentachlorophenol.
Mass balance shown is the sum of PCP and tetrachlorophenols.
(Exp. 15B, February 21-22, 1990, VSS=742 mg/L)

2,3,4,5-TeCP began to appear after two hours, accumulated to a maximum concentration of 0.32 umol/L after 12 hours, and then began to disappear. All 2,3,4,5-TeCP was removed from the reactor after one day. Therefore, 2,3,4,5-TeCP was both produced and degraded during the course of the experiment. The production of 2,3,4,5-TeCP is attributed to the removal of an ortho chlorine from PCP and its replacement by a hydrogen atom.

2,3,5,6-TeCP accumulated rapidly in the reactor to a maximum molar concentration of about three-fourths that of the initial PCP concentration. The accumulation of 2,3,5,6-TeCP ceased when PCP had been removed from the reactor. Thus, removal of a para chlorine from PCP to produce 2,3,5,6-TeCP proceeded simultaneously with the removal of an ortho chlorine to form 2,3,4,5-TeCP. The measured combined mass of PCP and the three tetrachlorophenols held relatively constant until PCP was removed from the reactor, then began to fall. This loss of mass is attributed to degradation of tetrachlorophenols to trichlorophenols that were not included in the mass balance calculations. If trichlorophenols are taken into account, 95.2% of the initial mass of PCP was recovered as metabolites after 25 hours.

2,3,4,6-TeCP was present in the sludge at the start of the experiment, apparently produced by dechlorination of PCP at the meta position in the mother reactor, but did not accumulate during the batch experiment. Because there was no accumulation of 2,3,4,6-TeCP, the data suggest that 2,3,4,6-TeCP was degraded at a much faster rate than it was produced by dechlorination of PCP. The reason for the accumulation of 2,3,4,6-TeCP in the mother reactor is problematic.

The development of split degradation pathways has generally not been observed in previous laboratory experiments, although it has been documented in rice paddy soils exposed to PCP (Ide et al., 1972; Kuwatsuka and Igarashi, 1975). Microorganisms in the sludge acquired the ability to dechlorinate PCP at all three chlorine positions after

exposure to PCP for a period of about six weeks. This ability may result from the growth of individual species or consortia of bacteria which dechlorinate exclusively at these positions, or from the development of enzyme systems that catalyze removal of chlorines from certain molecular sites. The fact that previous attempts to isolate individual species of reductive dechlorinators have failed argues strongly for a consortium of species. Additionally, sequences of PCP metabolites that correspond to those observed in this study were reported for experiments performed using three sludges acclimated to 2-CP, 3-CP, and 4-CP respectively (Mikesell and Boyd, 1986), which suggests that enzyme systems capable of catalyzing dechlorination at a particular position are developed in response to exposure to compounds containing chlorines at those sites.

Reductive dechlorination of the three tetrachlorophenols were investigated in separate batch experiments. 2,3,4,5-TeCP was rapidly degraded and accompanied by the production of less than stoichiometric amounts of 3,4,5-TCP (Figure 2.5a). Only 68% of the initial mass of 2,3,4,5-TeCP was recovered as total chlorophenols after 36 hours. 2,3,4,5-TeCP has been previously reported to be the principal initial metabolite for PCP treated in an upflow anaerobic sludge blanket reactor (Woods et al., 1989). Other possible metabolites of 2,3,4,5-TeCP (2,3,4-trichlorophenol (2,3,4-TCP), 2,4,5-TCP, and 2,3,5-TCP) were not detected during this or repeated experiments. 2,3,4,5-TeCP also appears to inhibit methanogenesis: experiments performed with 2,3,4,5-TeCP at concentrations ranging from 5 to 20 umol/L (not shown) were accompanied by reduced or no methane production.

2,3,4,6-TeCP was completely removed from the reactor within one day accompanied by the accumulation of nearly equal amounts of both 2,4,6-trichlorophenol (2,4,6-TCP) and 2,4,5-TCP (Figure 2.5b). Therefore, 2,3,4,6-TeCP was dechlorinated at the ortho and meta positions. 2,3,4-TCP and 2,3,6-trichlorophenol

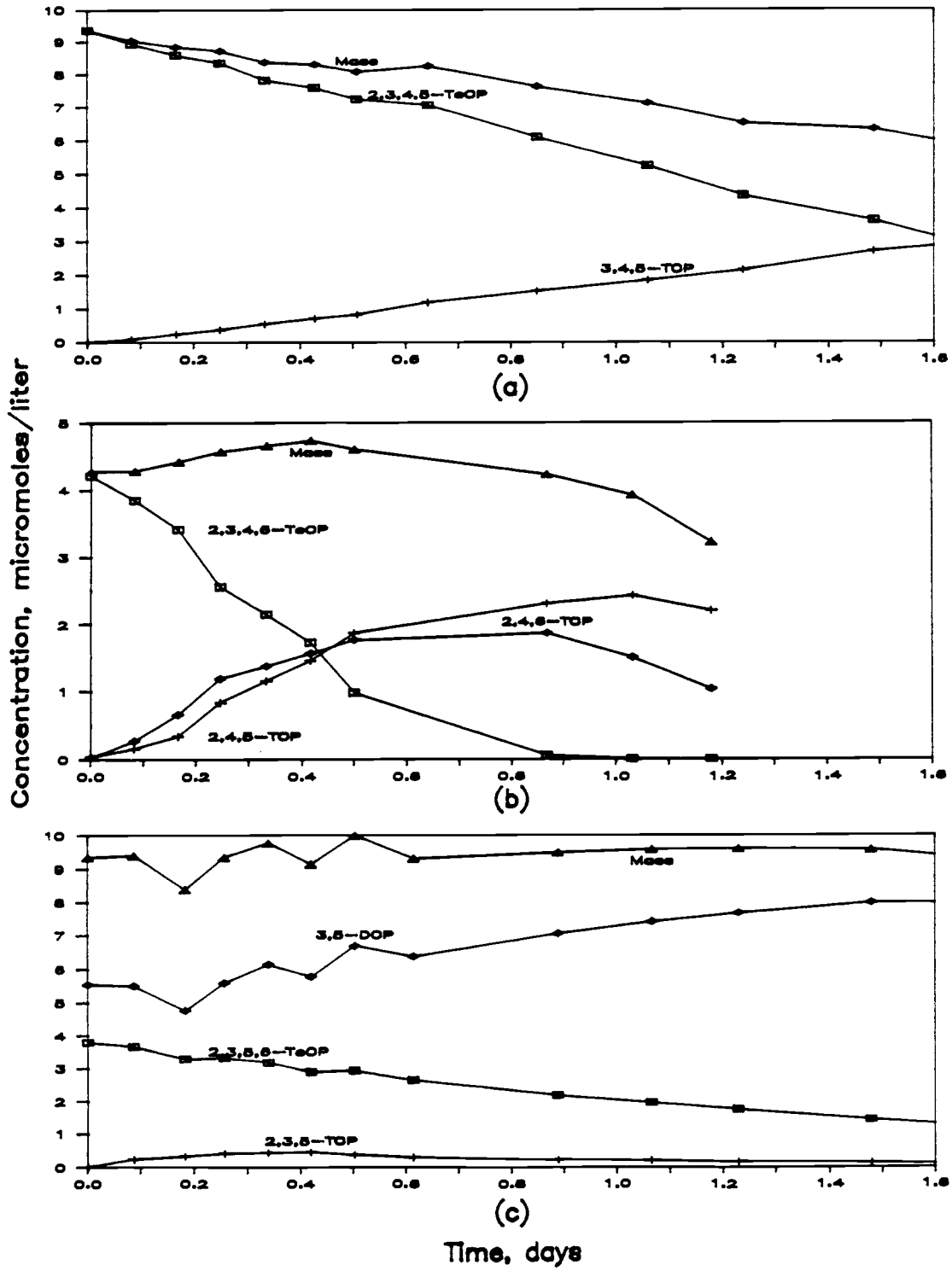

Figure 2.5. Progress curves for the degradation of tetrachlorophenols.
a. 2,3,4,5-TeCP (Exp. 7, December 18-20, 1989, VSS=522 mg/L).
b. 2,3,4,6-TeCP (Exp. 11, January 26-28, 1990, VSS=782 mg/L).
c. 2,3,5,6-TeCP (Exp. 16, March 21-23, 1990, VSS=915 mg/L).

(2,3,6-TCP) were not detected. Summation of all congeners of chlorophenols after 29 hours resulted in the recovery of 107.4% of the initial mass of 2,3,4,6-TeCP. Both metabolites accumulated until 2,3,4,6-TeCP had been largely destroyed, thus demonstrating the tendency for reductive dechlorination reactions to be sequential in nature. The transformation of 2,3,4,6-TeCP to 2,4,5-TCP has not been previously reported.

2,3,5,6-TeCP was also removed from the reactor at a steady but slower rate than the other tetrachlorophenols (Figure 2.5c). 2,3,5-TCP began to accumulate immediately and the concentration then began to decline after 10 hours with a concomitant increase in the amount of 3,5-dichlorophenol (3,5-DCP) in the reactor. 2,3,6-TCP was not detected. Thus, 2,3,5,6-TeCP was dechlorinated at an ortho position to form 2,3,5-TCP, and 2,3,5-TCP was degraded by the removal of a para chlorine to form 3,5-DCP. 103.7% of the initial mass of 2,3,5,6-TeCP was recovered as all chlorophenols after 36 hours.

Progress curves for separate batch experiments of the reductive dechlorination of trichlorophenols are shown in Figure 2.6. 3,4,5-TCP was completely removed from the reactor within two days, accompanied by an equivalent increase in the concentration of 3,5-DCP (Figure 2.6a). Thus, 3,4,5-TCP was dechlorinated at the para position, as has been demonstrated by previous workers (Mikesell and Boyd, 1985; Mikesell and Boyd, 1986). Transformation of 3,4,5-TCP to 3,4-DCP has been previously observed in unacclimated sludge in a reactor (Woods et al., 1989) and in soils amended with sewage sludge (Mikesell and Boyd, 1988). A small amount of 3,4-dichlorophenol (3,4-DCP) also accumulated during this experiment, suggesting that dechlorination at the meta position also occurred. The total mass of chlorophenols was 101.6% of the initial mass of 3,4,5-TCP after 36 hours.

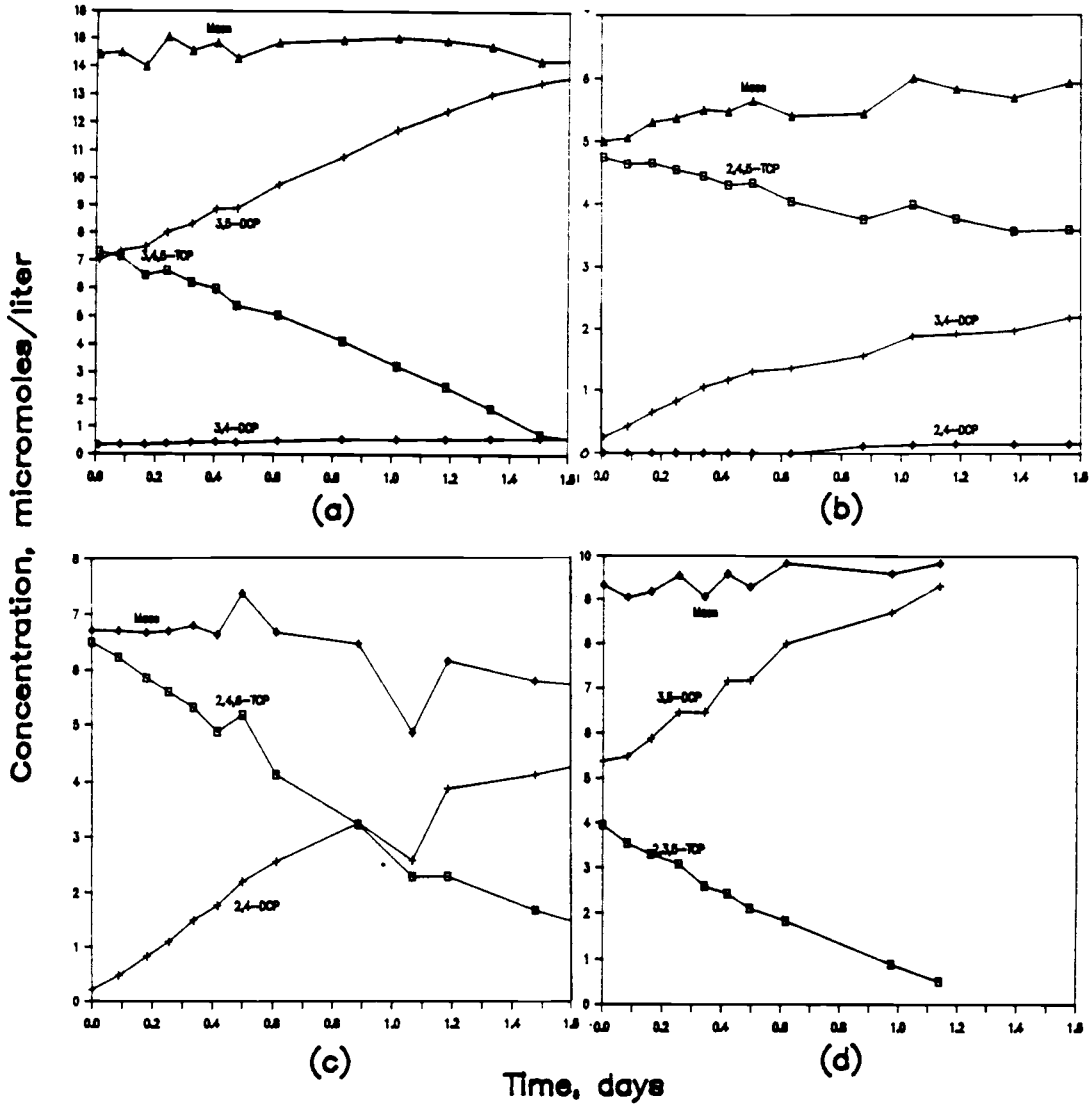

Figure 2.6. Progress curves for the degradation of trichlorophenols.
a. 3,4,5-TCP (Exp. 10, January 2-4, 1990, VSS=593 mg/L).
b. 2,4,5-TCP (Exp. 12, January 26-28, 1990, VSS=772 mg/L).
c. 2,4,6-TCP (Exp. 17, March 21-23, 1990, VSS=905 mg/L).
d. 2,3,5-TCP (Exp. 18, April 1-2, 1990, VSS=1024 mg/L).

2,4,5-TCP was slowly removed from the reactor accompanied by the production of 3,4-DCP (Figure 2.6b), reflecting dechlorination at the ortho position. 2,4-DCP was also detected in the reactor and began accumulating after 14 hours. 2,5-DCP was not detected. After 36 hours, the mass of all chlorophenols was 119 .5% of the initial mass of 2,4,5-TCP. 2,4,5-TCP was the most recalcitrant of the trichlorophenols studied in these experiments, the reason for which is not known.

2,4,6-TCP was degraded rapidly and was accompanied by the production of 2,4-DCP (Figure 2.6c), revealing that 2,4,6-TCP was dechlorinated at the ortho position, as has been previously reported (Mikesell and Boyd, 1985; Woods et al., 1989). 92.3% of the initial mass of 2,4,6-TCP was recovered unaltered or as 2,4-DCP after 36 hours. 2,6-dichlorophenol (2,6-DCP) was not detected in this experiment.

2,3,5-TCP was also rapidly degraded accompanied by production of a slightly excess molar amount of 3,5-DCP (Figure 2.6d). Total chlorophenols equaled 106.7% of the initial mass of 2,3,5-TCP after 24 hours. 2,5-dichlorophenol (2,5-DCP) and 2,3-dichlorophenol (2,3-DCP) were not detected. Thus, 2,3,5-TCP was dechlorinated by the removal of an ortho chlorine. This pathway has not been previously reported.

Batch reactor experiments were performed to evaluate the degradation of the three dichlorophenols observed in the pathways. Progress curves for these experiments (Appendix G) reveal the relative recalcitrance of these compounds. Only 2,4-DCP was degraded within the two days of the experiment. A metabolite was not detected. Batch experiments were then performed using 60-ml serum bottles (not shown) to test the degradation of 2,4-DCP, 3,4-DCP, and 3,5-DCP over a period of three weeks. 2,4-DCP was partially removed during this time and 4-CP accumulated, indicating removal of the ortho chlorine. 3,4-DCP and 3,5-DCP were not significantly dechlorinated during this time interval.

Based on the results of the various experiments, the complete pathways observed for the reductive dechlorination of PCP are depicted in Figure 2.7. Of the possible polychlorinated phenolic congeners, only five are not included in the pathways: 2,3-DCP, 2,5-DCP, 2,6-DCP, 2,3,4-TCP, and 2,3,6-TCP. Of the five, only 2,3,6-TCP has been previously reported as a metabolic product of PCP (Murthy et al., 1979).

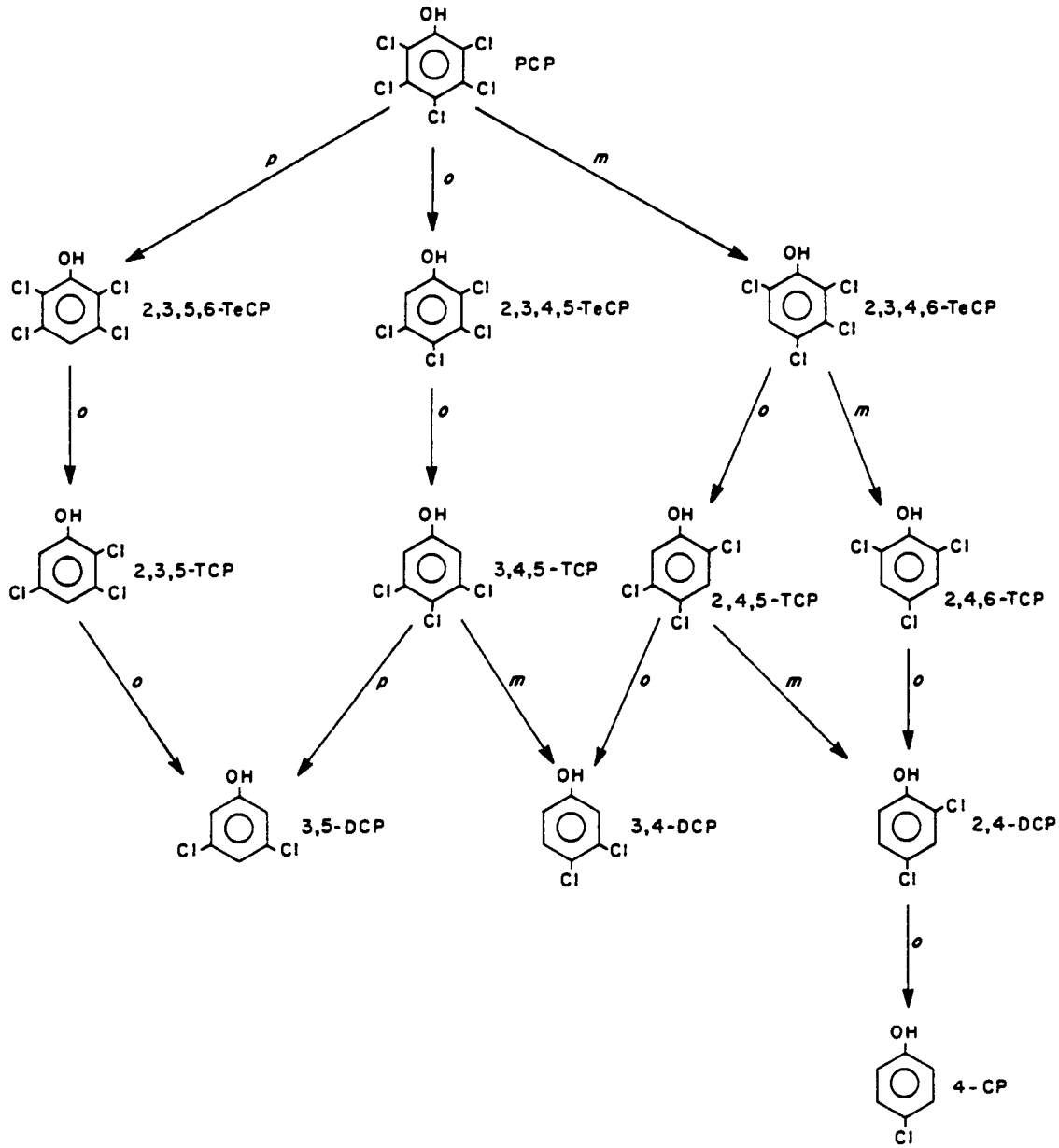

Figure 2.7. Summary of observed biodegradation pathways.

2.5 SUMMARY AND CONCLUSIONS

Acclimation of anaerobic sewage sludge to PCP greatly increased the rate at which PCP was biodegraded to lesser chlorinated phenols. Additionally, acclimation produced a microbial consortium with the ability to remove chlorines from the ortho, meta, and para positions.

The complete pathways observed are shown in Figure 2.7. PCP was dechlorinated at three positions to produce 2,3,4,5-TeCP, 2,3,4,6-TeCP, and 2,3,5,6-TeCP. Dechlorination of each succeeding metabolite was examined in batch experiments. 2,3,4,5-TeCP was dechlorinated at the ortho position to form 3,4,5-TCP which then gave 3,5-DCP and minor 3,4-DCP as persistent products. 2,3,4,6-TeCP produced both 2,4,6-TCP and 2,4,5-TCP. 2,4,6-TCP was dechlorinated in successive steps to 2,4-DCP and 4-CP, and 2,4,5-TCP produced both 3,4-DCP and 2,4-DCP. 2,3,5,6-TeCP produced 2,3,5-TCP which yielded 3,5-DCP.

The metabolites observed were consistent with those reported by earlier researchers for a mixture of three sludges acclimated to individual monochlorophenols. These results, coupled with the previous inability to isolate individual species of bacteria that dechlorinate chlorophenols, suggest that a consortium of microorganisms is responsible for the reductive dechlorination of pentachlorophenol. Presumably, the development of specific enzyme systems that catalyze dechlorination at the three chlorine positions results from acclimation of the bacteria to compounds containing those chlorines.

3,4,5-TCP was not dechlorinated in the presence of 2,3,4,5-TeCP, and 2,4,6-TCP and 2,4,5-TCP were not degraded simultaneously with 2,3,4,6-TeCP. However, degradation of 2,3,5-TCP to form 3,5-DCP occurred simultaneously with the transformation of 2,3,5,6-TeCP to 2,3,5-TCP. Accordingly, these results suggest

that the presence of the parent chlorophenol inhibits ortho and meta dechlorination of corresponding metabolites, but may not inhibit para dechlorination.

3,5-DCP accumulated in the mother reactor and was the primary metabolic product observed during these experiments. Therefore, this congener can be expected to accumulate in anaerobic digesters receiving wastes containing PCP. Similar PCP degradation pathways as those observed in this study were reported for rice paddy soils exposed to PCP, suggesting that the same sequence of metabolic products can be expected in oxygen-deficient aquifer systems contaminated with PCP from wood-treating activities.

2.6 ACKNOWLEDGEMENTS

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APPENDICES

Appendix A. Preparation of Feed Media.

The solution was prepared using a modification of the anaerobic feed given by Owen et al. (1979). Stock solutions of the various minerals and vitamins were prepared as follows:

Solution	Component	Conc., g/L
S3	$(NH_4)_2HPO_4$	26.7
S4	$CaCl_2 \cdot 2H_2O$	16.707
	NH_4Cl	26.600
	$MgCl_2 \cdot 6H_2O$	120.089
	KCl	86.713
	$MnCl_2 \cdot 4H_2O$	1.338
	$CoCl_2 \cdot 6H_2O$	2.001
	H_3BO_3	0.388
	$CuCl_2 \cdot 2H_2O$	0.181
	$Na_2MoO_4 \cdot 2H_2O$	0.173
	$ZnCl_2$	0.141
	$NiCl_2 \cdot 6H_2O$	1.010
S7	Biotin	0.00201
	Folic acid	0.00205
	Pyridoxine hydrochloride	0.01001
	Riboflavin	0.00508
	Thiamin	0.00505
	Nicotinic acid	0.00501
	Pantothenic acid	0.00500
	B_{12}	0.00012
	p-aminobenzoic acid	0.00505
	Thioctic acid	0.00503

To make 12 liters of feed, the following formula was used:

100 ml S4
90 ml S7
46.7 grams anhydrous sodium acetate
48 grams sodium bicarbonate
33 ml S3
30 ml glacial acetic acid
1200 ml saturated PCP solution
distilled water to make 12 liters

10 liters of distilled water were added first, followed by the acetic acid, sodium acetate, S4, S3, and S7 and PCP solutions. Distilled water is then added to fill to 12 liters, and finally the sodium bicarbonate added.

270 microliters each of 500 gr/L $Na_2S \cdot 9H_2O$ and 370 gr/L $FeCl_2 \cdot 4H_2O$ were added directly to the reactor daily.

Appendix B. Experimental Protocol

1. Prepare a solution containing the chlorophenols of interest; analyze for chlorophenol concentrations on the gas chromatograph; calculate the volume of solution needed for the experiment.
2. Calibrate pH electrode; seal pH and Eh electrode ports with vacuum grease when replacing electrodes; replace sample port septum.
3. Run gas partitioner standard curves for N_2, CH_4, O_2, and CO_2.
4. Record gas composition and pH of mother reactor.
5. Run initial COD determinations on filtered and unfiltered mother reactor liquid.
6. Purge batch reactor with N_2 gas; analyze headspace gas on the gas partitioner to verify the absence of oxygen.
7. Add 5.213 grams of sodium acetate (anhydrous) to the calculated volume of chlorophenol solution to give an initial acetate concentration of 1500 mg/L in the batch reactor.
8. Clamp off feed and effluent lines to the mother reactor; shut off feed pump; continue stirring.
9. Connect the drain port of the mother reactor to the influent valve of the batch reactor using tygon tubing.
10. Open gas relief and influent valves and close the sample valve on batch reactor.
11. Gently purge mother reactor headspace with N_2 gas using the sample port while filling the batch reactor; leave approximately 1/2 inch of headspace.
12. Place batch reactor on magnetic stirrer set at intensity 10 in a 31° C environmental chamber; connect electrode leads to meters and the gas relief valve to a wet test gas meter. The temperature of the environmental chamber should be within 1° C of that of the mother reactor chamber.
13. Allow at least 12 hours for the consortia to equilibrate.
14. Add the appropriate volume of chlorophenol/acetate solution to the batch reactor using a glass syringe after the Eh has dropped to -330 or below; record volume added in the notebook.
15. Sample immediately and at intervals appropriate for the experimental duration and goals.
16. To sample, first record pH, Eh, gas production, and temperature; use a water-immersed thermometer located at the same height as the reactor.
17. Withdraw 20 ml of liquid sample from the sample port using a 20-ml glass syringe with 18 gauge, 6" needle; flush the needle and syringe with water after sampling to clean.
18. Filter 3 ml of sample through a pre-washed and weighed Gelman type A/E glass fiber filter using a Millipore filtration apparatus, discard this portion, then filter the remaining sample; place filter paper in the drying oven for solids analysis.
19. Extract 10 ml of sample into hexane by following the Acetylation/extraction Protocol; fill 2 ml amber autosampler and storage vials with teflon caps with hexane collected with a new pasteur pipette.
20. Place the remaining filtrate in a 10 ml amber vial with teflon cap; freeze for later analysis for acetate using the ion chromatograph.
21. Record all data in the notebook; staple any data sheets into the notebook and use carbon paper.
22. Analyze headspace gas at intervals throughout the experiment.
23. At the conclusion of the experiment, repeat COD determinations and complete solids determinations on the filter papers used.

24. Transfer the consortia back to the mother reactor by pumping into the influent line.
25. Run chlorophenol samples on GC the using the the Gas Chromatograph Protocol and acetate samples on the ion chromatograph; save hexane extracts in a refrigerator in the dark; refreeze remaining acetate samples.

Appendix C. Acetylation/extraction Protocol.

1. Clean glassware by acid-washing in 50% H_2SO_4 solution, rinsing at least three times with water, three times with distilled water, and three times with hexane (for separatory funnels).
2. Add 50 ml glass-distilled water to the separatory funnel using a 50-ml volumetric pipette.
3. Add 25 microliters of ISTD 1 (2,6-DBP) or 50 microliters of ISTD 2 (2,4,6-TBP) to the funnel using dedicated 50 microliter syringes.
4. Add the sample using a clean and dry volumetric pipette.
5. Add 1 ml of .72 gr/ml K_2CO_3 solution to the funnel using a dedicated 1 ml volumetric pipette.
6. Add 1 ml of acetic anhydride to the funnel using a dedicated 1 ml volumetric pipette.
7. Shake the funnel for exactly two minutes, venting every 10-15 seconds.
8. After 20 minutes, add 5 ml of hexane to the funnel using a dedicated 5 ml volumetric pipette.
9. Shake the funnel for exactly two minutes, venting every 15-30 seconds.
10. Withdraw water from the funnel until the hexane layer is easy to withdraw without contaminating the hexane extract with water. Use a disposable pasteur pipette to transfer the hexane.
11. Transfer the hexane to a new, amber glass vial with teflon-lined cap that has been labeled with the sample number, date, and your initials. Always fill the vial to the same level. Store the samples in a vial file in the refrigerator in the dark until analysis on the GC.
12. Record all data in a research notebook.

Appendix D. Gas Chromatograph Protocol.

1. Record sample order in the notebook. Sample order is as follows: two hexane rinses, standard curves, hexane rinse, samples. Place a hexane rinse after every 5 samples and include a standard (in the middle of the standard curve range) every ten samples. Use a hexane rinse both before and after the standard. End with two more hexane rinses.
2. Load the samples into the autosampler rack in the prerecorded order.
3. Check the gas tanks to make sure that the regulator is open and that the tank contains at least 500 psi gas. Adjust the regulators to the following pressures, if necessary: helium, 60 psi, argon/methane, 40 psi.
4. Fill the autosampler solvent rinse A and B vials with hexane.
5. Edit the method and INET sequences on the integrator to select the temperature program and number of samples to run, by keying the following sequence: "edit" "seq" "enter" "3" "enter" "(method name)" "enter" "1" "enter" "(first and last sample, enter for all other questions)" "enter".
6. Print the operating conditions and temperature program by keying "list" "meth" "enter".
7. Start the analyses by keying "seq" "start".
8. After all samples have been run, list the method again.
9. Keep the sample set as one continuous roll of paper.

Appendix E. Retention times and relative retention times for chlorophenols.

Times listed (in minutes) are for the short temperature program as described in Section 2.2.

congener	retention time, min	ISTD	relative retention time[1]
2-CP	11.610	2,6-DBP	.625
3-CP	12.160	2,4,6-TBP	.507
4-CP	12.303	2,6-DBP	.663
2,3-DCP	15.695	2,4,6-TBP	.653
2,4-DCP	14.939	2,6-DBP	.803
2,5-DCP	14.916	2,4,6-TBP	.621
2,6-DCP	14.445	2,4,6-TBP	.601
3,4-DCP	16.322	2,6-DBP	.876
3,5-DCP	15.240	2,4,6-TBP	.634
2,3,4-TCP	19.756	2,4,6-TBP	.882
2,3,5-TCP	18.545	2,4,6-TBP	.772
2,3,6-TCP	18.339	2,4,6-TBP	.764
2,4,5-TCP	18.695	2,4,6-TBP	.778
2,4,6-TCP	17.253	2,6-DBP	.928
3,4,5-TCP	20.119	2,6-DBP	1.081
2,3,4,5-TeCP	23.175	2,6-DBP	1.245
2,3,4,6-TeCP	21.756	2,4,6-TBP	.906
2,3,5,6-TeCP	21.652	2,4,6-TBP	.902
PCP	25.955	2,6-DBP	1.395

[1]Retention time divided by the retention time of the internal standard (ISTD). Typical retention times for the internal standards are 18.59 min for 2,6-DBP and 24.01 for 2,4,6-TBP.

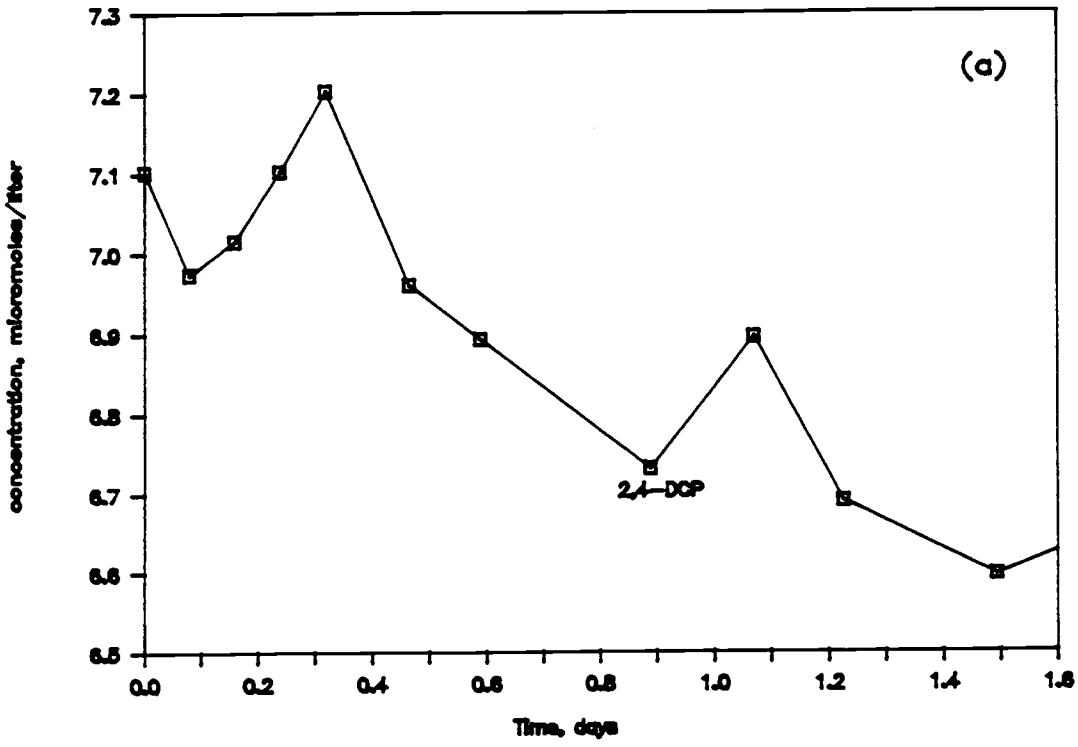
Appendix F. Filenames, notebook page number, and solids concentrations for all experiments.

Exp. #	filename	compounds/ analysis[1]	notebook page #	VSS, mg/L
1	EXP12.WK1	PCP, CP	5	254
	IC-EX12.WK1	PCP, AC	14	
2	EXP12.WK1	2345, CP	5	254
	IC-EX12.WK1	2345, AC	14	
3	EXP34.WK1	PCP, 2345, CP	21	364
	IC-EX34.WK1	PCP, 2345, AC	29	
4	EXP34.WK1	PCP, 2345, CP	21	364
	IC-EX34.WK1	PCP, 2345, AC	29	
5	EX56.WK1	PCP, CP	32	489
	IC-EX56.WK1	PCP, AC	39	
6	EX56.WK1	2345, CP	32	516
	IC-EX56.WK1	2345, AC	39	
7	EXP78.WK1	2345, CP	40	522
	IC-EX78.WK1	2345, AC	45	
8	EXP78.WK1	2345, CP	40	507
	IC-EX78.WK1	2345, AC	45	
9	EXP910.WK1	2345, CP	49	529
	IC-EX910.WK1	2345, AC	55	
10	EXP910.WK1	345, CP	49	593
	IC-EX910.WK1	345, AC	55	
11	EXP1112.WK1	2346, CP	57	782
	EX11B.WK1	2346, CP	57	
12	EXP1112.WK1	245, CP	57	772
13	EX1314.WK1	24, 35, CP	65	782
14	EX1314.WK1	34, CP	65	681
15	EX15.WK1	PCP, CP	71	742
	EX15B.WK1	PCP, CP	71	
16	EX16B.WK1	2356, CP	77	915
17	EX17.WK1	246, CP	77	905
18	EX18.WK1	235, CP	84	1024

[1]Compound (s) injected into the batch reactor; CP=GC analysis of chlorophenols, AC= IC analysis of acetate

Appendix G. Progress Curves for the Degradation of Dichlorophenols

(a) Degradation of 2,4-DCP (Exp. 13a, February 8-10, 1990, VSS=782 mg/L).
(b) Degradation of 3,5-DCP (Exp. 13b, February 8-10, 1990, VSS=782 mg/L).
(c) Degradation of 3,4-DCP (Exp. 14b, February 8-10, 1990, VSS=681 mg/L).



Appendix G - continued