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DISSERTATION

MICROBE-LEVEL INVESTIGATION OF SEQUENTIAL ANAEROBIC/AEROBIC
BIOREMEDIATION OF
POLYCHLORINATED BIPHENYLS IN SOIL

Submitted by

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Department of Chemical and Bioresource Engineering

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2000

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June 1, 2000

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OUR SUPERVISION BY JULIA BULL ROGERS ENTITLED
MICROBE-LEVEL INVESTIGATION OF SEQUENTIAL
ANAEROBIC/AEROBIC BIOREMEDIATION OF
POLYCHLORINATED BIPHENYLS IN SOIL
BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF THESIS

MICROBE-LEVEL INVESTIGATION OF SEQUENTIAL ANAEROBIC/AEROBIC BIOREMEDIATION OF POLYCHLORINATED BIPHENYLS IN SOIL

Environmental polychlorinated biphenyl (PCB) contamination is often comprised of mixtures of congeners with a range of degree of chlorination. Complete biodegradation of PCB mixtures is rarely achieved, since highly chlorinated PCBs are not metabolized under aerobic conditions and less chlorinated congeners are not degraded by anaerobic microorganisms. Sequential anaerobic-aerobic treatment for PCB contamination is promising, yet little is known about specific parameters important for its successful outcome. A combination of engineering and microbial ecology was used in this work to understand the underlying mechanisms in the bioremediation of PCBs.

Laboratory-scale, PCB-spiked, soil slurry microcosms were used to investigate the effects of engineering parameters on PCB biodegradation and the microbial community in each phase and in the overall sequential process. The overall goal of this work was to not only effect greater overall PCB degradation in the soil microcosms but to gain knowledge of the microbial populations responsible for this degradation so that this information could be utilized for other sites.

In microcosms incubated anaerobically, dechlorination of several hexachlorobiphenyls to penta- and tetrachlorobiphenyls by indigenous organisms was detected in soil microcosms within 12 weeks. The dechlorination pattern was strongly

dependent on the congener being degraded in addition to the electron donor utilized. Phospholipid fatty acid/fatty acid methyl ester (PLFA/FAME) analysis indicated small differences in community structure between different electron donor treatments. Biphenyl-amended microcosms achieved the highest extent of dechlorination with 22.5% dechlorination achieved over the 60-wk anaerobic phase. Unamended, butyrate-, methanol-, butyrate+biphenyl-, and methanol+biphenyl-amended microcosms exhibited less dechlorination over the 60-wk anaerobic phase with unamended microcosms exhibiting the highest initial rate of dechlorination. Use of a denaturing gradient gel electrophoresis method combined with sequencing showed that most of the cloned sequence types were related to the 16S rRNA sequences of aerobic species, and several to acidiphilic bacteria. In addition, several sequence types were similar to known microorganisms involved in either iron reduction or iron oxidation. These microbial types fit the soil characteristics; i.e., slightly acidic and high in iron.

Both 2,3',4'-PCB and biphenyl were completely degraded in aerobic microcosms in 7 wk. Microcosms not amended with biphenyl showed decreased degradation rates compared with those amended with biphenyl during the aerobic phase. Although total biomass remained constant in all treatments (as shown by PLFA), changes in microbial community composition occurred in biphenyl-amended microcosms: the anaerobic microbial community decreased in size and there was a subsequent increase in aerobic lipid biomarkers. Soil incubated with no amendment showed no difference in community composition from time zero samples.

A key question in developing a sequential remediation treatment was how to switch from anaerobic to aerobic treatment conditions. Aerobic microorganisms in

microcosms treated with both air and hydrogen peroxide were able to partially degrade 2,3',4'-PCB in 7 wk. In all treatments, the rate and extent of degradation were less than that observed in the aerobic treatment alone. It was hypothesized that this was due to the harsher method used to initiate anaerobic conditions before the oxygenation treatment and not due to the oxygenation treatment itself. No difference in degradation rates or microbial populations was observed between treatments, but a slightly higher rate of dissolved oxygen (DO) utilization in H₂O₂-treated microcosms prompted the use of a 0.25% H₂O₂ treatment in the sequential experiments.

The sequential treatment process was more effective for overall PCB removal than either the anaerobic or aerobic processes alone. A total reduction in PCB mass of 57.5% in sequential microcosms was observed versus 27% and 25% for anaerobic and aerobic microcosms alone. Because the soil was aerobic at the onset of the experimentation, highly reducing conditions were never obtained. In fact, degradation of the trichlorobiphenyl and biphenyl occurred during the first 12 wk of the anaerobic phase, essentially making the treatment process an aerobic/anaerobic/aerobic process. The less-reducing conditions of the anaerobic process allowed the aerobic microorganisms to survive the 60 wk and rebound to degrade the less chlorinated congeners produced. Few changes were observed in community structure between the anaerobic and aerobic phase samples.

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CHAPTER 1

INTRODUCTION

Bioremediation is a useful method for cleanup of sites contaminated with hazardous chemicals that utilizes microorganisms to facilitate the removal of pollutants. Contamination of natural environments with chlorinated compounds has caused challenges for bioremediation of these sites. Often, contamination occurs as mixtures of compounds with a range of degree of chlorination. Complete biodegradation of these mixtures is rarely achieved, since highly chlorinated compounds are not readily metabolized under aerobic conditions and less chlorinated congeners are not often degraded by anaerobic microorganisms. Sequential anaerobic-aerobic treatment for this type of mixture contamination is promising, yet little is known about specific parameters important for their successful bioremediation. Most of the work in this area has focused on a “black box” approach to enhancement of chlorinated mixture transformation with little insight into the actual microbial processes at work.

Information about the microbial community dynamics that reveals which organisms are dominant and which may be out-competed under given environmental conditions must be obtained. As a first step in the development of economical and effective sequential remediation strategies, this research addressed several unanswered questions. Which

microorganisms are critical for each phase of treatment? How does each engineered process parameter affect the activity of these vital populations? Is it possible to stimulate chlorinated chemical mixture transformation in every situation with the right approach? The approach used in this work is a blend of microbial ecology and engineering that addresses these questions to design a bioremediation process “from the microbe up”.

The goal of this work was to directly correlate changes in the microbial community to the observed enhancement of transformation of one type of chlorinated mixture contamination (polychlorinated biphenyls) due to the manipulation of treatment variables. In this work, the treatment variables investigated were the use of electron donor additions during the anaerobic phase, the method used for oxygenation between phases, and use of biphenyl to stimulate aerobic PCB degradation. The correlations obtained will allow for development of better remediation strategies of PCB contaminated soil as well as provide insight into remediation of other chlorinated mixtures.

To correlate changes in microbial community composition and functional gene quantities with observed changes in chemical transformation, microbiological methods are needed that do not rely on standard culturing techniques which are known to describe less than 1% of the total microbial population. Two such techniques, a genotypic (denaturing gradient gel electrophoresis in combination with sequencing) and phenotypic (phospholipid fatty acid analysis) microbiological technique were utilized for this work. Information from PLFA analysis has been used to determine the amount of viable microbial biomass and to provide insights into the microbial community composition. DGGE was utilized to track changes in microbial community composition and to direct

development of a quantitative polymerase chain reaction method. Changes in community composition were used to understand and correlate changes in PCB bioremediation.

This focus on the microbial populations responsible for PCB transformations and the factors affecting their activity is a novel approach for improving engineered sequential PCB treatment. Not only will application of the developed correlations allow us to engineer processes with improved PCB transformation rates but these results will also have significance for developing sequential anaerobic-aerobic treatment processes for other chlorinated contaminants, which are a ubiquitous problem in soils and sediments across the nation.

CHAPTER 2

BACKGROUND

2.A. Polychlorinated Biphenyls

2.A.1. General Characteristics

The term polychlorinated biphenyl (PCB) refers to a man-made class of compounds produced by chlorination of a biphenyl molecule (Figure 2.1).

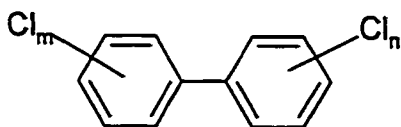


Figure 2.1 Polychlorinated biphenyl structure

More than 200 isomers are possible; however, not all possible isomers are likely to be formed during a manufacturing process. The most common isomers produced are those that have either an equal number of chlorine atoms on both rings or a difference of only one chlorine atom between rings. PCB mixtures were produced under a variety of trade names with the chlorine content of these commercial mixtures varying from 18 to 79 percent. These commercial mixtures contained up to 70 PCB congeners per mixture. The physical properties of PCBs vary not only with the degree of chlorination, but also the position of the chlorines on the biphenyl ring. PCB mixtures have a high thermal

stability, are resistant to oxidation, acids, and bases, and have excellent electrical insulating and dielectric characteristics (28). American PCB production occurred under the trade name Aroclor, with mixtures used as a coolant/dielectric for transformers and capacitors, as heat transfer fluids, and as protective coatings for woods when low flammability was important. Between 1930 and 1960 the use of PCBs widened to include use in paints, inks, dusting agents and pesticides.

Based on their frequency at National Priority List sites and their classification as probable carcinogens, PCBs are ranked sixth on the 1997 Agency for Toxic Substances and Disease Registry/Environmental Protection Agency hazardous substance priority list. Environmental PCB contamination is often derived from one or more of the commercial PCB mixtures, each of which is composed of a different percentage of the 209 possible PCB congeners. Though the production of PCBs was banned in 1976, an estimated one-half billion pounds remain in storage, in landfills or as environmental contamination (2). Additional PCB contamination continues to occur through the leaking of transformers by corrosion, natural disasters (e.g. lightning strikes), and mobilization through dredging of sediments. Incineration is currently the primary technology used for remediation of PCB contaminated soils and sediments. With costs ranging from \$500 to \$800 per ton of solid waste, incineration is significantly more costly than a typical in-situ bioremediation effort (\$175 per ton solid waste) (1). PCBs are ubiquitous pollutants; they have been found in nearly all marine plant and animal species, fish, mammals, bird, and humans (47). Two major health tragedies due to PCB contamination instigated the concern over PCB contamination and was the catalyst for the ban in their production. The first of these was a mass food poisoning caused by the ingestion of a commercial

brand of rice oil contaminated with PCBs, polychlorinated dibenzofurans, and polychlorinated quaterphenyls. The poisoning, which occurred in Western Japan in 1968 involved more than 1,600 people who experienced fatigue, joint swelling and pain, anemia, acneform eruptions and swelling of the eyes (47). The second event was the contamination of the Hudson River by two General Electric plants manufacturing PCB-containing capacitors. PCB discharge from these two plants led to massive contamination of the Hudson basin. This contamination problem damaged the ecosystem and is still prevalent today (3, 31, 65).

2.A.2. Health Effects of Polychlorinated Biphenyls

Exposure to each of the different congeners carries a different associated risk to human health. The two main routes of exposure to PCBs are through the environment (primarily due to fish consumption) and a person's work place (47). This exposure can occur by ingestion, inhalation and dermal contact. Because of their physical and chemical properties, polychlorinated biphenyls persist in the environment and bioaccumulate in fish, birds and other organisms; PCBs accumulated in one organism are passed on to another as they move up the food chain (73). This tendency to bioaccumulate stems from the high lipid-water partition ratio of PCBs that causes them to accumulate in fatty tissues.

The relative acute toxicity of PCBs is not the primary health concern; of more concern is the metabolization and redistribution of the metabolites into adipose tissue. Exposure of fetuses and children to PCBs has been linked to substandard juvenile growth, developmental delays and behavioral problems during later years. Other adverse health

effects linked to PCB exposure are cancer and effects on the cardiovascular, hepatic, immune, musculoskeletal, endocrine, gastrointestinal, and dermal systems. It has been shown that PCBs can covalently bind to DNA *in vivo* and *in vitro*, that PCB mixtures exhibit mutagenic activity and that the more highly chlorinated mixtures are hepatocarcinogens in rodents (28).

The effect on human and animal health depends not only on the degree of chlorination, but also on the chlorine substitution pattern. Some chlorine substitution patterns allow the biphenyl molecule to become coplanar which increases the health risk associated with a particular congener.

2.B. Remediation Technologies for PCB Contamination

Because of their chemical properties, PCBs are not broken down readily in the environment and accumulate in soil, sediment and water. For sites with PCB contamination, possible remediation methods include chemical, thermal, and biochemical processes.

2.B.1. Incineration and Chemical Methods

To date, the primary mode for remediation of PCB contaminated waste has been incineration. In the United States, incinerators are strictly regulated to assure that the PCBs are completely destroyed. Most incineration operations have a retention time of fifteen to ninety minutes with a gas phase operation temperature that varies from 650-1200 °C (86). While most incineration processes remove 99.999% of PCBs, they are energy intensive and require monitoring and treatment of waste gases. PCBs may also be

legally destroyed in the boilers used by utilities to generate electricity (47). Another physical method used for PCB remediation is supercritical fluid extraction (SFE). This technique uses supercritical CO₂ (CO₂ at high pressures) to quickly extract the PCBs from soils and sediments (34). However, the PCBs are not destroyed by SFE; they have simply been concentrated to a smaller volume of pollutant in a different medium. The SFE process requires an additional destruction method such as incineration. The advantage is that less energy is required to incinerate the smaller volume of waste, making this an improvement over incineration alone.

Chemical techniques for destruction of PCBs include, chlorinolysis, catalytic dehydrochlorination, ozonation, wet air oxidation, reaction with sodium naphthalide, and reaction with molten sodium (73). In the top few centimeters of soil, photolysis is a significant chemical degradation process. However, the majority of released PCBs are expected to accumulate in aquatic sediments where they are shielded from photolytic breakdown (73).

2.B.2. Microbial Biodegradation

2.B.2.a. Introduction

The microbial degradation of polychlorinated biphenyls has been extensively documented since 1973, beginning with work by Ahmed and Focht, which demonstrated the use of two *Achromobacter* species for degradation of several PCB congeners (4). Complete mineralization of PCBs to carbon dioxide, chloride, and water requires both removal of chlorines and breakup of the biphenyl ring. While both of these processes have been shown to occur, questions remain as to the origin of microbial degradative

action on PCBs, as is the case with many halogenated xenobiotics. The presence of natural halogenated compounds is common; more than 700 halogenated natural compounds were identified by 1986 (50). These compounds are thought to be important in the adaptation of organisms to the degradation of halogenated xenobiotics. For example, Hernandez et al. found the presence of terpenes to enhance transformation of PCBs and postulated that these natural compounds may be the original substrates for biphenyl-degrading bacteria (68). Because of the diverse nature of this class of chemicals, one would expect a similarly diverse set of microorganisms able to carry out the degradation of the polychlorinated biphenyls.

The persistence of halogenated pollutants is related to the number, type, and position of the halogen substituents (50). In general, those compounds with few numbers of substituents are thought to be more degradable, while those with a greater number of substituents are less biodegradable. Because PCB contamination occurs as a mixture of these less and highly degradable compounds, a biodegradation approach able to degrade all mixture components is challenging to develop. There are two main routes for degradation of polychlorinated biphenyls: anaerobic dechlorination and aerobic degradation.

2.B.2.b. Anaerobic Dechlorination

Under anaerobic conditions, reductive dechlorination occurs by removal of halogen substituents from a molecule with concurrent addition of hydrogen to the molecule (Figure 2.2). Dehalogenation is an important mechanism for the biodegradation of many highly chlorinated pollutants, including PCBs, hexachlorobenzene,

tetrachloroethene, chlorotoluenes, and pentachlorophenol (39, 49). Pollutants are generally rendered less toxic and more biodegradable through dehalogenation. Dechlorination can also occur under aerobic conditions, although reports of this phenomenon are rare (36, 74).

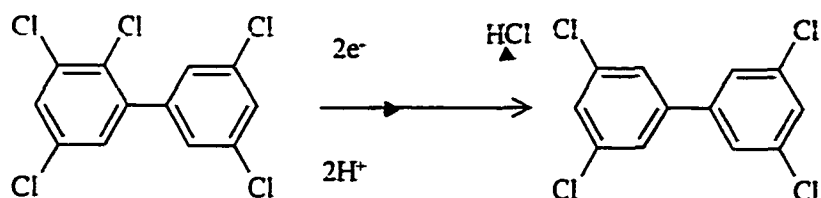


Figure 2.2 Aryl reductive dechlorination of PCBs

Several mechanisms of dehalogenation are known so far; the one shown to be important for anaerobic dechlorination of PCBs is reductive dechlorination. Reductive dechlorination is the only known anaerobic biotransformation pathway of highly chlorinated PCB congeners. In this reaction, the PCB molecule serves as an electron acceptor; as the degree of chlorination decreases, so do the redox potential and the rate of dechlorination. This results in accumulation of less chlorinated congeners that are more resistant to dechlorination. Two mechanisms are believed possible: 1) a one-step transfer of 2 electrons and one proton, both provided by a reduced organic substrate and 2) a two-step reduction by an electron donor with the proton source being provided by the solvent (often H₂O). Nies and Vogel found evidence for a 2-step mechanism in reductive dechlorination of 2,3,4,5,5-pentachlorobiphenyl for which the proton source was water (104).

The carbon-halogen bond cleavage can occur either by specific enzymes (dehalogenases) or by enzymes with relaxed substrate specificity that allow fortuitous dehalogenation of the substrate analog (16). Fortuitous dehalogenation is the dehalogenation of the pollutant by a microorganism that is unable to use the substrate as a source of carbon or energy. An example of cometabolic (or fortuitous dechlorination) was observed by Fathepure and Boyd in the dechlorination of PCE by methanogens (49). PCB dechlorination carried out by methanogens and sulfate-reducers may also fall into this category.

The potential of any compound to serve as an electron acceptor for the production of energy depends on the Gibbs free energy of formation of the compound. Several studies have shown that a variety of chlorinated compounds, including chlorobenzenes, chlorobenzoates and chlorophenols, can support reductive dechlorination as electron acceptors (42). Homes et al. estimated the Gibbs free energy of formation for each of the 209 possible polychlorinated biphenyl congeners. Table 2.1 shows the Gibbs free energy of formation of select PCB congeners (those used in this work) as well as those for other potential electron acceptors for comparison. All values were calculated with hydrogen as the electron donor.

By comparing the Gibbs free energy of formation of substrates and products in aqueous solution, an estimation of the amount of energy available from each reaction can be calculated. Using hydrogen as the electron donor under hydrogen partial pressures reasonable for environmental conditions, the energy available for dechlorination of PCBs ranges between 128 to 151 kJ/reaction (72). This amount of energy is theoretically enough to allow organisms to obtain energy from this process. In all of these cases,

reduction dechlorination is more favorable than sulfate reduction or methanogenesis. If more than one electron acceptor is present in a given environment, the compound that will be preferentially used by the microorganisms is determined by the potential energy yield of the reaction. The more energy an electron acceptor provides, the more favorable the reaction, thus this electron acceptor will be used by the microorganisms.

Table 2.1 Gibbs free energies of formation for select chlorinated compounds and inorganic electron acceptors

Compound	ΔG_f (kJ/mol)	Reference
2,2',5-trichlorobiphenyl	-211.7	(72)
3,3',4,4'-tetrachlorobiphenyl	-188.7	(72)
2,2',3,3',4,4'-hexachlorobiphenyl	-167.8	(72)
2,2',4,4',5,5'-hexachlorobiphenyl	-151.8	(72)
2,3',4,4',5,5'-hexachlorobiphenyl	-150.1	(72)
Sulfate (SO ₄ ²⁻)	-33.9	(87)
Nitrate (NO ₃ ⁻)	-240	(87)
Fe(III)	-228.3	(87)

2.B.2.b.1. Microorganisms

Most reports of reductive dechlorination involve mixtures of microorganisms; only a few examples of pure cultures able to carry out this process have been reported (50, 124). Many microorganisms may in fact be able to carry out the dechlorination process. However, current culture methods may be able to isolate only a small subset of these. The majority of pure cultures reported to catalyze reductive dehalogenation are those able to dehalogenate alkyl halides (50, 81). This group of microorganisms is phylogenetically diverse, including eukaryotes and prokaryotes, strict anaerobes, facultative anaerobes and aerobes. Most were previously isolated strains that were

subsequently tested and found competent for dehalogenation. This dechlorination is believed to correspond to the use of the acetyl coenzyme A pathway (50).

One anaerobe that has been shown to gain energy from reductive dechlorination of aromatics is *Desulfomonile tiedjei*, a “dehalorespirer”. This organism is able to catalyze the reductive dechlorination of chlorophenols, benzamides and halobenzoates (38). In addition, it is capable of the dechlorination of perchloroethylene. A few bacterial strains are also able to aerobically carry out aryl reductive dehalogenation, but this process has not been observed for highly halogenated compounds. *D. tiedjei* is a sulfate-reducing bacterium that was isolated on 3-chlorobenzoate and preferentially dehalogenates aromatic substrates at the *meta* position (38). The hydrogen atom that replaces the chlorine substituents originates from water. Some other organisms able to dechlorinate chloroaromatic rings are *Desulfitobacterium hafniense* that dechlorinates tri- and di-chlorophenols (126), *Azotobacter chroococcum* MSB-1 that reductively dechlorinates 2,4-D to 4-chlorophenoxyacetic acid (50), and several *Clostridium* species involved in γ -hexachlorocyclohexane (γ -HCH) dechlorination (67). γ -HCH dechlorination was supported by the electron donors glucose, pyruvate and formate but not ethanol or acetate and was dependent on H_2 production from the fermentation of these substrates.

Little is known about the diversity of PCB-dechlorinating organisms; in fact, no single organism capable of PCB dechlorination has been isolated. Most examples of reductive dehalogenation have been described for complex mutualistic communities (93). It is probable that dechlorination activity is dependent on the metabolic activities of nondechlorinating members of the microbial community. For example,

nondechlorinating microorganisms such as fermenters might produce substrates required for dechlorination or poise the H_2 concentration at a favorable level for dechlorination. Thus, a single organism may be very difficult to isolate until all nutritional requirements are understood; such was the case for *D. tiedjei*, which grew very slowly until these conditions were identified (38).

Highly reducing methanogenic cultures have been shown to readily dechlorinate PCBs (101) and, in a recent study by Zwiernik et al., sulfate reducers were shown to be responsible for extensive PCB dechlorination (142). However, a study by Kim and Rhee describing the population dynamics of PCB dechlorinators, sulfate-reducers, and methanogens showed that at least some of the PCB dechlorinators were a population distinct from the methanogens and sulfate reducers (79). Treatments that inhibited either methanogens or sulfate reducers indicated that sulfate reducers were not directly involved in the dechlorination of PCBs. However, the inhibition of methanogens decreased the extent of dechlorination and changed the dechlorination pattern observed. MPN analysis corroborated the hypothesis that there were two different dechlorinating populations, one that required the presence of methanogens and the other that dechlorinated independently. The independent dechlorinators were postulated to be dehalorespirers that could derive energy from the dechlorination of PCBs and would have a competitive advantage over other organisms in PCB-rich environments (32).

Most successful enrichment cultures capable of PCB dechlorination have been conducted using sediment slurries, where the sediment presumably supplied the carbon source required (39) (51, 118). More recently, Wu et al. reported the development of an enrichment culture in sediment-free medium with unique dechlorinating capabilities

(138). The established culture was found to attack only doubly flanked chlorines in the *para* position. Before this, only *ortho*-dechlorinating cultures had been successfully transferred to a sediment-free medium. The authors also noted that this dechlorination occurred in the absence of methanogenesis, indicating that dehalorespirers were responsible (138). However, more specific information on the members of the microbial consortium was not given. May et al. transferred a dechlorinating enrichment consortium to anaerobic slants prepared with mineral medium and river sediments without PCBs. One colony was picked from a slant and transferred to a sterile anaerobic sediment slurry was able at first to produce methane and dechlorinate four PCB congeners used to develop the original enrichment. However, when the dechlorinating culture was plated on solid media a second and third time the colony lost both its methanogenic and dechlorination activity (90).

2.B.2.b.2. Patterns of Dechlorination

Different patterns of PCB dechlorination have been observed in various cultures (19, 127, 139). The patterns are referred to as *o*, *m*, or *p* dechlorination based on the position of the chlorine substituent removed with respect to the carbon bond (Figure 2.3). Up to 10 different dechlorination patterns have been described in the literature, with each pattern having a different range of congeners it is able to dechlorinate. The cause of these specificities could be at the level of enzymes, organisms, or broad physiological groups. However, it is difficult to determine the mechanism responsible for the different patterns as no single organism has been isolated that carries out these processes.

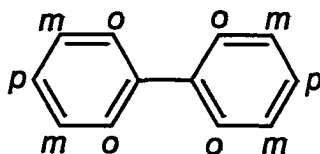


Figure 2.3 Position labeling on PCBs

The first reports of *in situ* dechlorination described occurred in the Hudson River and showed only *meta* and *para* dechlorination of PCBs (31). Brown et al. observed several distinct dechlorination patterns with respect to the congeners dechlorinated and the position at which they were dechlorinated. Brown and colleagues designated these different patterns by letters and by 1987 had observed other patterns at several different locations (32). These patterns were distinguished by the correlation of the disappearance of specific congeners with increases in others and on the ability to carry out a mass balance on the process. Pattern recognition is difficult to distinguish with studies on select congeners, and thus studies with full Aroclors are ideal to determine which dechlorination pattern is active.

Two of the described dechlorination patterns have been labeled M and Q. These patterns exhibit the most extensive dechlorination of *meta* and *para* chlorines, respectively (16). Both remove chlorines without the presence of an adjacent chlorine, in contrast to all of the other patterns that have this requirement. Process M has been observed in sediment inoculated with microorganisms from Silver Lake sediment (111), and from sediment microorganisms from the Hudson River (140). Process Q has also been observed in Hudson River sediments (112). Table 2.2 shows a summary of the dechlorination patterns and their reactivity to different substituents. Processes M and N

remove exclusively *meta* chlorines and Process P removes only *para* chlorines. The other processes remove both *meta* and *para* chlorines, but with different specificities. A combination of two or more of these processes would be ideal for degradation of complete Aroclor mixtures. For example, processes M and Q combined could carry out virtually complete removal of *meta* and *para* chlorines. The sources of the sediments with which these studies have been performed are the Hudson River, Silver Lake, New Bedford Harbor or Woods Pond (111, 112, 140).

The presence of several of these dechlorination patterns at a number of diverse locations suggests that the enzymes responsible for PCB dechlorination may also serve some other important function making them common among microorganisms. Another scenario is that these enzymes, with nearly identical substrate specificity on different Aroclor mixtures, evolved separately in response to PCBs that have only been in the environment for 60 yrs. Several groups have observed that the reactivity of a *meta* or *para* chlorine is partly determined by the number and position of the chlorines on the same ring, as well as those on the opposite ring (16, 134). For example, doubly flanked chlorines are more reactive than singly flanked chlorines, which are more reactive than unflanked chlorines, and isolated chlorines on monochlorophenyl rings are least reactive (Fig 2.4).

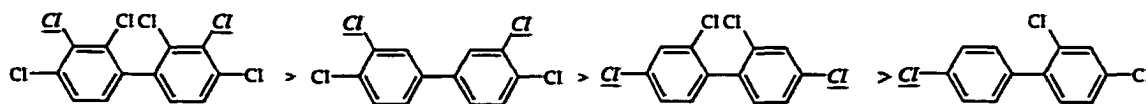


Figure 2.4 Reactivity of chlorine substituents

Cases of *ortho* dechlorination have been reported infrequently, but this pattern of dechlorination does occur (43, 80). Van Dort and Bedard observed sequential *ortho* and then *meta* dechlorination of 2,3,5,6-tetrachlorobiphenyl in Woods Pond sediment slurries (127). Montgomery and Vogel reported dechlorination of 2,3,5,6-tetrachlorobiphenyl by an anaerobic phototrophic enrichment culture (96). 68% of the dechlorination occurred at the *ortho* position to di- and tri-chlorobiphenyls from the tetrachlorobiphenyl. However, the reports have not yet been labeled by a letter in the same way that the previously discovered *meta* and *para* patterns have. To date, no reports of *in situ* *ortho* dechlorination have been made; all of the information on this pattern has been gained with enrichment cultures.

Table 2.2 Chlorophenyl reactivity patterns of various PCB Dechlorination Processes (16)

Dechlorination Reaction	M	Q	C	H	H'	P	N
3 → 0	?		+				
4 → 0		+	+				
23 → 2	+	+	+		+		
24 → 2		+	+				
25 → 2	+		+				
34 → 3		+	+	+	+	+	
34 → 4	+		+				+
234 → 2		+	+				
234 → 23		?				+	
234 → 24	+	?		+	+		+
236 → 26	+	?	+		?		+
245 → 2			+				
245 → 24	?						+
245 → 25		+	+	+	+	+	
2345 → 24	NA ^a	NA	NA				+
2345 → 235	NA	NA	NA	+	+	+	

a Data not available

The microorganisms responsible for specific dechlorination patterns have not been conclusively established; however, some evidence exists for specific microorganisms in several patterns. The Process M dechlorination is believed to be carried out by sulfate-reducing spore-formers (140). When molybdate, an inhibitor of sulfate-reducers, was added, no dechlorination was observed. However, dechlorination was observed when pasteurized cultures were used: indicating spore-formers were carrying out the process. In addition, these same cultures were tested with specific antibiotics that indicated that they were also gram-negative microorganisms. However, other studies have not been performed to confirm this result.

Another study by Ye et al. (1994) provides evidence that process H dechlorination is carried out by methanogens. Work by May and co-workers indicated a simultaneous loss of methanogenic and dechlorinating activity in process H dechlorinating colonies plated on solid agar supports this theory.

2.B.2.b.3. Time Course of Dechlorination

Dechlorination of PCBs *in situ* occurs very slowly. Boyle et al. were able to calculate a rate of dechlorination of 2,3,6-trichlorobiphenyl to 2,6-dichlorobiphenyl per dechlorinating cell using a most probable number technique to determine the concentration of dechlorinating microorganisms. This rate was determined to be 1.13 pmol/day per dechlorinating cell; ten times slower than that reported for the dechlorination of 3-chlorobenzoic acid by *D. tiedjei* (27, 123).

2.B.2.b.4. Factors affecting rate and extent of PCB dechlorination

Many environmental factors important to successful bioremediation in general also apply to the specific case of degradation of polychlorinated biphenyls. Some of these factors include redox level, temperature, pH, available carbon, adsorption to soil particles, and trace metals. No detailed study has investigated all these factors, but some information on their effects on PCB dechlorination is known. PCB dechlorination is believed to be associated only with low redox levels, such as those associated with sulfate-reducing or methanogenic conditions (16).

The effect of temperature on PCB dechlorination is relatively unknown, although dechlorination occurs *in-situ* in the Hudson River where sediment temperatures are below 12 °C for half the year (31). A laboratory experiment by Tiedje et al. observed dechlorination at 12 °C, a dechlorination rate twice as fast at 25 °C and no dechlorination at 37 °C (125). Wu and co-workers investigated not just the effect of temperature on the rate of dechlorination but also the effect on the pattern of dechlorination observed (137). Up to 6 different temperature dependent patterns were observed that were attributed to different polychlorinated biphenyl-dechlorinating populations with different selectivities at each temperature. For example, at 15 °C *ortho* dechlorination dominated while at 20 °C, *para* dechlorination dominated (137).

2.B.2.b.4.a. Alternate Electron Acceptors

In reductive dehalogenation, the highly halogenated molecule may serve as an electron acceptor. Microorganisms with the ability to use PCBs as an electron acceptor (dehalorespiration) might have a selective growth advantage. The presence of electron

acceptors that compete with PCBs can significantly influence the rate and extent of dehalogenation. This might occur by affecting the flow of energy via intracellular channeling of electrons or interspecific competition for electron donors. However, the relationship between competing electron acceptors and dechlorination is complex. For example, sulfate, an electron acceptor used by sulfate-reducing bacteria, has been observed to both inhibit and stimulate dechlorination in different studies (21). Alder et al. used sediment slurries from the Hudson River, Silver Lake, and New Bedford Harbor to investigate dechlorination under both sulfidogenic and methanogenic conditions. Under sulfidogenic conditions, no dechlorination was observed throughout 17 months of incubation (5). However, under methanogenic conditions, dechlorination was observed in all three types of sediments with the highest dechlorination rates observed in Hudson River sediments. Morris et al. (1992) investigated the effects of sulfate, nitrate, ferric oxyhydroxide, pyruvate and BESA on the dechlorination of Aroclor 1242 in sediment slurries. Those cultures amended with either pyruvate (methanogenic) or nitrate (nitrate-reducing) showed the most extensive dechlorination (39% in 12 wks). Those sediments amended with ferric oxyhydroxide showed somewhat slower dechlorination (34% in 12 wks) while those amended with sulfate had significantly slower dechlorination (11% in 12 wks) (98). Unfortunately, none of these studies attempted to determine what effect the amendment had on the microbial population composition, and thus it is hard to extrapolate these results to other situations. A critical factor in whether a certain electron acceptor is inhibitory to PCB dechlorination is the presence or absence of populations capable of using the alternate electron acceptor. In addition, certain electron acceptors

may induce growth of a nondehalogenating population. These nondehalogenaters may outcompete dehalogenaters because of their higher growth rate (93).

2.B.2.b.4.b. Electron Donors

In addition to an electron acceptor, biochemical reactions require an electron donor. In anaerobic systems, the electron donor can take many forms, e.g. hydrogen, natural organic matter. In some systems, natural organic matter in the soil can act as an electron donor. The primary means by which enhancement of anaerobic dechlorination has been investigated is the addition of an organic electron donor such as lactate, glucose, methanol, or other readily degraded compounds (80, 103). The addition of an electron donor should theoretically enhance PCB dechlorination by providing the reducing equivalents needed for PCB reduction and/or to support growth of the dechlorinating populations, thus increasing PCB dechlorination. However, little research has been done to understand why a certain electron donor enhances dechlorination in some environments and not others.

Addition of an electron donor will affect PCB transformation in complex ways based on environment-specific factors. Environments will differ not only in the fermentation pathways present to degrade the electron donor but also in the effect a certain addition will have on the microbial community. A certain electron donor may select for a microbial population that may or may not have a positive effect on PCB dechlorination. Alder et al. found that additions of volatile fatty acids stimulated dechlorination in carbon-limited Hudson River sediment slurries, but not in Silver Lake or New Bedford Harbor slurries that were carbon rich (5). Nies and Vogel performed a

study where several different electron donors were added to anaerobic sediment enrichments and then monitored for patterns and rate of dechlorination of PCB congeners. They observed an initial rate of dechlorination that was similar for acetate-, glucose-, and methanol-amended slurries, while those amended with acetone had a lower initial rate (103). However, those slurries amended with acetone continued to dechlorinate over a longer period of time with less carbon added, indicating that for long-term studies, acetone was the best substrate. Although each electron donor enhanced dechlorination to a different extent, the individual congeners that were dechlorinated were similar for each treatment.

Löffler et al. demonstrated that evidence of halorespiration could be determined by H_2 concentration measurements. In addition, the presence of halorespiring bacteria could be determined in mixed cultures even though no pure cultures were able to be cultured (87). A disadvantage of this method is that the presence of manganese or iron reduction can interfere with the determination of halorespiration as they have similar hydrogen concentrations. A model by Lovley et al. predicts steady-state threshold concentration of H_2 in the following order: acetogens>methanogens>sulfate reducers>Fe(III) reducers>Mn(IV) reducers>denitrifiers. Based on this model, chlororespiring microorganisms should reduce H_2 concentrations to levels very close to iron reducers and manganese reducers. Thus, dechlorinating bacteria should outcompete methanogens for H_2 at low H_2 concentrations while methanogens would prevail at high H_2 levels (118). Thus, a deliberate choice of electron donor has significant implications for which microbial population will be selected based on the rate at which H_2 is released by the fermentation of the electron donor.

In addition, the H_2 concentration is not a predictor of the *extent* of dechlorination, only that halorespiration is occurring. Also, this method might not be a good indicator of cometabolic dechlorination, which may occur under methanogenic or sulfate-reducing conditions. Additions of various nutrients to act as electron donors may also effect other aspects of the dechlorination process. In some cases, these compounds may serve to support the growth of dehalogenating organisms, or may stimulate dehalogenation by supporting the growth of nondechlorinating populations required for a syntrophic association with the dechlorinators.

2.B.2.b.4.c. Priming Dechlorination Activity

Often, researchers have investigated the use of alternative compounds to “prime” dehalogenation or enrich for dehalogenating microorganisms. Possible mechanisms for the observed dechlorination enhancement due to priming are the selection and enrichment of a microbial population with a preferred substrate or by the induction of PCB-dechlorinating enzyme activity without growth stimulation. A study by Kim and Rhee showed that the number of PCB-dechlorinating microorganisms increased 200 fold as a result of priming with other PCBs (79). Wu et al. (1997) observed an even larger increase in the level of PCB dechlorinating microorganisms (as evidenced by most probable number techniques) using bromobiphenyls as priming agents (137). These compounds have been shown to induce dechlorination of the compounds of interest even when the priming compounds themselves were not transformed (93). The more typical case where both the priming agent and the halogenated compound of interest are dehalogenated has been reported often (18, 19, 93, 127). Deweerd and Bedard found that

PCB dechlorination continued long after the priming agents, halobenzoates, had been depleted. This indicates that the enriched dehalogenating populations survived long after the halobenzoates were gone (37).

2.B.2.b.4.d. PCB Concentration

The correlation between PCB concentration and the rate and extent of dechlorination is also not completely understood. This correlation can most easily be investigated in laboratory studies where all other factors can be controlled. In one such study, when Aroclor 1242 was added to anaerobic sediment slurries, dechlorination was greatest in both rate and extent at 700 mg PCBs/kg (sediment dry weight), and undetectable at 14 mg/kg (112). This study indicated a critical concentration of PCBs below which dechlorination cannot occur. The critical concentration may be based on the threshold for enzyme induction of dehalogenases or on the need for the microbial growth rate on PCBs to be greater than the death rate. Rhee et al. also observed a persistence of low levels of several congeners even after 24 months of incubation (113). This is an important concern for environmental PCB remediation, as low-level contamination is widespread. More recent work by Abramowicz et al. showed that dechlorination occurred at total PCB concentrations as low as 20 mg/kg (3). Above this critical concentration, higher PCB concentrations appear to increase the dechlorination rate. This may be attributed to the growth of dechlorinating populations above this critical concentration that in turn are able to dechlorinate at a higher rate. However Abramowicz and co-workers also showed that the dechlorination rate leveled off above a certain concentration. The authors attributed this to a dependency of the rate of dechlorination

on the aqueous concentration of PCBs in sediment pore water (3). Some conflicting results have been obtained when using surfactant to enhance biodegradation.

The issue of PCB concentration effect is closely related to the concept of bioavailability. Most analyses assume that only the soluble fraction of the PCBs is available to microorganisms. In addition to being relatively insoluble in water, PCBs sorb very strongly to sediments and soils, thus diffusion of the PCBs from the matrix to the aqueous phase may control the rate of dechlorination (115). PCBs in contaminated media may be so strongly sorbed to the polymeric humic matrix of aged organic matter that it is unavailable for microbial use.

2.B.2.b.5. Abiotic Dechlorination

Reductive dechlorination is typically believed to be biologically dependent, but not necessarily biologically catalyzed. In addition to reductive dechlorination by microorganisms either cometabolically or for energy benefit, researchers have shown that microbial corrinoids can catalyze reductive dechlorination of not only PCBs, but also 1,2-dichloroethane, perchloroethylene and hexachlorobenzene (59). One such corrinoid, vitamin B₁₂, consists of a cobalt atom coordinated at four positions by the nitrogen of a corrin ring. It exists in the +3 state under normal redox conditions but under reduced conditions it can exist in a +2 or +1 state. Corrinoids are widely distributed among bacterial species. As the conditions become more reducing, vitamin B₁₂ becomes a stronger electron donor. In this reaction, the proton added to the biphenyl ring in place of the chlorine substituents is extracted from water (11).

Two different studies have shown that vitamin B₁₂ can catalyze the reductive dechlorination of PCBs (Woods et al., 1999; Assaf-Anid et al., 1992). Neither study attempted to show that vitamin B₁₂ released by anaerobic microorganisms was the cause of observed dechlorination in an actual soil microcosm study. In both studies, the amount of dechlorination observed was dependent on the redox state of the vitamin B₁₂. When dithiothreitol was used as a reducing agent, dechlorination of pentachlorobiphenyls on the order of 3.5% was obtained (Assaf-Anid et al. 1992). When titanium citrate was used (providing much lower redox conditions) nearly 40% dechlorination of the same pentachlorobiphenyl was observed by a different research group (Woods et al. 1999). Using vitamin B_{12s}, chloride was removed equally from all three chloride substitution positions, *ortho*, *meta*, and *para*; the product distributions agreed with calculations based on free energies of formation.

The studies by Woods et al., Assaf-Anid et al., and Gantzer et al. suggest that the dechlorination reactions carried out by vitamin B₁₂ are limited to dechlorination of aromatic rings containing five or six chlorines. This has not been shown to apply to microbially mediated dechlorination; dechlorination of PCBs to dichlorobiphenyls has been observed. However, a study by Woods and Smith found dechlorination of several trichlorophenols to dichlorophenols using vitamin B_{12s} (136). These authors also found that a thermodynamic correlation using Gibbs free energy values and redox potential was able to describe nearly all of the regiospecificity of the vitamin B_{12s} induced dechlorination of chlorophenols.

2.B.2.c. Aerobic Degradation of PCBs

Aerobic PCB degradation by microorganisms is well documented starting with the first reported evidence by Ahmed and Focht in 1973 of the PCB-degrading ability of two *Achromobacter* species (4, 13). Ahmed and Focht showed that degradation of the unsubstituted aromatic ring of select PCB congeners occurred and that this degradation led to a build-up of chlorobenzoic acids. This led to the conclusions that PCB congeners with low numbers of chlorine substituents are oxidized by addition of oxygen to the biphenyl ring. Biphenyl compounds with fewer chlorine substituents are more reduced and therefore more susceptible to oxidation. Some bacterial isolates are able to degrade congeners with up to seven chlorines, but in most studies those congeners with five or more chlorine atoms have been resistant to aerobic attack (62). Other structural features besides the degree of chlorination appear to affect the biodegradability of PCBs. In an early paper by Furukawa et al., it was reported that PCBs containing two chlorines on either the *ortho* position of a single ring (2',6'') or on both rings (2,2'') were much less susceptible to biodegradation (58). This phenomenon was also observed by Crawford et al. and is believed to be due to a blocking effect of the enzyme responsible for the first step in the breakdown of less chlorinated congeners.

2.b.2.c.1. Biphenyl Dioxygenase Pathway

The primary enzymatic pathway utilized for the aerobic breakdown of PCBs is shown in Figure 2.5 with the corresponding genes labeled in each step. This multi-component enzyme system was shown to be similar to other multi-component aromatic hydrocarbon dioxygenases by Gibson and co-workers (64). Biphenyl dioxygenase

catalyzes the first step in the process to produce a chloro-biphenyl-2,3-dihydrodiol. This chromosomally encoded, single copy gene occurs in many pseudomonads and other closely related genera (45). It is also able to catalyze the breakage of the biphenyl ring of hydroxylated biphenyls and chlorohydroxybiphenyls (119). The second step in the pathway occurs by attack of a dihydrodiol dehydrogenase (bphB) to produce 2,3-dihydroxybiphenyl. This compound is subsequently cleaved via another dioxygenase reaction (bphC) between carbon atoms 1 and 2 (*meta*-cleavage) to produce 2-hydroxyl-6-oxo-6-phenylhexa-2,4-dienoic acid. The bphC gene encodes a 2,3-dihydroxybiphenyl dioxygenase that has been shown by Furukawa et al. to be extremely variable between PCB degradative strains (56). The last step in the pathway is an oxidation of the *meta*-cleavage product to produce a chlorinated benzoic acid and a smaller aliphatic fragment. This final gene (*bphD*) encodes for a hydrolase. Most organisms capable of degrading PCBs are unable to degrade the resulting chlorobenzoic acids; this step is carried out by other microorganisms in the community.

Work by several groups suggests that aromatic ring dioxygenases, including biphenyl dioxygenase, may have evolved from a common ancestor (57, 102). Furukawa et al. observed homology between the *tod* and *bph* operon and found a similar gene organization between the two.

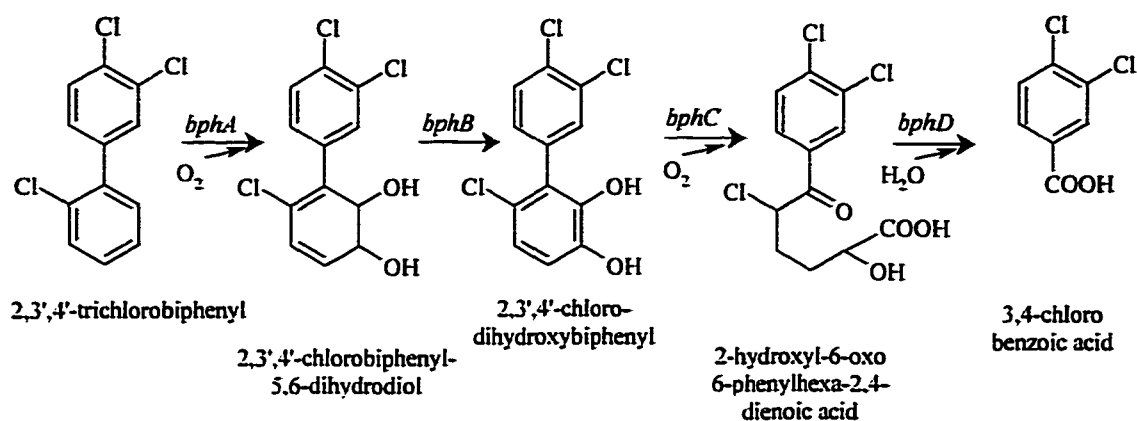


Figure 2.5 Biphenyl dioxygenase pathway for degradation of 2,3',4'-trichlorobiphenyl

Some aerobic PCB degradation occurs through a cometabolic process wherein the enzyme 2,3-biphenyl dioxygenase is induced by another compound (typically biphenyl or monochlorinated biphenyl) (20, 33, 53). Early work by Baxter et al. found that some congeners that were degraded slowly when present as the sole carbon source were degraded much more rapidly in the presence of biphenyl or less chlorinated congeners (14). In addition, acetate and carbohydrates have been shown to induce the 2,3-biphenyl dioxygenase enzyme.

While the 2,3-dioxygenase attack has been most extensively researched and described in the literature, other metabolic pathways for the aerobic degradation of PCBs have been elucidated. Several organisms have been shown to contain multiple *bphC* genes (12, 89, 116). Seto and co-workers observed at least four genes encoding 2,3-dihydroxybiphenyl *meta*-cleavage activity. A mutant deficient in the *bphC* gene was able to transform 4-chlorobiphenyl to 4-chlorobenzoate, indicating that the genes responsible for ethylbenzene catabolism may also catalyze PCB degradation (116).

2.B.2.c.2. Microorganisms

Many organisms have been isolated that have a wide substrate range for less chlorinated polychlorinated biphenyls. Members of a wide range of bacterial genera are capable of degrading PCBs, including *Acinetobacter*, *Pseudomonas*, *Rhodococcus*, and *Arthrobacter*. In addition to these bacteria, white-rot fungi have been shown to degrade low levels of PCBs but with a smaller congener range than bacteria (141). A study by Pellizari and co-workers found that PCB degradative capability did not correlate well with the presence of PCBs in the matrix from which they were isolated, indicating considerable diversity of PCB degradative genes in nature (108). Several exceptional strains of bacteria have been isolated that can biodegrade a wide range of congeners, sometimes including penta- and hexachlorobiphenyls. The first of these, *Alcaligenes eutrophus* H850, was found to attack PCBs that had no unchlorinated 2,3 sites and could hydroxylate at positions 3,4 on the phenyl ring (15). *A. eutrophus* H850 also produced a unique metabolite, 2',3'-dichloroacetophenone, in addition to chlorobenzoic acids. In addition to an exceptional ability to degrade tetra-, penta-, and several hexachlorobiphenyls, it can also degrade Aroclor mixtures.

Another strain with similar degradative capabilities and breadth is *Pseudomonas* sp. LB400, a gram-negative organism isolated from PCB-contaminated sediment from the Hudson River Bay (23). Using sequence homology of the *bph* operon, it has been shown that there are at least two different classes of PCB genes (95). Mondello and co-workers found that the *Pseudomonas* LB400 operon only demonstrated homology with the operon from *Alcaligenes eutrophus* H850, which interestingly also has a substrate

range similar to the LB400. No homology with 6 other strains was observed. In addition, the biphenyl dioxygenase operon of LB400 was shown to have significant homology to components of the toluene dioxygenase genes from *P. putida* (46). The amazing substrate specificity of the LB400 and H850 was found to be due to only slight differences in nucleotide sequences of these strains operon compared to that other strains, for example *Pseudomonas* KF707 (60). Gibson and co-workers found evidence that single amino acid substitutions may play a pivotal role in determining congener specificity. Using site-directed mutagenesis, Erickson and Mondello were able to modify the LB400 bphA gene so that it displayed broader congener specificity with increased activity for several congeners characteristically used by the KF707 (46).

In addition to these well-characterized strains, many environmental isolates have been shown to degrade PCBs aerobically. Williams and co-workers isolated several PCB-degrading strains from PCB-contaminated Hudson River sediment (135). Each isolate was characterized by its 16S rRNA gene sequences. Several isolates had identical 16S rDNA sequences to *A. eutrophus* H850, indicating that they are likely different strains of the same species. These isolates also exhibited a similar substrate range to H850; they have strong abilities to degrade a broad group of congeners, but are unable to degrade congeners substituted at both *para* positions (135). In addition, the authors were able to determine that two previously isolated strains, *Corynebacterium* sp. strain MBI and *Arthrobacter* sp. strain M5, were members of the same genus and had both originally been mis-identified. These two strains were determined to be members of the *Rhodococcus* genus; they are closely related to another PCB-degrading isolate, *Rhodococcus* sp. strain RHA1, and have similar degradative capabilities. Strain RHA1

degrades both *ortho*- and *meta*-chlorine substituted PCBs with high activity levels (116). Bedard and co-workers developed an assay to rapidly screen and characterize microorganisms for their ability to degrade polychlorinated biphenyls (17). To test each microorganism, they used defined mixtures of PCB congeners ranging from dichloro- to hexachlorobiphenyls that represented the different classes of PCBs (e.g., congeners chlorinated on a single ring, or those blocked at 3,4 sites). Using this method, they tested 25 environmental isolates and were able to identify strains with superior PCB-degradative ability.

The enzymatic pathway shown in Figure 2.5 is often described as the upper pathway and the pathway required for the breakdown of chlorobenzoic acids is referred to as the lower pathway. Few pure cultures have been isolated that are able to degrade both PCBs and the chlorobenzoic acids produced from breakage of the biphenyl ring (9). In fact, Guilbeaut et al. found that the buildup of chlorobenzoic acids inhibited further PCB degradation (62). Work by Pettigrew et al. investigated a 3-membered bacterial consortium able to completely mineralize 4-chlorobiphenyl (4-CB). It included three organisms: a *Pseudomonas testosteroni* that catalyzed the breakdown of the 4-CB to 4-chlorobenzoic acid, an *Arthrobacter* species that degraded the 4-chlorobenzoic acid, and a third strain whose role was not elucidated (109).

2.B.2.c.3. Enhancement of Aerobic Degradation

Because aerobic PCB degradation has been shown to be cometabolic in many studies, use of a substrate analog such as biphenyl has been investigated as a means to improve the aerobic degradation process. Guilbeault et al. showed that analog

enrichment selected for oxygenases with increased activities toward the less chlorinated PCB congeners (62). However, they observed no enhancement towards more highly chlorinated congeners. Focht and Brunner observed roughly a 50% conversion of PCBs to $^{14}\text{CO}_2$ in soil microcosms with biphenyl enrichment whereas only a 2% conversion to $^{14}\text{CO}_2$ was observed in unamended microcosms (53). The use of naturally occurring substrates with structures similar to biphenyl for the enhancement of PCB degradation was investigated by Hernandez et al. (68). Soils amended with orange peels, eucalyptus leaves, pines needles and ivy leaves had higher levels ($10^8/\text{g}$) of biphenyl utilizing bacteria than did unamended soils ($10^3/\text{g}$) and had complete degradation of PCBs within six months which was not observed in unamended controls. These results indicate that the enzymes used for biphenyl degradation are designed to attack natural substrates and is an important finding for implementation of *in situ* biodegradation for PCBs. The addition of biphenyl to contaminated soil is unreasonable, as it is a priority pollutant itself, but these naturally occurring substrates could easily be added to soil that is to be remediated.

Several researchers have investigated bioaugmentation of soil microcosms with either naturally occurring microorganisms with superior PCB degradative abilities or with microorganisms genetically engineered to enhance their degradative abilities (29, 53, 94). One of these groups, Mokross et al., constructed a hybrid *Pseudomonas* strain that had the upper pathway of a *Pseudomonas putida* strain isolated from a biphenyl enrichment combined with the lower pathway from another *Pseudomonas* species (94). The constructed hybrids were able to completely mineralize 3-chlorobiphenyl. In later work, this group was able to generate hybrid strains that could mineralize 2-, 3-, 4-, 2,4-, and 3,4-chlorobiphenyl (66). Focht and Brunner inoculated soil microcosms with

Acinetobacter strain P6, a microorganism able to degrade more highly chlorinated congeners, but did not observe an overall increase in mineralization of PCBs. However, they did observe a higher rate and extent of degradation of more highly chlorinated PCB congeners (53). In a second study, this same group observed a 20% increase in degradation when analog enrichment with biphenyl was combined with inoculation with *A. strain* P6 (33). An aerobic slurry reactor treatment using a combination of two bacterial strains, ENV307 and ENV360, achieved 80% degradation of PCBs in soils containing 300 ppm PCBs. These two strains are complementary in their substrate specificities and can attack tetra-, penta-, and hexachlorobiphenyls (71). While many of these results seem promising, none have been performed under conditions simulating a real environment, and thus their applicability to *in situ* applications cannot be determined.

Lajoie and co-workers investigated bioaugmentation with a strain of *Pseudomonas* engineered to express the *bph* genes without the presence of biphenyl. This strain, that utilizes a water-soluble surfactant as a selective growth substrate, was added to soil systems and increased the PCB degradation in these systems (82). Focht and co-workers demonstrated that the addition of chlorobenzoate-degrading bacteria increased the extent of mineralization of PCBs in soils where no indigenous chlorobenzoate degraders were present; this effect was thought to be due to the elimination of inhibition of the PCB degraders by the chlorobenzoates (70). Springael and co-workers addressed the issue of PCB contamination in mixtures with heavy metals by expressing the PCB catabolic genes in a heavy metal resistant strain, *Alcaligenes eutrophus* (120). Further studies are needed with this strain to determine if it is able to

express both the catabolic and metal resistance markers under conditions representative of real situations.

Only a limited number of studies have been done under actual field conditions to look at full-scale bioremediation of PCBs. In one such study, McDermott and co-workers used a landfarming technique in which contaminated surface soils were well mixed with nutrients and inoculated with PCB-degrading bacteria. The test plot achieved up to 20% degradation over the four-month test while no significant degradation was observed in a control plot. This amount of degradation was far less than that observed in similar laboratory-scale investigations. It was unclear whether the nutrients, the inoculation, or some combination of both had enhanced the degradation. The reason behind this was postulated to be the decreased bioavailability in the large-scale treatment (91).

Another approach to enhancing aerobic degradative activity is the addition of surfactants to solubilize the PCBs so they become more bioavailable to the microorganisms. Billingsley et al. compared the effect of the presence of different ionic and anionic surfactants on the degradation of several PCBs by *Pseudomonas* LB400 (22). Each of the surfactants was found to at least partially solubilize the PCBs above the surfactant's critical micelle concentration. None of the surfactants was observed to cause a decrease in the numbers of *P. LB400*. The presence of anionic surfactants increased PCB transformation while nonionic surfactants inhibited PCB degradation (22). Another chemical treatment to enhance PCB degradation was investigated by Aronstein and Rice (10). Using Fenton's reagent to break down some of the PCBs, followed by inoculation

with *P. sp.* strain LB400 and *Alcaligenes eutrophus* strain H850, overall degradation of 2-chlorobiphenyl increased 2.9 and 7.4 times, respectively.

2.B.2.d. Sequential Processes for Remediation

While both anaerobic and aerobic PCB degradation have been shown to be important in environmental PCB transformation, neither an anaerobic nor an aerobic degradation scheme *alone* is ideal for remediation of highly chlorinated pollutants. Often, anaerobic dechlorination is able to dechlorinate the substrate only to a certain point, leaving compounds that are sometimes more toxic than the original substrate. For example, perchloroethylene is dechlorinated to trichloroethylene, dichloroethylene and sometimes vinyl chloride. Vinyl chloride is more harmful to human and ecological health than is perchloroethylene and is persistent under anaerobic conditions. However, it is readily degraded under aerobic conditions, and thus a sequential treatment process is desirable.

This same strategy applies to polychlorinated biphenyls. While neither an anaerobic or aerobic scheme alone is ideal for complete mineralization of PCBs, a *combination* of the two has great potential for success at degrading PCB mixtures. The anaerobic process removes chlorines from highly chlorinated congeners but is unable to act upon the less chlorinated congeners that are the products of this process. The aerobic degradation process can break the biphenyl ring of less-chlorinated congeners but is unable to act upon the highly chlorinated congeners. In addition, a sequential treatment scheme that involves alternating anaerobic and aerobic periods may ensure that less highly chlorinated intermediates do not accumulate. In some cases these intermediates

are more toxic than the parent compound and may inhibit dehalogenation at higher concentrations (97). Figure 2.6 depicts a sequential treatment scheme where an anaerobic dechlorination stage is followed by an aerobic degradation stage. One of the biggest challenges of these types of remediation strategies is that with the two different metabolic regimes come two different microbial communities that must be understood. In addition, perturbations made to the system under the anaerobic phase affect the viability and composition of the aerobic microbial community. It may also be necessary to understand the effect that aerobic conditions have on anaerobic organisms if the sequential process is to be repeated for more than one cycle.

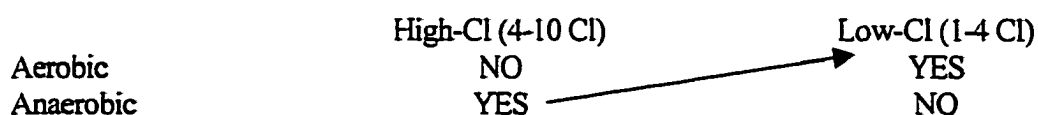


Figure 2.6 Sequential anaerobic/aerobic treatment scheme

Anid et al. did preliminary work investigating methods for inducing anaerobic dechlorination and switching from anaerobic to aerobic culture for degradation of PCBs (7). Sequential treatment was performed in batch serum bottle experiments and showed accumulation of several di-*ortho* substituted congeners. In another work, Anid et al. used a laboratory apparatus that more closely resembled conditions encountered in the Hudson River bottom sediments (7). In this work, they observed slower rates of dechlorination than in slurry, batch incubations but found that the overall sequential process degraded a higher overall percentage of PCBs than either the aerobic or anaerobic process alone.

Evans et al. observed a 70% reduction of PCBs in soil in a sequential process inoculated with river sediment organisms in serum bottle experiments (48). Also using a sequential approach, Shannon and co-workers observed more than 80% degradation of PCBs from sediment contaminated with 1240 ppm of Aroclor 1248 (71). A major field study was also conducted in the Hudson River to determine if indigenous aerobic microorganisms could degrade PCBs present in anaerobically dechlorinated sediments (65). Large caissons were prepared *in situ* and were amended with biphenyl and oxygen. Both mixed and unmixed treatments were investigated. Stimulation of degradation of less chlorinated congeners with a concomitant production of chlorobenzoic acids was observed in nearly all treatments with a 37-55% loss over the 73 day study. This study indicated that indigenous aerobic microorganisms could be stimulated after prolonged anaerobic conditions. These studies indicate the utility of a sequential process for PCB remediation. However, connections between engineering process variables (electron donor addition, cometabolite addition) and microbial population responses were not established. Without these connections and the use of an experimental set-up that begins to approximate natural systems, it is impossible to maintain that the observed results will be applicable to other systems.

2.B.2.d.1. Initiation of Aerobiosis

To carry out a successful sequential anaerobic/aerobic process, the conditions in the experimental system, or natural environment, must be switched from anaerobic to aerobic conditions. This requires the addition of oxygen to the system that can be done through addition of air, pure oxygen or hydrogen peroxide. Oxygen is sparingly soluble

in water (~8.5 mg/L under 1 atm air, 44 mg/L under 1 atm oxygen), making it difficult to introduce enough oxygen for aerobic microorganisms to survive. This is particularly true if the quantity of the contaminant is great and the microorganisms achieve high growth rates. To resolve this problem, hydrogen peroxide (H_2O_2) is often added as a substitute for oxygen or air sparging. H_2O_2 decomposes to oxygen and water and is very soluble in water, making the possible O_2 concentration achievable essentially limitless.

However, the addition of H_2O_2 can be toxic to some cells; as the H_2O_2 decomposes, free radicals are produced that can damage cells. Some cells have developed enzymes to defend against H_2O_2 and to repair associated damages.

Many studies have looked at the use of H_2O_2 to stimulate aerobic degradation of various compounds. These studies sometimes give conflicting results with respect to the utility of H_2O_2 additions. Anid et al. observed that rates of oxygen utilization were proportional to the added amount of H_2O_2 and an increase in microbial plate counts in all samples amended with H_2O_2 (8).

2.C. *Micobial Ecology*

In any bioremediation scheme, the catalytic entity responsible for the breakdown of the pollutants is a biological catalyst (e.g., microorganisms, plants). In *in situ* remediation efforts, several lines of evidence are required to show that degradation of the pollutants of interest is actually occurring. One of these is a decrease in pollutant concentration with a subsequent increase in degradation products. A second line of evidence is the presence and activity of microorganisms able to carry out the transformation. In addition to proving that degradation is occurring, knowledge of the

microorganisms involved is essential to development of a better remediation scheme. Environmental matrices contain an abundance of microorganisms, most of which are unknown to researchers. These microorganisms occur as mixed cultures of many different types of organisms. These microbial communities can be as complex and diverse as the environmental matrices in which they reside (40).

The different microbial groups in a particular environmental niche can interact, in both positive and negative ways. Knowledge of these interactions is important for predicting biodegradation rates and patterns in complex environments. The composition of the microbial mixture and the substrate degradation are linked. Certain pollutants and natural compounds will select for specific microbial populations; these changes in microbial population composition then affect the rate and extent of degradation of the substrates. To begin understanding the interactions between microbes and the effect these interactions have on the degradation of pollutants of interest, we must first know what microorganisms are present and how their concentrations change throughout a remediation process.

2.C.1. Methods for Enumerating Indigenous Microorganisms

To develop reliable and improved remediation strategies, methods are needed to determine the concentrations of different groups of microorganisms in environmental samples. Many times, before the concentrations can be determined, researchers must determine what types of organisms are indigenous to a certain environmental matrix. Environmental organisms pose particular problems in culturing and enumeration. Traditional methods, including plate-counting techniques and most probable number

procedures, are only applicable to culturable populations. However, numerous studies have demonstrated the futility of using traditional culture-based methods for enumerating environmental organisms (107). Typical estimates are that up to 99% of the existing bacterial species have not been cultured to date. Thus, viable plate counts are rarely quantitative in natural systems.

Some of the reasons for this include our lack of knowledge of the optimum growth medium for these organisms and the interdependence of most microbial consortia. Traditional culture-based techniques always introduce a bias towards culturable microorganisms, and are unable to enumerate microorganisms that are viable but unable to replicate due to stressful conditions (130). In addition to identifying what microorganisms are present in a certain environment, it is important in remediation design to understand which microorganisms are carrying out the action of interest as not all phenotypes will be expressed in a given environment. Thus enumeration of both genotypic and phenotypic group characteristics is critical.

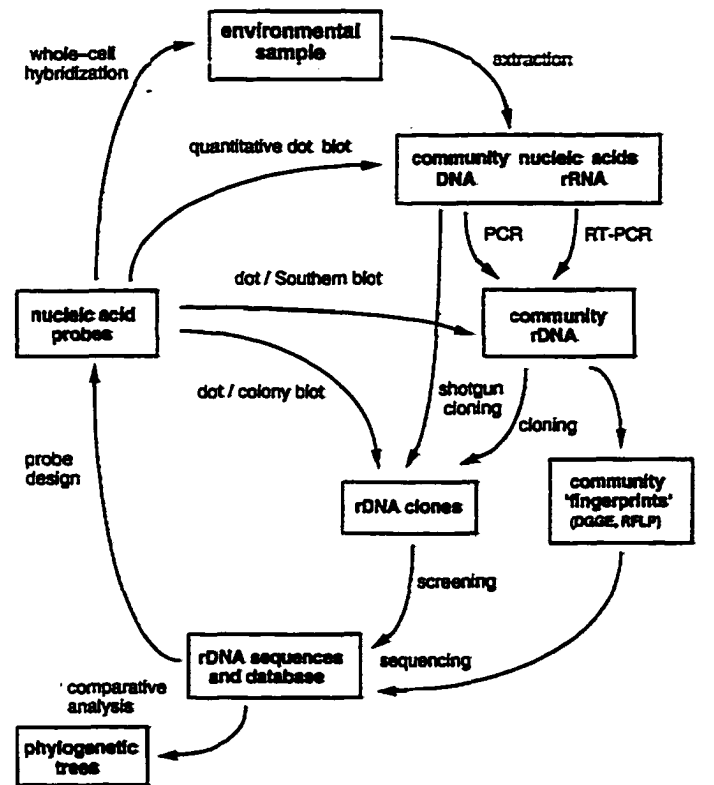
Non-culture based methods rely on identifying microorganisms on the basis of chemical compounds present in the bacterial cells. These chemical compounds can include nucleic acids (genetic based) and carbohydrates and lipids (phenotypic based).

2.C.1.a Genetic-Based Methods

Genetic-based techniques, which rely on DNA sequence differences between species, provide results that are independent of growth conditions and media used. Using these techniques, the microorganisms need not be cultured at all; the DNA or RNA can be isolated directly from samples. Many groups have used genetic techniques to identify

specific microbial groups or genes in environmental applications (6, 15, 35, 52, 78).

Figure 2.7 shows the variety of different methods that can be used to explore the diversity and concentration of microorganisms in an environmental sample.



Arrows indicate the interconnected utility of methods, experimental materials, and information in the study of microbial ecosystems. RT-PCR, reverse transcriptase to copy rRNA into DNA, followed by PCR; DGGE, denaturing gradient gel electrophoresis; RFLP, restriction fragment length polymorphism.

Figure 2.7 Molecular microbial ecology methods for detecting and enumerating environmental microorganisms. (107)

Each of the methods shown begins with extracting microbial DNA directly from the environmental matrix. To begin the process, community nucleic acids are either cloned to determine specific sequences of microorganisms present, or used in a PCR reaction for use in the denaturing gradient gel electrophoresis (DGGE) or restriction fragment length

polymorphism (RFLP) methods. None of this first round of methods is quantitative, but they can be coupled with other methods to provide quantitative data about the microorganisms in a sample. Specific microbial sequences obtained from cloning can then be used to develop probes for either DNA hybridization, which determines the amount of a specific sequence in a particular sample, or for whole-cell hybridization with a fluorescently labeled probe that can be used for visual quantitative analysis of microorganisms in environmental samples. DGGE allows separation of DNA fragments based on the electrophoretic mobility of partially denatured DNA strands and gives an estimate of the community diversity. RFLP is a similar method, but relies on identification based on the restriction endonuclease digestion pattern rather than sequence data.

Biases can be introduced in the initial extraction of DNA from the sample. Some problems encountered are with the reproducible lysis of all cells in a sample as well as with the extraction of intact nucleic acids. In addition, methods based on the polymerase chain reaction as a first step tend to overestimate the abundance of a detected population since PCR fails to detect some DNA fragments that are hard to amplify.

16s ribosomal RNA (rRNA) genes have been widely used for construction of oligonucleotide probes and PCR primers. Different regions of the 16s rRNA have evolved at different rates; this allows differentiation at phylogenetic levels ranging from the subspecies to the kingdom level. As bacteria evolved into different domains, classes, genera and species, these events were recorded in the sequence of rRNAs. These differences in sequence (molecular signatures) form a basis for identifying microorganisms (24). However, microorganisms can have identical 16S rRNA sequences

but also have different physiological activity. For example, Watanabe et al. observed that although two phenol-degrading microorganisms in activated sludge had the identical 16S rDNA sequence; one was a floc-forming bacterium while the other was a non-flocculating bacterium (131).

2.4.2.a.1. Polymerase Chain Reaction

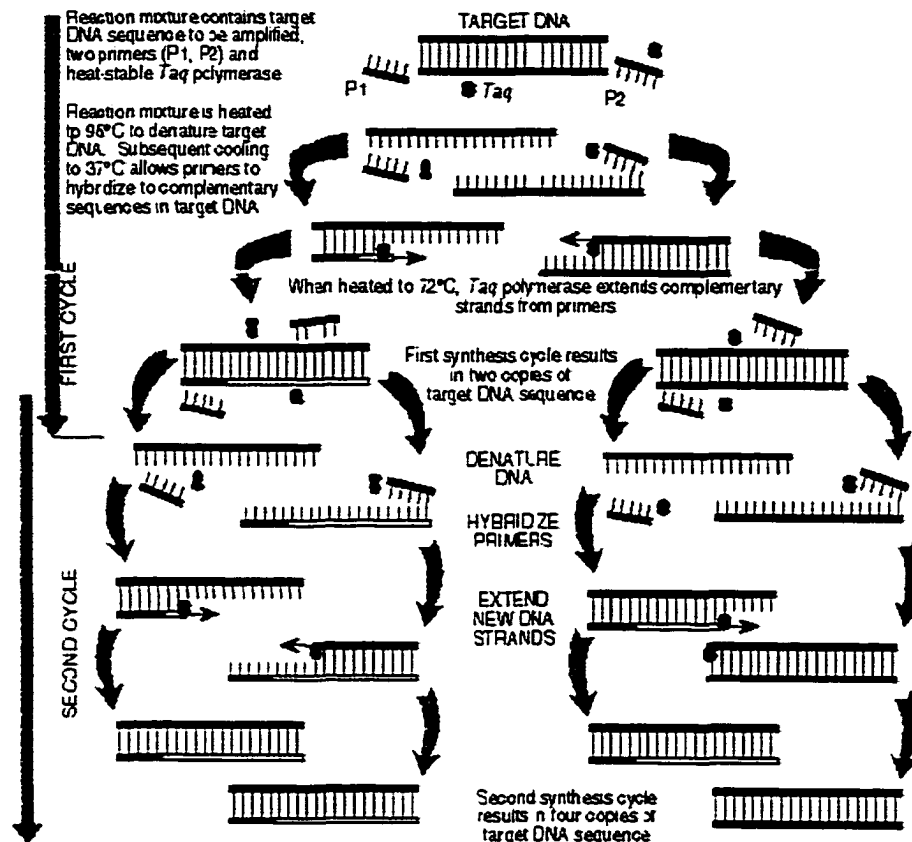
The polymerase chain reaction (PCR) allows the detection of a single target gene of interest through exponential amplification for easier detection. Polymerase chain reaction-based methods depend on differences in the genetic makeup of different species of microorganisms. The rRNA operon is often used as it consists both of highly conserved and highly variable regions. The highly conserved regions can be used as primers to amplify the highly variable regions.

PCR allows amplification of specific DNA by the use of repetitive enzymatic synthesis using two oligonucleotide primers that flank the target DNA of interest. A single PCR amplification cycle consists of three steps and is shown in Figure 2.8. First, the double stranded template DNA is denatured at a high temperature (94 °C). The oligonucleotide primers then anneal to the complementary flanking regions, one on each DNA strand. Finally, primer extension occurs. In this step, DNA complementary to the target region of interest is synthesized. Extension is carried out from the 3' end of each primer and results in a double stranded copy of target DNA from each strand. Taq DNA polymerase catalyzes this primer extension. A thermostable DNA polymerase isolated from *Thermus aquaticus* is used because it can endure repeated heating to 94 °C without loss of activity. In addition, the enzyme lacks any endo- or exonuclease activity. During

each cycle, the number of copies of DNA is doubled. Each step occurs at a defined temperature and time interval.

The strength of PCR methods is their extreme sensitivity to small quantities of DNA of interest. The degree of specificity of primers used in PCR can be varied by either choosing unique DNA sequences for highly specific primers or by using “universal” primers that amplify a DNA sequence conserved in all bacteria. A large set of such universal primers are specific to the 16s rRNA, so theoretically all species in a sample can be amplified. These PCR products can then be used to determine which microorganisms are in a sample and whether or not they correspond to known organisms. To do these comparisons, homologous nucleotide sequences from different organisms are compared and the number of differences in nucleotide sequences is determined. The fewer the differences, the more closely related are the two microorganisms. This approach allows researchers to begin to understand the identity of organisms in their environmental samples.

DNA Amplification Using Polymerase Chain Reaction



Source: DNA Science, see Fig. 13.

Figure 2.8 The polymerase chain reaction, "PCR: Polymerase Chain Reaction (<http://www-biology.ucsd.edu/others/dsmith/classes/pcr.html>)

Pulliam-Holoman et al. monitored changes in a PCB *ortho*-dechlorinating microbial community by analyzing total community 16S rDNA (110). Sequences associated with the highest rates of PCB dechlorination had high sequence similarities to the delta, low G+C gram positive and *Thermotogales* subgroups that include sulfur-, sulfate- and/or iron-respiring bacterial species. Thus, PCB dechlorination may have been carried out by either sulfate- or iron-reducing bacteria. Analysis of microbial communities from aerobic biphenyl-degrading enrichment cultures was undertaken by

Wagner-Dobler and associates (129). By comparing 16S rRNA gene sequences of isolates cultured from enrichments, they found that gram-positive bacteria dominated the microcosms. *R. opacus*, a known polychlorinated biphenyl degrader, was particularly abundant. In addition, *Alcaligenes* sp., *Terrabacter* sp. and *Bacillus thuringiensis* were dominant members of the culturable community. The similarity in dominant populations between samples was surprising as the enrichments were prepared from soils and sediments from a diverse set of environments.

In addition to determining the identity of different microorganisms, analysis of 16S rDNA fragments from PCR can give an indication of the concentration of specific organisms and also help infer the physiology of microorganisms in certain environments. The number of times a particular sequence is present as a percentage of the total number of sequences cloned can be computed to give its relative concentration in the sample as a whole. Work by Dojka et al. used rDNA clone libraries to investigate microbial diversity in samples from an aquifer contaminated with waste fuels and chlorinated solvents (41). Samples were taken from 3 sites with different proposed terminal electron acceptors; a methanogenic zone, a methanogenic-sulfate reducing zone, and an iron reducing-sulfate reducing zone. Differences in microbial population composition and concentrations were found between the sites. The microbial composition between the methanogenic and methanogenic-sulfate reducing zones were very similar, indicating that these zones were really under the same redox conditions and not two separate zones as previously thought. In addition, physiological interactions between two sequence types could be inferred based on their phylogenetic associations. It was hypothesized that acetoclastic methanogenesis was occurring in syntrophic association with acetate and hydrogen

production occurring by anaerobic oxidation of organic acids. Use of reverse transcription-PCR (PCR amplification from extracted RNA as opposed to DNA) was used by Nogales and co-workers to identify metabolically active populations in soil highly polluted with PCBs by cloning and sequencing of RT-PCR products (105).

While the analysis of 16S rDNA sequences has amazing potential to allow for an accurate description of environmental microbial consortia, these methods have limitations and pitfalls. One of these limitations is the generation of laboratory artifacts caused by the introduction of contaminating DNA during experimental procedures (121). Because PCR is so sensitive, any trace contaminating DNA will be amplified and could be mistaken as originating from the environmental sample. This issue is of particular concern in low-biomass environments where the contaminating DNA may be nearly as abundant as the native DNA. Tanner and co-workers investigated this problem by cloning and sequencing PCR amplified 16S rDNA fragments obtained in the absence of template DNA (121). They observed contaminating DNA from the genera *Duganella*, *Acinetobacter*, *Eschericia* and *Leptothrix* among others. The authors recommend running similar controls in each laboratory to detect and analyze potential sources of contaminations. Another potential source of bias in PCR methods originates because different organisms rDNA genes can have varying amplification efficiencies and can be affected by the physiological state of the cells at the time of extraction.

2.C.1.a.2. Quantitative PCR

While PCR is currently the most sensitive method for the detection of specific DNA sequences in environmental samples, a major disadvantage of traditional PCR

methods is that they are not quantitative. It is often necessary to determine not only the presence or absence of particular DNA sequences but also their concentration. The nature of the PCR causes it to be extremely dependent on the initial concentration of template DNA. Because of the exponential nature of PCR, small differences in amplification efficiency can result in dramatic differences in product yield (76). In order to account for these differences, quantitative PCR methods were developed primarily for use in the clinical arena. The four quantitative PCR methods currently in use are: 1) Quantitation using an external standard; 2) Co-amplification of an internal standard; 3) MPN-PCR; and 4) Competitive PCR (76). Using these methods, it is possible to detect and enumerate low concentrations of microorganisms, with great specificity.

Two types of quantitative PCR methods have been utilized for estimating abundances of uncultured soil bacteria; most probable number (MPN) – PCR and competitive PCR (75, 77, 85, 117). The first two methods (quantitation using an external standard and co-amplification using an internal standard) are not as applicable to environmental samples. Tube-to tube variability is more common in environmental samples than in clinical samples. Many environmental samples contain material that can inhibit PCR amplification and the concentration of these substances can vary widely between samples affecting the PCR efficiency. Therefore, using quantitation by extrapolation to an external standard is not well suited for environmental applications; any initial sample differences will cause a large difference in the final PCR product yield due to varying amplification efficiencies. Because different PCR templates have highly different amplification efficiencies, co-amplification with an internal standard can encounter the same difficulties as using an external standard curve.

MPN – PCR is a version of the traditional MPN culture-based technique in that it relies on diluting samples to extinction with several replicates of each dilution. Instead of looking for the presence or absence of growth in culture media, MPN-PCR uses PCR with specific primers and depends on the presence or absence of amplification product. Although this approach is relatively simple methodologically, it requires a large number of replicates and dilutions making it extremely labor intensive. In addition, the MPN method is by nature relatively imprecise.

Competitive PCR

In competitive PCR (cPCR), an internal standard (competitor) with a known DNA sequence and mass is added to the sample prior to PCR. During the PCR reaction, the competitor and target DNA are amplified together, and the ratio of the final mass of competitor to its initial mass is compared with the final mass of the target sequence. This allows for enumeration of the starting mass of the target DNA. It is important that the competitor have a size and primer-binding site similar to those of the target DNA (76). This allows the competitor and target DNA to compete evenly for reagents (e.g. enzymes, primers, dNTPs) for amplification. Any variable affecting the amplification efficiency is assumed to have the same effect on amplification of both the competitor and the target DNA sequence. To quantitate the DNA in a sample absolutely, appropriate standard curves are necessary. To prepare these curves, the competitor DNA is added at a constant known concentration to a dilution series of template DNA. The ratio of the final yield of target sequence to competitor should be equal to the ratio of the initial target to the initial competitor concentrations.

While the development of competitors is more complex than preparation for the MPN-PCR method, once a competitor has been generated multiple samples can be analyzed relatively easily (84). Competitors can be generated by a variety of different methods. The most commonly used methods are deletion-type, nonhomologous, and intron competitors. In deletion-type competitors, the competitor has the same primer binding sites as the target sequence, but is shorter by 100-200 bp. This type of competitor can be constructed by restriction enzyme digestion of the target sequence with subsequent re-ligation of the fragments. Low stringency annealing can be used to produce nonhomologous competitors (76). An intron competitor is the opposite of a deletion type; an internal fragment is added so that the competitor can be recognized based on its larger size compared to the target after amplification.

Methods for accurately quantitating the DNA yields of target and competitor after amplification are: image digitizing of DNA bands on gels; laser densitometric quantitation of DNA bands on gels; phosphorimaging of ^{32}P -labeled DNA bands; enzyme-linked colorimetric detection; and enzyme-linked chemiluminescent detection. For example, after the cPCR has been performed, the amplification products are separated by agarose gel electrophoresis. The gel is stained with ethidium bromide, which can be visualized using a UV-transilluminator. A picture of the gel is saved on disk and digitized using a software program such as Bio Rad's QuantityOne or Image Quant by Molecular Dynamics. The same program is then able to quantitate the amount of signal in each band to produce standard curves of target to competitor.

Several groups have used competitive PCR to track the concentration of microorganisms introduced into a specific environment. Watanabe et al. used cPCR to

enumerate the amount of a bacterial population introduced into an activated-sludge microbial community (131). Their work utilized the *gyrB* gene, which encodes the subunit B protein of DNA gyrase, to produce a strain-specific monitoring system. Using this method, they observed different population dynamics of two phenol-degrading strains introduced into activated sludge under different phenol-loading conditions. Leser et al. investigated the survival of a genetically engineered *Pseudomonas* strain capable of growth on various chloroaromatics into marine ecosystem microcosms (85). Within two days after the microbial introduction, cells became unculturable but were detected by the cPCR method.

Lee et al. were the first to estimate the abundance of an uncultured soil bacterium using a cPCR method (84). Microbial DNA from a field soil was directly isolated and a clone library containing 16S rDNA sequences was constructed. One member of the clone library was then chosen for enumeration by a cPCR method. Recent work by Mesarch and co-workers utilized a cPCR approach to determine the number of dioxygenase genes in petroleum-contaminated soils. The primers were developed and tested using dioxygenase gene sequences from 8 different organisms that utilized dioxygenases for a variety of different carbon sources, including toluene, phenol, biphenyl and benzene (92). While the presence of a catabolic gene does not necessarily correlate to expression, it does suggest the potential for metabolic activity.

2.C.1.a.3. Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) can be used to resolve PCR-amplified regions of genes based solely on differences in nucleotide sequences (106).

This method generates profiles or “fingerprints” of microbial communities that can be used to identify differences in community structure over time or space that occur in response to environmental perturbations. Each DNA fragment on the gel is believed to represent a single or a few phylogenetically distinct populations. Using this information, one can estimate the diversity of a bacterial population. DGGE has been utilized for a wide variety of different applications, from understanding the bacterial biofilm encrusting small marine creatures to investigating a co-culture carrying out sulfate reduction (61, 69, 100, 122).

In a denaturing gradient gel, double-stranded DNA is subjected to increasingly denaturing conditions throughout the electrophoresis that causes it to melt in discrete segments. These segments are called “melting domains” and the melting temperature of each domain is sequence-specific. As the DNA melts, it becomes branched; this reduces its mobility in the polyacrylamide gel. In DGGE, the denaturing conditions are created by a combination of uniform temperature and a linear denaturant gradient formed with urea and formamide. DGGE can be performed with the gradient either perpendicular or parallel to the direction of electrophoresis (Figure 2.9). PCR-amplified DNA is typically loaded onto the gel. Parallel lanes are used to analyze multiple samples on the same gel. DNA bands in DGGE can be visualized using ethidium bromide, fluorescent stains, and silver staining; the last of these is the most sensitive.

While the DGGE method is ideal for detecting differences in microbial populations, the method is not quantitative. Therefore, when quantitative information is needed, DGGE must be used as a complement to quantitative methods, such as cPCR, to relate community structure to function as a bioindicator of environmental changes.

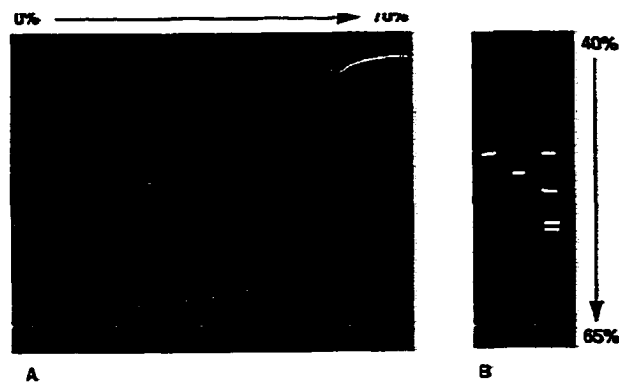


Figure 2.9 Denaturing gradient gel electrophoresis. (A) perpendicular denaturing gradient gel and (B) parallel denaturing gradient gel. DCODE Manual.

By comparing bands on a DGGE gel between samples, differences in population composition can be identified. Once bands have been identified as different from sample to sample, these bands can be removed from the gel and sequenced. This sequence can be compared to all known sequences by searching a database, such as GenBank, to determine the phylogenetic identity of that band. Using this new knowledge about the identity of the organisms in a sample, DNA probes or primers can be produced to allow the researcher to more quantitatively follow the concentrations of each type of organism. DGGE has the advantage of being relatively rapid for the comparison of different time point samples. The cloning approach, where 16S rRNA clone libraries are produced directly from PCR-amplified DNA samples, is very time-consuming and labor intensive and less practical for multi-sample analysis.

Beyond the sources of potential bias described in the PCR section, the DGGE process can also contribute to differences in the interpretation of community complexity. One limitation of DGGE is that DNA fragments up to only 500 bp can be separated (100). This constraint limits the amount of information available for phylogenetic

analysis or probe design. In addition, there is a limited number of bands that can be resolved using DGGE. Torsvick et al. found that there can be up to 10^4 different genomes present in soil samples. DGGE is unable to resolve this large number of bands. Several studies have shown that DGGE will detect only those populations making up >1% of the total community (99, 100). These limitations can be avoided by using PCR products obtained with group-specific primers or by fractionating the DNA according to its %G+C content and then performing PCR-DGGE on each fraction separately (30, 106).

The first use of DGGE of PCR-amplified 16S rDNA fragments was to profile the community complexity of a microbial mat and bacterial biofilms (100). Ovreas et al. used DGGE to determine the community structure of bacterioplankton in Lake Saelenvannet using PCR primers specific to 16s rRNA (106). They observed that bacterial diversity decreased with depth, while *Archaeal* diversity increased with depth. DGGE soon began to be used as a tool in bioremediation studies to determine the effect different remediation strategies had on indigenous microbial populations. Rooney-Varga et al. used DGGE with PCR amplification of either bacterial or *Geobacteraceae*-specific primers to investigate the microorganisms involved in anaerobic benzene degradation in a petroleum-contaminated aquifer (114). Bands were sequenced and revealed the enrichment of microorganisms in the *Geobacter* group in benzene-degrading sediments that was not observed in non-benzene degradation sediments. This genus contains the only known organisms that can oxidize aromatic compounds with the simultaneous reduction of Fe(III) and indicated a potential role of these organisms in benzene degradation at this site. DGGE was used in combination with a phospholipid fatty acid analysis (see next section) method to identify the bacterial community members

responsible for decontamination of an oil spill site (88). A coastal oil spill was simulated and four treatments were investigated. In all cases where oil was applied, significant differences in community structure were observed from the initial population as evidenced by both DGGE banding patterns and changes in the PLFA content. After band sequencing, it was found that oil treatment encouraged the growth of gram-negative organisms within the α -proteobacteria phylum. This group of organisms was never observed in control treatments. In addition, Macnaughton and co-workers hoped to use the changes in microbial community structure to define an end-point for removal of the oil. However, the two methods differed in this regard. By week 14, PLFA analysis indicated the microbial population was returning to its original configuration, yet the DGGE banding patterns remained quite different.

Wawer et al. compared DGGE patterns from PCR products from genomic DNA and mRNA extracted from bioreactor samples incubated with hydrogen. The presence of at least 2 *Desulfovibrio* populations was observed. However, only one population expressed the (NiFe) hydrogenase gene, which is an indicator of growth on hydrogen (132). This combination of DNA and mRNA DGGE allowed the researchers to distinguish between populations that were merely present and those that were carrying out the reaction of interest. This approach has great potential to determine not only what organisms were present but also which organisms are active at a particular site.

DGGE techniques can also be a useful tool for monitoring simpler mixtures of microorganisms. Teske et al. were able to identify two distinct 16S ribosomal DNA bands using DGGE, indicating two different bacterial components, from a coculture capable of sulfate reduction (122). These bands were sequenced and identified as a

Desulfovibrio stain and an *Arcobacter* strain. After the identification of the two bacteria in their coculture, Teske and co-workers were able to design more selective conditions for the isolation of both strains in pure culture, which they were unable to isolate previously.

2.C.1.b. Phenotypic-Based Methods

2.C.1.b.1. Phospholipid Fatty Acid Analysis

Another non-culture based method relies on the use of membrane lipid components to enumerate and describe microbial communities. This method requires neither culturing of the microorganisms nor dislodging the microorganisms from the soil surface, bypassing much of the difficulties encountered with traditional techniques. The method, termed a phospholipid fatty acid analysis, can be used as a determinant of the total viable biomass concentration in a soil, water, or culture sample. Bacteria synthesize more than 200 fatty acids and related lipids (24) that are defined as such by their solubility in an organic solvent. Lipids are the principal chemical component of all membranes, including both the cytoplasm and individual organelles within a cell. These lipids are organized as bilayers in bacterial cell membranes, with their polar head groups facing away from each other and their non-polar side chains facing each other (Figure 2.10). In addition to their role as structural components of microorganisms, lipids also serve as energy storage compounds, are able to solubilize water-insoluble substrates for growth, and interact with protein molecules to make them less soluble in water to help with association with the membrane (25).

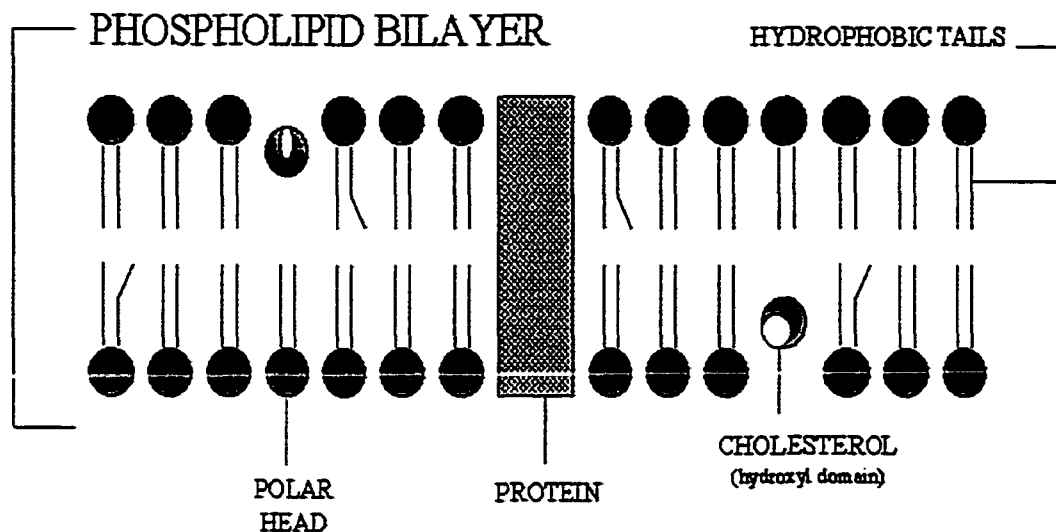


Figure 2.10 Bacterial membrane structure (www.uwyo.edu/ag/anisci/wjm/repro/fig2-25.htm).

Phospholipids make up a significant fraction of the membrane of bacterial cells and consist of a straight chain of hydrocarbon (hydrophobic) with a carboxyl group (hydrophilic) at one end. Eukaryotic cells consist of 98% phospholipids, while 50% of the lipids of other organisms are phospholipids. When a cell dies, the phosphate group is cleaved from the phospholipid leaving diglyceride fatty acids (Figure 2.11). Thus, the amount of phospholipid extracted from a sample at a given time point is indicative of only those cells with an intact cellular membrane. Studies have also shown that there is a very rapid turnover of phospholipids in living cells and in killed cells added to the environment (128) making this method an accurate way to quantitate only viable cells.

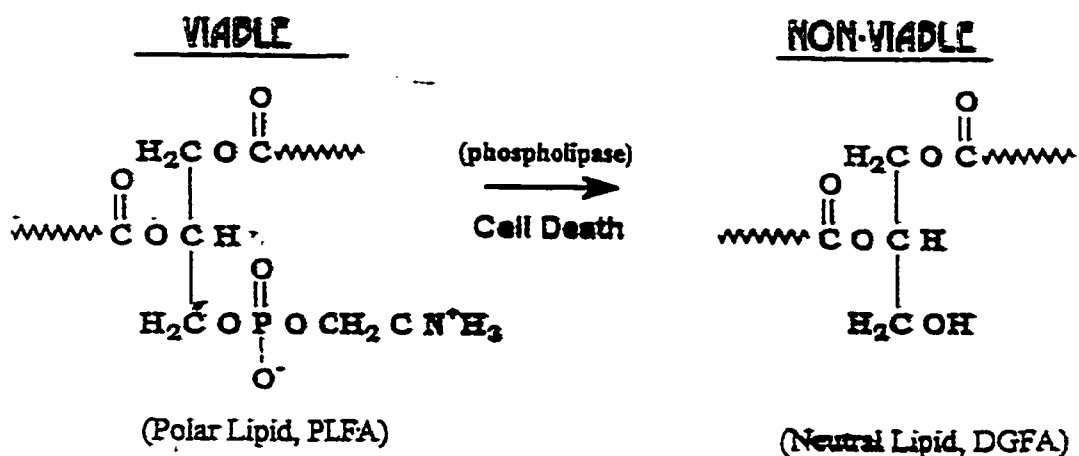


Figure 2.11 Cleavage of phosphate group from phospholipid fatty acid upon cell death (133).

The PLFA analysis can be used to determine not only the amount of viable microbial biomass (an indication of microbial activity) but also to provide insight into the microbial community composition. The value of this analysis arises from the fact that different microorganisms have different combinations of fatty acids present in microbial cellular membranes. In some cases, certain microorganisms can be related to “biomarker” fatty acids that are present only in that particular group of microbes. However, all microorganisms contain a mixture of fatty acids in common, so identifications for a specific microbial group is difficult. The relationships between specific microbial groups and fatty acids have been investigated in pure culture studies, enrichment studies and manipulated field studies (26, 44, 63). Bacteria have been shown to alter their membrane fatty acids in response to environmental stress. Thus, certain “stress signatures” have been recognized that are common to most bacteria undergoing environmental or nutritional stress (e.g. an increase in the ratio of *cis* to *trans* isomers).

To carry out this method, the phosphate group can be cleaved from the phospholipid fatty acid to produce fatty acid methyl esters (FAMES) that are readily volatilized following methylation. Separation and quantitation of the FAMES can be carried out on a gas chromatograph with either flame ionization detection or mass spectrometry. Identification of each FAME occurs by comparison to known standards and by investigation of the mass spectral pattern.

While PLFA analysis can provide important information about microorganisms in an environmental sample, it is most powerful when used to compare differences between samples. Lipid accumulation can signal growth of microorganisms, but many eukaryotic microorganisms have been shown to increase their lipid content if they become depleted of a particular nutrient such as magnesium or phosphate (25). Fries et al. used FAME analysis in conjunction with genomic fingerprinting to determine the succession of dominant toluene- and phenol-degrading strains in an aquifer tested for degradation of trichloroethylene (55). FAME analysis found that six of the seven originally dominant microbial groups were still dominant after treatment with 1,1-DCE. In contrast to this, Franzmann and co-workers observed no significant differences in microbial population composition as determined by FAME analysis between samples taken from hydrocarbon-contaminated sediments and samples taken from uncontaminated sites. They found that microbial profiles corresponded more to the sample's distance to the rhizosphere than to the effects of the hydrocarbon plume (54).

A combination of PLFA-FAME and nucleic acid hybridization was used to characterize the response of a freshwater microbial community to PAH contamination (83). The site specifics (similar flow and sediment characteristics at both contaminated

and uncontaminated sampling sites) allowed a direct analysis of the impact of PAHs on sediment communities. The microbial population concentrations determined by PLFA analysis at contaminated sites were 10 to 100 times greater than at uncontaminated sites. In addition, PLFA analysis revealed that at higher PAH concentration, the microbial community structure was altered. Hybridization of probes specific to PAH degradation genes also indicated higher levels of these genes at contaminated sites. This information combined showed both a phenotypic and genotypic response to PAH contamination.

2.D. References

1. 1993. Bioremediation Report. King Publishing Group.
2. **Abramowicz, D. A.** 1990. Aerobic PCB biodegradation and anaerobic PCB dechlorination in the environment. *Environmental Health Perspectives*. **103**:97-100.
3. **Abramowicz, D. A., M. J. Brennan, H. M. V. Dort, and E. L. Gallagher.** 1993. Factors influencing the rate of polychlorinated biphenyl dechlorination in Hudson River sediments. *Environmental Science and Technology*. **27**:1125-1131.
4. **Ahmed, M., and D. D. Focht.** 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. *Canadian Journal of Microbiology*. **19**:47-52.
5. **Alder, A. C., M. M. Haggblom, S. R. Oppenheimer, and L. Y. Young.** 1993. Reductive dechlorination of polychlorinated biphenyls in anaerobic sediments. *Environmental Science and Technology*. **27**:530-538.

6. **Amman, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria on multispecies biofilms. *Applied and Environmental Microbiology*. **58**:614-623.
7. **Anid, P. J., L. Nies, and T. M. Vogel.** 1991. Sequential anaerobic-aerobic biodegradation of PCBs in the river model, p. 428-436. *In* R. E. Hinchee and R. F. Olfenbuttel (ed.), *On-site Bioreclamation Processes for Xenobiotic and Hydrocarbon Treatment*. Butterworth-Heinemann, Columbus.
8. **Anid, P. J., B. P. Ravest-Webster, and T. M. Vogel.** 1993. Effect of hydrogen peroxide on the biodegradation of PCBs in anaerobically dechlorinated river sediments. *Biodegradation*. **4**:241-248.
9. **Arensdorf, J. J., and D. D. Focht.** 1995. A meta cleavage pathway for 4-chlorobenzoate, an intermediate in the metabolism of 4-chlorobiphenyl by *Pseudomonas cepacia* P166. *Applied and Environmental Microbiology*. **61**:443-447.
10. **Aronstein, B. N., and L. E. Rice.** 1995. Biological and integrated chemical-biological treatment of PCB congeners in soil/sediment-containing systems. *Journal of Chemical Technology and Biotechnology*. **63**:321-328.
11. **Assaf-Anid, N., L. Nies, and T. M. Vogel.** 1992. Reductive dechlorination of a polychlorinated biphenyl congener and hexachlorobenzene by Vitamin B₁₂. *Applied and Environmental Microbiology*. **58**:1057-1060.
12. **Asturias, J. A., and K. N. Timmis.** 1993. Three different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the gram-positive

- polychlorobiphenyl-degrading bacterium *Rhodococcus globerulus* P6. *Journal of Bacteriology*. 175:4631-4640.
13. **Barriault, D., and M. Sylvestre.** 1993. Factors affecting PCB degradation by an implanted bacterial strain in soil microcosms. *Canadian Journal of Microbiology*. 39:594-602.
 14. **Baxter, R. A., R. E. Gilbert, R. A. Lidgett, J. H. Mainprize, and H. A. Vodden.** 1975. The degradation of polychlorinated biphenyls by microorganisms. *Science of the Total Environment*. 4:53-61.
 15. **Becker-Andre, M., and K. Hahlbrock.** 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Research*. 17:9437-9446.
 16. **Bedard, D. L., and J. F. Quensen III.** 1995. Microbial reductive dechlorination of polychlorinated biphenyls, p. 127-216. *In* L. Y. Young and C. E. Cerniglia (ed.), *Microbial Transformation and Degradation of Toxic Organic Chemicals*. Wiley-Liss.
 17. **Bedard, D. L., R. Unterman, L. H. Bopp, M. J. Brennan, M. L. Haberl, and C. Johnson.** 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. *Applied and Environmental Microbiology*. 51:761-768.
 18. **Bedard, D. L., and H. M. Van Dort.** 1998. Complete reductive dehalogenation of brominated biphenyls by anaerobic microorganisms in sediment. *Applied and Environmental Microbiology*. 64:940-947.

19. **Bedard, D. L., H. M. Van Dort, R. J. May, and L. A. Smullen.** 1997. Enrichment of microorganisms that sequentially *meta*, *para*-dechlorinate the residue of Aroclor 1260 in Housatonic river sediment. *Environmental Science and Technology*. **31**:3308-3313.
20. **Bedard, D. L., R. E. Wagner, M. J. Brennan, M. L. Haberl, and J. F. Brown.** 1987. Extensive degradation of aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. *Applied and Environmental Microbiology*. **53**:1094-1102.
21. **Beeman, R. E., and J. M. Suflita.** 1987. Microbial ecology of a shallow unconfined ground water aquifer polluted by municipal landfill leachate. *Microbial Ecology*. **14**:39-54.
22. **Billingsley, K. A., S. M. Backus, and O. P. Ward.** 1999. Effect of surfactant solubilization on biodegradation of polychlorinated biphenyl congeners by *Pseudomonas* LB400. *Applied Microbiology and Biotechnology*. **52**:255-260.
23. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. *Journal of Industrial Microbiology*. **1**:23-29.
24. **Bottger, E. C.** 1996. Approaches for identification of microorganisms. *ASM News*. **62**:247-250.
25. **Boulton, C. A., and C. Ratledge.** 199. Biosynthesis of Fatty Acids and Lipids.
26. **Bowman, J. P., J. H. Skerratt, P. D. Nichols, and L. I. Sly.** 1991. Phospholipid fatty acid and lipopolysaccharide fatty acid signature lipids in methane-utilizing bacteria. *FEMS Microbiology Ecology*. **85**:15-22.

27. **Boyle, A. W., C. K. Blake, W. A. Price II, and H. D. May.** 1993. Effects of polychlorinated biphenyl congener concentration and sediment supplementation on rates of methanogenesis and 2,3,6-trichlorobiphenyl dechlorination in an anaerobic enrichment. *Applied and Environmental Microbiology*. 59:3027-3031.
28. **Boyle, A. W., C. J. Silvin, J. P. Hassett, J. P. Nakas, and S. W. Tanenbaum.** 1992. Bacterial PCB biodegradation. *Biodegradation*. 3:285-298.
29. **Brenner, V., J. J. Arensdorf, and D. D. Focht.** 1994. Genetic construction of PCB degraders. *Biodegradation*. 5:359-377.
30. **Brinkhoff, T., and G. Muyzer.** 1997. Increased species diversity and extended habitat range of sulfur-oxidizing *Thiomicrospira* spp. *Applied and Environmental Microbiology*. 63:3789-3796.
31. **Brown, J. F., R. E. Wagner, D. L. Bedard, M. J. Brennan, J. C. Carnahan, R. J. May, and T. J. Tofflemire.** 1984. PCB transformations in upper Hudson sediments. *Northeast Environmental Science*. 3:167-179.
32. **Brown, J. F., R. E. Wagner, H. Feng, D. L. Bedard, M. J. Brennan, J. C. Carnahan, and R. J. May.** 1987. Environmental dechlorination of PCBs. *Environmental Toxicology and Chemistry*. 6:579-593.
33. **Brunner, W., F. H. Sutherland, and D. D. Focht.** 1985. Enhanced biodegradation of polychlorinated biphenyls in soil by analog enrichment and bacterial inoculation. *Journal of Environmental Quality*. 14:324-328.
34. **Chen, P., W. Zhou, and L. L. Tavlarides.** 1997. Remediation of polychlorinated biphenyl contaminated soils/sediments by supercritical fluid extraction. *Environmental Progress*. 16:227-235.

35. **Cigolini, J. F., A. K. Goyal, and G. J. Zylstra.** 1997. Presented at the 97th General Meeting, American Society for Microbiology, Miami Beach.
36. **Criddle, C. S., P. L. McCarty, M. C. Elliott, and J. F. Barker.** 1990. Reductive dehalogenation of carbon tetrachloride by *Escherichia coli* K-12. *Applied and Environmental Microbiology*. **56**:3240-3246.
37. **Deweerd, K. A., and D. L. Bedard.** 1999. Use of halogenated benzoates and other halogenated aromatic compounds to stimulate the microbial dechlorination of PCBs. *Environmental Science and Technology*. **33**:2057-2063.
38. **DeWeerd, K. A., and J. M. Suflita.** 1990. Anaerobic aryl reductive dehalogenation of halobenzoates by cell extracts of "*Desulfomonile tiedjei*". *Applied and Environmental Microbiology*. **56**:2999-3005.
39. **DiStefano, T. D., J. M. Gossett, and S. H. Ziner.** 1991. Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. *Applied and Environmental Microbiology*. **57**:2287-2292.
40. **Dobbins, D. C.** 1992. Subsurface, terrestrial microbial ecology and biodegradation of organic chemicals: A review. *Critical Reviews in Environmental Control*. **22**:67-136.
41. **Dojka, M. A., P. Hugenholtz, S. K. Haack, and N. R. Pace.** 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Applied and Environmental Microbiology*. **64**:3869-3877.

42. **Dolfing, J., and B. K. Harrison.** 1992. Gibbs Free Energy of formation of halogenated aromatic compounds and their potential role as electron acceptors in anaerobic environments. *Environmental Science and Technology*. **26**:2213-2218.
43. **Dort, H. M. V., and D. L. Bedard.** 1991. Reductive ortho-dechlorination and meta-dechlorination of a polychlorinated biphenyl congener by anaerobic microorganisms. *Applied and Environmental Microbiology*. **57**:1576-1578.
44. **Dowling, N. J. E., F. Widdell, and D. C. White.** 1986. Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulphate-reducers and other sulphide-forming bacteria. *Journal of General Microbiology*. **132**:1815-1825.
45. **Erb, R. W., and I. Wagner-Dobler.** 1993. Detection of polychlorinated biphenyl degradation genes in polluted sediments by direct DNA extraction and polymerase chain reaction. *Applied and Environmental Microbiology*. **59**:4065-4073.
46. **Erickson, B. D., and F. J. Mondello.** 1992. Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain LB400. *Journal of Bacteriology*. **174**:2903-2912.
47. **Erickson, M. D.** 1991. Physical, chemical, commercial, environmental, and biological properties, p. 5-53. *In* M. D. Erickson (ed.), *Analytical Chemistry of PCBs*, vol. 1. Butterworth Publishers, Boston.
48. **Evans, B. S., C. A. Dudley, and K. T. Klasson.** 1996. Sequential anaerobic-aerobic biodegradation of PCBs in soil slurry microcosms. *Applied Biochemistry and Biotechnology*. **57/58**:885-894.

49. **Fathepure, B. Z., and S. A. Boyd.** 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. *FEMS Microbiology Letters*. **49**:149-156.
50. **Fetzner, S., and F. Lingens.** 1994. Bacterial dehalogenases: Biochemistry, genetics, and biotechnological applications. *Microbiological Reviews*. **58**:641-685.
51. **Findlay, R. H., M. B. Trexler, J. B. Guckert, and D. C. White.** 1990. Laboratory study of disturbance in marine sediments: response of a microbial community. *Marine Ecology Progress Series*. **62**:121-133.
52. **Fleming, J. T., J. Sanseverino, and G. S. Sayler.** 1993. Quantitative relationship between naphthalene catabolic gene frequency and expression in predicting PAH degradation in soils at town gas manufacturing sites. *Environmental Science and Technology*. **27**:1068-1074.
53. **Focht, D. D., and W. Brunner.** 1985. Kinetics of biphenyl and polychlorinated biphenyl metabolism in soil. *Applied and Environmental Microbiology*. **50**:1058-1063.
54. **Franzmann, P. D., B. M. Patterson, T. R. Power, P. D. Nichols, and G. B. Davis.** 1996. Microbial biomass in a shallow, urban aquifer contaminated with aromatic hydrocarbons: analysis by phospholipid fatty acid content and composition. *Journal of Applied Bacteriology*. **80**:617-625.
55. **Fries, M. R., G. D. Hopkins, P. L. McCarty, L. J. Forney, and J. M. Tiedje.** 1997. Microbial succession during a field evaluation of phenol and toluene as the

- primary substrates for trichloroethene cometabolism. *Applied and Environmental Microbiology*. **63**:1515-1522.
56. **Furukawa, K., and N. Arimura.** 1987. Purification and properties 2,3-dihydroxybiphenyl dioxygenase from polychlorinated biphenyl-degrading *Pseudomonas pseudoalcaligenes* and *Pseudomonas aeruginosa* carrying the cloned bphC gene. *Journal of Bacteriology*. **169**:924-927.
57. **Furukawa, K., J. Hirose, A. Suyama, T. Zaiki, and S. Hayashida.** 1993. Gene components responsible for discrete substrate specificity in the metabolism of biphenyl (bph operon) and toluene (tod operon). *Journal of Bacteriology*. **175**:5224-5232.
58. **Furukawa, K., N. Tomizuka, and A. Kamibayashi.** 1979. Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. *Applied and Environmental Microbiology*. **38**:301-310.
59. **Gantzer, C. J., and L. P. Wackett.** 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environmental Science and Technology*. **25**:715-722.
60. **Gibson, D. T., D. L. Cruden, J. D. Haddock, G. J. Zylstra, and J. M. Brand.** 1993. Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. LB400 and *Pseudomonas pseudoalcaligenes* KF707. *Journal of Bacteriology*. **175**:4561-4564.
61. **Gillan, D. C., A. G. C. L. Speksnijder, G. Zwart, and C. D. Ridder.** 1998. Genetic diversity of the biofilm covering *Montacuta feruginosa* (Mollusca, Bivalvia) as evaluated by denaturing gradient gel electrophoresis analysis and

- cloning of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology*. 64:3464-3472.
62. **Guilbeault, B., M. Sondossi, D. Ahmad, and M. Sylvestre.** 1994. Factors affecting the enhancement of PCB degradative ability of soil microbial populations. *International Biodeterioration and Biodegradation*. 33:73-91.
63. **Haack, S. K., H. Garchow, D. A. Odelson, L. J. Forney, and M. J. Klug.** 1994. Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. *Applied and Environmental Microbiology*. 60:2483-2493.
64. **Haddock, J. D., L. M. Nadim, and D. T. Gibson.** 1993. Oxidation of biphenyl by a multicomponent enzyme system from *Pseudomonas* sp. Strain LB400. *Journal of Bacteriology*. 177:20-26.
65. **Harkness, M. R., J. B. McDermott, D. A. Abramowicz, J. J. Salvo, W. P. Flanagan, M. L. Stephens, F. J. Mondello, R. J. May, J. H. Lobos, K. M. Carroll, M. J. Brennan, A. A. Bracco, K. M. Fish, G. L. Warner, P. R. Wilson, D. K. Dietrich, D. T. Lin, C. B. Morgan, and W. L. Gately.** 1993. In situ stimulation of aerobic PCB biodegradation in Hudson River sediments. *Science*. 259:503-507.
66. **Havel, J., and W. Reineke.** 1991. Total degradation of various chlorobiphenyls by cocultures and in vivo constructed hybrid pseudomonads. *FEMS Microbiology Letters*. 78:163-170.

67. **Heritage, A. D., and I. C. MacRae.** 1977. Identification of intermediates formed during degradation of hexachlorocyclohexanes by *Clostridium sphenoides*. *Applied and Environmental Microbiology*. **33**:1295-1297.
68. **Hernandez, B. S., S. C. Koh, M. Chial, and D. D. Focht.** 1997. Terpene-utilizing isolates and their relevance to enhanced biotransformation of polychlorinated biphenyls in soil. *Biodegradation*. **8**:153-158.
69. **Heuer, H., M. Krsek, P. Baker, K. Smalla, and E. M. H. Wellington.** 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology*. **63**:3233-3241.
70. **Hickey, W. J., D. B. Searles, and D. D. Focht.** 1993. Enhanced mineralization of polychlorinated biphenyls in soil inoculated with chlorobenzoate-degrading bacteria. *Applied and Environmental Microbiology*. **59**:1194-1200.
71. **Hinchee, R. H., A. Leeson, L. Semprini, and S. K. Ong.** 1994. Evaluating polychlorinated biphenyl bioremediation process: from laboratory feasibility testing to pilot demonstrationsf, p. 354-358. *In* R. H. Hinchee, A. Leeson, L. Semprini, and S. K. Ong (ed.), *Bioremediation of Chlorinated and PAH Compounds*. Lewis Publishers, Boca Raton.
72. **Holmes, D. A., B. K. Harrison, and J. Dolfing.** 1993. Estimation of Gibbs free energies of formation for polychlorinated biphenyls. *Environmental Science and Technology*. **27**:725-731.

73. **Hooper, S. W., C. A. Pettigrew, and G. S. Sayler.** 1990. Ecological fate, effects and prospects for the elimination of environmental polychlorinated biphenyls (PCBs). *Environmental Toxicology and Chemistry*. 9:655-667.
74. **Horowitz, A., J. M. Suflita, and J. M. Tiedje.** 1983. Reductive dehalogenations of halobenzoates by anaerobic lake sediment microorganisms. *Applied and Environmental Microbiology*. 45:1459-1465.
75. **Jansson, J.** 1995. Tracking genetically engineered microorganisms in nature. *Current Opinions in Microbiology*. 6:275-283.
76. **Jansson, J. K., and T. Leser.** 1996. Quantitative PCR of environmental samples, p. 2.7.4 1-19, *Molecular Microbial Ecology Manual*. Kluwer Academic Publishers, Netherlands.
77. **Johnsen, K., O. Enger, C. S. Jacobsen, L. Thirup, and V. Torsvik.** 1999. Quantitative selective PCR of 16S ribosomal DNA correlates well with selective agar plating in describing population dynamics of indigenous *Pseudomonas* spp. in soil hot spots. *Applied and Environmental Microbiology*. 65:1786-1789.
78. **Ka, J. O., P. Burauel, J. A. Bronson, W. E. Holben, and J. M. Tiedje.** 1995. DNA probe analysis of microbial community selected in field by long-term 2,4-D application. *Soil Science Society of America Journal*. 59:1581-1587.
79. **Kim, J., and G.-Y. Rhee.** 1997. Population dynamics of polychlorinated biphenyl-dechlorinating microorganisms in contaminated sediments. *Applied and Environmental Microbiology*. 63:1771-1776.

80. **Klasson, K. T., J. W. Barton, B. S. Evans, and M. E. Reeves.** 1996. Reductive microbial dechlorination of indigenous polychlorinated biphenyls in soil using a sediment-free inoculum. *Biotechnology Progress*. **12**:310-315.
81. **Kulakova, A. N., M. J. Larkin, and L. A. Kulakov.** 1997. The plasmid-located haloalkane dehalogenase gene from *Rhodococcus rhodochrous* NCIMB 13064. *Microbiology*. **143**:109-115.
82. **Lajoie, C. A., A. Layton, and G. S. Sayler.** 1994. Cometabolic oxidation of polychlorinated biphenyls in soil with a surfactant-based field application vector. *Applied and Environmental Microbiology*. **60**:2826-2833.
83. **Langworthy, D. E., R. D. Stapleton, G. S. Sayler, and R. H. Findlay.** 1998. Genotypic and phenotypic responses of a riverine microbial community to polycyclic aromatic hydrocarbon contamination. *Applied and Environmental Microbiology*. **64**:3422-3428.
84. **Lee, S.-Y., J. Bollinger, D. Bezdicsek, and A. Ogram.** 1996. Estimation of the abundance of an uncultured soil bacterial strain by a competitive quantitative PCR method. *Applied and Environmental Microbiology*. **62**:3787-3793.
85. **Leser, T. D., M. Boye, and N. B. Hendriksen.** 1995. Survival and activity of *Pseudomonas* sp. Strain B13(FR1) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and its effect on the indigenous bacterioplankton. *Applied and Environmental Microbiology*. **61**:1201-1207.
86. **Leuser, R. M., L. A. Velazquez, A. Cohen, and J. Janssen.** 1990. Remediation of PCB soil contamination by on-site incineration. *Journal of Hazardous Materials*. **25**:375-385.

87. **Loffler, F. E., J. M. Tiedje, and R. A. Sanford.** 1999. Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology. *Applied and Environmental Microbiology*. **65**:4049-4056.
88. **Macnaughton, S. J., J. R. Stephen, A. D. Venosa, G. A. Davis, Y. J. Chang, and D. C. White.** 1999. Microbial population changes during bioremediation of an experimental oil spill. *Applied and Environmental Microbiology*. **65**:3566-3574.
89. **Maeda, M., S.-Y. Chung, E. Song, and T. Kudo.** 1995. Multiple genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase in the gram-positive polychlorinated biphenyl-degrading bacterium *Rhodococcus erythropolis* TA421, isolated from a termite ecosystem. *Applied and Environmental Microbiology*. **61**:549-555.
90. **May, H. D., A. W. Boyle, W. A. Price II, and C. K. Blake.** 1992. Subculturing of a polychlorinated biphenyl-dechlorinating anaerobic enrichment on solid media. *Applied and Environmental Microbiology*. **58**:4051-4054.
91. **McDermott, J. B., R. Unterman, M. J. Brennan, R. E. Brooks, D. P. Mobley, C. C. Schwartz, and D. K. Dietrich.** 1989. Two strategies for PCB soil remediation: Biodegradation and surfactant extraction. *Environmental Progress*. **8**:46-51.
92. **Mesarch, M. B., C. H. Nakatsu, and L. Nies.** 2000. Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR. *Applied and Environmental Microbiology*. **66**:678-683.

93. **Mohn, W. W., and J. M. Tiedje.** 1992. Microbial Reductive Dehalogenation. *Microbiological Reviews.* **56**:482-507.
94. **Mokross, H., E. Schmidt, and W. Reineke.** 1990. Degradation of 3-chlorobiphenyl by in vivo constructed hybrid pseudomonads. *FEMS Microbiology Letters.* **71**:179-186.
95. **Mondello, F. J., M. P. Turcich, J. H. Lobos, and B. D. Erickson.** 1997. Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. *Applied and Environmental Microbiology.* **63**:3096-3103.
96. **Montgomery, L., and T. M. Vogel.** 1992. Dechlorination of 2,3,5,6-tetrachlorobiphenyl by a phototrophic enrichment culture. *FEMS Microbiology Letters.* **94**:247-250.
97. **Morris, J. G.** 1994. Obligately anaerobic bacteria in biotechnology. *Applied Biochemistry and Biotechnology.* **48**:75-106.
98. **Morris, P. J., W. W. Mohn, J. F. Quensen, J. M. Tiedje, and S. A. Boyd.** 1992. Establishment of a polychlorinated biphenyl-degrading enrichment culture with predominately meta dechlorination. *Applied and Environmental Microbiology.* **58**:3088-3094.
99. **Murray, A. E., J. T. Hollibaugh, and C. Orrego.** 1996. Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient electrophoresis of 16S rDNA fragments. *Applied and Environmental Microbiology.* **62**:2676-2680.

100. **Muyzer, G., E. C. d. Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA. *Applied and Environmental Microbiology*. **59**:695-700.
101. **Natarajan, M. R., W.-M. Wu, J. Nye, H. Wang, L. Bhatnagar, and M. K. Jain.** 1996. Dechlorination of polychlorinated biphenyl congeners by an anaerobic microbial consortium. *Applied Microbiology and Biotechnology*. **46**:673-677.
102. **Neidle, E. L., C. Hartnett, L. N. Ornston, A. Bairoch, M. Rekik, and S. Harayama.** 1991. Nucleotide sequences of the *Acinetobacter calcoaceticus* benABC genes for benzoate 1,2-dioxygenase reveal evolutionary relationships among multicomponent oxygenases. *Journal of Bacteriology*. **173**:5385-5395.
103. **Nies, L., and T. M. Vogel.** 1990. Effects of organic substrates on dechlorination of Aroclor 1242 in anaerobic sediments. *Applied and Environmental Microbiology*. **56**:2612-2617.
104. **Nies, L., and T. M. Vogel.** 1991. Identification of the proton source for the microbial reductive dechlorination of 2,3,4,5,6-pentachlorobiphenyl. *Applied and Environmental Microbiology*. **57**:2771-2774.
105. **Nogales, B., E. R. B. Moore, W. R. Abraham, and K. N. Timmis.** 1999. Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. *Environmental Microbiology*. **1**:199-212.

106. **Ovreas, L., L. Forney, F. L. Daae, and V. Torsvik.** 1997. Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-Amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology*. **63**:3367-3373.
107. **Pace, N. R.** 1996. New perspectives on the natural microbial world: Molecular microbial ecology. *ASM News*. **62**:463-470.
108. **Pellizari, V. H., S. Bezborodnikov, J. F. Q. III, and J. M. Tiedje.** 1996. Evaluation of strains isolated by growth on naphthalene and biphenyl for hybridization of genes to dioxygenase probes and polychlorinated biphenyl-degrading ability. *Applied and Environmental Microbiology*. **62**:2053-2058.
109. **Pettigrew, C. A., A. Breen, C. Corcoran, and G. S. Sayler.** 1990. Chlorinated biphenyl mineralization by individual populations and consortia of freshwater bacteria. *Applied and Environmental Microbiology*. **56**:2036-2045.
110. **Pulliam Holoman, T. R., M. A. Elbersen, L. A. Cutter, H. D. May, and K. R. Sowers.** 1998. Characterization of a defined 2,3,5,6-tetrachlorobiphenyl-*ortho*-dechlorinating microbial community by comparative sequence analysis of genes coding for 16S rRNA. *Applied and Environmental Microbiology*. **64**:3359-3367.
111. **Quensen III, J. F., S. A. Boyd, and J. M. Tiedje.** 1990. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. *Applied and Environmental Microbiology*. **56**:2360-2369.

112. **Quensen III, J. F., J. M. Tiedje, and S. A. Boyd.** 1988. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. *Science*. 242:752-754.
113. **Rhee, G.-Y., R. C. Sokol, C. M. Bethoney, and B. Bush.** 1993. A long-term study of anaerobic dechlorination of PCB congeners by sediment microorganisms: pathways and mass balance. *Environmental Toxicology and Chemistry*. 12:1829-1834.
114. **Rooney-Varga, J. N., R. T. Anderson, J. L. Fraga, D. Ringelberg, and D. R. Lovley.** 1999. Microbial communities associated with anaerobic benzene degradation in a petroleum-contaminated aquifer. *Applied and Environmental Microbiology*. 65:3056-3063.
115. **Scow, K. M., and J. Hutson.** 1992. Effect of diffusion and sorption on the kinetics of biodegradation: theoretical considerations. *Soil Science Society of America Journal*. 56:119-127.
116. **Seto, M., E. Masai, M. Ida, T. Hatta, K. Kimbara, M. Fukuda, and K. Yano.** 1995. Multiple polychlorinated biphenyl transformation systems in the gram-positive bacterium *Rhodococcus* sp. Strain RHA1. *Applied and Environmental Microbiology*. 61:4510-4513.
117. **Simon, L., R. C. Levesque, and M. Lalonde.** 1992. Rapid quantitation by PCR of endomycorrhizal fungi colonizing roots. *PCR Methods and Applications*. 2:76-80.
118. **Smatlak, C. R., and J. M. Gossett.** 1996. Comparative kinetics of hydrogen utilization for reductive dechlorination of tetrachloroethene and methanogenesis

- in an anaerobic enrichment culture. *Environmental Science and Technology*. **30**:2850-2858.
119. **Sondossi, M., M. Sylvestre, D. Ahmad, and R. Masse.** 1991. Metabolism of hydroxybiphenyl and chloro-hydroxybiphenyl by biphenyl/chlorobiphenyl degrading *Pseudomonas testoseroni* strain B-356. *Journal of Industrial Microbiology*. **7**:77-88.
 120. **Springael, D., L. Diels, and M. Mergeay.** 1994. Transfer and expression of PCB-degradative genes into heavy metal resistant *Alcaligenes eutrophus* strains. *Biodegradation*. **5**:343-357.
 121. **Tanner, M. A., B. M. Goebel, M. A. Dojka, and N. R. Pace.** 1998. Specific ribosomal DNA sequences from diverse environmental settings correlate with experimental contaminants. *Applied and Environmental Microbiology*. **64**:3110-3113.
 122. **Teske, A., P. Sigalevich, Y. Cohen, and G. Muyzer.** 1996. Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Applied and Environmental Microbiology*. **62**:4210-4215.
 123. **Tiedje, J. M., S. A. Boyd, and B. Z. Fathepure.** 1987. Anaerobic degradation of chlorinated aromatic hydrocarbons. *Developments in Industrial Microbiology*. **27**:117-127.
 124. **Tiedje, J. M., J. F. Q. III, W. W. Mohn, J. P. Schimel, J. A. Cole, and S. A. Boyd.** 1991. Reductive dechlorination of chlorinated aromatic pollutants, p. 292-

307. *In* R. W. Rossmore (ed.), *Biodeterioration and Biodegradation*, vol. 8. Elsevier Applied Science, New York.
125. **Tiedje, J. M., J. F. Quensen, J. Chee-Sanford, J. P. Schimel, and S. A. Boyd.** 1993. Microbial reductive dechlorination of PCBs. *Biodegradation*. 4:231-240.
 126. **Utkin, I., C. Woese, and J. Wiegel.** 1995. Specificity of reductive dehalogenation of substituted *ortho*-chlorophenols by *Desulfitobacterium dehalogens*. *Applied and Environmental Microbiology*. 61:346-351.
 127. **Van Dort, H. M., L. A. Smullen, R. J. May, and D. L. Bedard.** 1997. Priming microbial meta-dechlorination of polychlorinated biphenyls that have persisted in Housatonic river sediments for decades. *Environmental Science and Technology*. 31:3300-3307.
 128. **Vestal, J. R., and D. C. White.** 1989. Lipid Analysis in Microbial Ecology. *BioScience*. 39:535-541.
 129. **Wagner-Dobler, I., A. Bennasar, M. Vancanneyt, C. Strompl, I. Brummer, C. Eichner, I. Grammel, and E. R. B. Moore.** 1998. Microcosm enrichment of biphenyl-degrading microbial communities from soils and sediments. *Applied and Environmental Microbiology*. 64:3014-3022.
 130. **Watanabe, K., and P. W. Baker.** 2000. Environmentally relevant microorganisms. *Journal of Bioscience and Bioengineering*. 89:1-11.
 131. **Watanabe, K., S. Yamamoto, S. Hino, and S. Harayama.** 1998. Population dynamics of phenol-degrading bacteria in activated sludge determined by *gyrB*-targeted quantitative PCR. *Applied and Environmental Microbiology*. 64:1203-1209.

132. **Wawer, C., M. S. M. Jetten, and G. Muyzer.** 1997. Genetic diversity and expression of the (NiFe) hydrogenase large subunit gene of *Desulfovibrio* spp. in environmental samples. *Applied and Environmental Microbiology*. **63**:4360-4369.
133. **White, D. C., and D. B. Ringelberg.** 1998. Signature lipid biomarker analysis, p. 255-272. *In* R. S. Burlage, R. Atlas, d. Stahl, G. Geesey, and G. Sayler (ed.), *Techniques in microbial ecology*, vol. 1. Oxford University Press, New York.
134. **Williams, W. A.** 1994. Microbial reductive dechlorination of trichlorobiphenyls in anaerobic sediment slurries. *Environmental Science and Technology*. **28**:630-635.
135. **Williams, W. A., J. H. Lobos, and W. E. Cheetham.** 1997. A phylogenetic analysis of aerobic polychlorinated biphenyl-degrading bacteria. *International Journal of Systematic Bacteriology*. **47**:207-210.
136. **Woods, S. L., and D. J. Trobaugh.** 1999. Polychlorinated biphenyl reductive dechlorination by Vitamin B₁₂: Thermodynamics and regiospecificity. *Environmental Science and Technology*. **33**:857-863.
137. **Wu, Q., D. L. Bedard, and J. Wiegel.** 1997. Effect of incubation temperature on the route of microbial reductive dechlorination of 2,3,4,6-tetrachlorobiphenyl in polychlorinated biphenyl (PCB)-contaminated and PCB-free freshwater sediments. *Applied and Environmental Microbiology*. **63**:2836-2843.
138. **Wu, Q., K. R. Sowers, and H. D. May.** 2000. Establishment of a polychlorinated biphenyl-dechlorinating microbial consortium, specific for doubly flanked

- chlorines, in a defined, sediment-free medium. *Applied and Environmental Microbiology*. **66**:49-53.
139. **Wu, Q., and J. Wiegel.** 1997. Two anaerobic polychlorinated biphenyl-dehalogenating enrichments that exhibit different *para*-dechlorination specificities. *Applied and Environmental Microbiology*. **63**:4826-4832.
140. **Ye, D. Y., J. F. Quensen, and S. A. Boyd.** 1992. Anaerobic dechlorination of polychlorobiphenyls (Aroclor 1242) by pasteurized and ethanol-treated microorganisms from sediments. *Applied and Environmental Microbiology*. **58**:1110-1114.
141. **Zeddel, A., A. Majcherczyk, and A. Huttermann.** 1994. Degradation and mineralization of polychlorinated biphenyls by white-rot fungi in solid-phase and soil incubation experiments. p. 436-440. *In* R. E. Hinchey, A. Leeson, L. Semprini, and S. K. Ong (ed.), *Bioremediation of Chlorinated and Polycyclic Aromatic Hydrocarbon Compounds*. Lewis Publishers, New York.
142. **Zwiernik, M. J., J. F. Quensen (III), and S. A. Boyd.** 1998. FeSO₄ amendments stimulate extensive anaerobic PCB dechlorination. *Environmental Science and Technology*. **32**:3360-3365.

CHAPTER 3

METHODS AND MATERIALS

3.A. Motivation and Approach

The purpose of this research was to investigate the bioremediation of polychlorinated biphenyls in soil and to understand the relationship between observed PCB degradation and changes in microbial community composition. Complete biodegradation of PCBs is difficult to achieve because of the need for more than one metabolic mode, the lack of appropriate electron donors and acceptors, and the possible inhibition by other chemicals. Although sequential anaerobic-aerobic treatment has been successful in several studies, a fundamental understanding of the mechanisms involved is necessary to reliably extrapolate results from site to site. To date, few studies of sequential PCB remediation have focused on the microbial processes responsible for the transformation, using an approach that investigates only changes in PCB degradation (a “black box” approach). Development of an effective in situ bioremediation process for PCB-contaminated sites requires detailed knowledge of the microorganisms present and how their chemical and physical environments affect their activity. The work described moves beyond a “black box” approach to address unanswered questions (Figure 3.1). Which microorganisms are critical for each phase of treatment? How does each engineered process parameter affect the activity of these vital populations? Is it

possible to stimulate PCB transformation in every situation with the right approach? This work utilizes a blend of microbial ecology and engineering to address these questions.

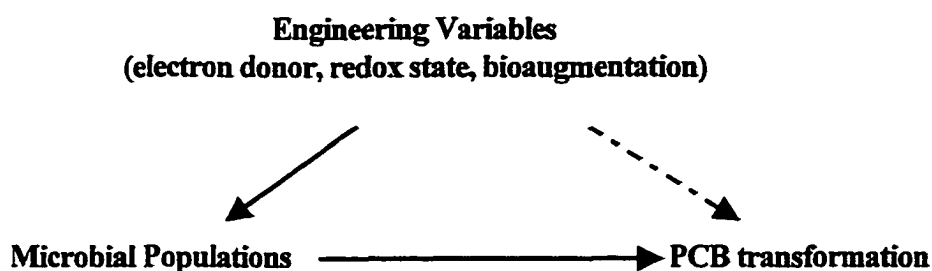


Figure 3.1 The relationship between microbial activity and PCB transformation. Solid lines represent direct effects and the dashed line represents an indirect effect. The dashed line is indicative of a “Black Box” approach to remediation.

3.A.1. Hypothesis and Specific Aims

The primary hypotheses of this research were:

- (1) Changes in microbial community composition due to manipulations of engineering variables can be correlated to observed reductions in PCB concentrations.*
- (2) The overall reduction in PCB concentration achieved by sequential anaerobic-aerobic treatment can be increased as compared to either process alone by basing engineering decisions on information about microbial changes.*

In order to provide meaningful data while having the advantage of the simplicity of laboratory experiments, soil slurry microcosms were utilized. This work was performed to determine whether the indigenous microorganisms are able to degrade PCBs in the soil samples of interest, aerobically, anaerobically or through a combination of both

treatments, as well as to provide insight into the factors dominating the degradation of PCBs in the system. These hypotheses were tested for a PCB-contaminated soil with application focused on in-situ bioremediation of vadose zone contamination.

To address these hypotheses, four specific objectives were developed and carried out:

- 1) Determine the effect of electron donor additions on dechlorination characteristics and composition of the microbial community.
- 2) Determine the effect of initiating aerobic conditions (after an anaerobic treatment phase) on the microbial population and its ability to carry out aerobic PCB biodegradation.
- 3) Determine the effect of biphenyl additions on aerobic PCB biodegradation and subsequent changes in the microbial community composition.
- 4) Based on the results from Specific Objectives 1-3, carry out a sequential bioremediation process and assess its effectiveness compared to each treatment alone.

Information about microbial community composition and activity was needed to correlate the effect of each engineering parameter (e.g., electron donor, biphenyl amendment, and oxygenation method) on PCB degradation. To do this, a combination of genotypic and phenotypic microbiological techniques was utilized to describe the microbial community. Phospholipid fatty acid analysis was used to determine total biomass levels and broad-based

changes in microbial community structure. A quantitative polymerase chain reaction procedure allowed for detection and quantitation of specific genera and degradative genes.

3.A.2. Experimental Design

Two phases of experiments were conducted for this work. A preliminary screening of two soils for the ability to degrade, both aerobically and anaerobically, polychlorinated biphenyls was followed by a full-scale treatability test investigating sequential treatment of the PCBs. The experimental design for the full-scale soil slurry microcosm studies is shown in Table 3.1. Each of the sixteen possible combinations shown was incubated simultaneously with duplicate microcosms for each treatment. In addition, a control that contained no polychlorinated biphenyls but was incubated under sequential treatment conditions was used to determine the effect that hexane addition (used for dosing of PCBs) had on the microbial population. This duplication allowed for statistical testing between treatments to evaluate the effect of each treatment on PCB degradation and microbial community composition.

Table 3.1 Experimental design for soil treatability studies. Numbers are treatment designations for identification

Metabolic Regime	No Additions	Biphenyl	Methanol	Butyrate	Methanol/Biphenyl	Butyrate/Biphenyl
Sterile Control		3	8	6	16	14
Anaerobic	4	-	7	5	-	-
Aerobic	1	2	-	-	-	-
Sequential	9	10	12	11	15	13

The soil slurry treatability tests were expected to provide an optimized method for initiating anaerobiosis and switching to aerobiosis. The effect of treatment conditions on the products obtained after each anaerobic and each aerobic phase was investigated. Combining the total biomass with population numbers of specific genera and degradative genes and PCB concentration data, the important parameters and considerations for developing a sequential bioremediation treatment for improved reduction of PCBs in soil systems was determined. These results are expected to have significance for developing sequential anaerobic/aerobic treatment processes for other chlorinated contaminants.

3.B. Chemicals

Not all of the 209 possible polychlorinated biphenyl congeners behave similarly in the environment. For example, several occur more frequently at contaminated sites and bioaccumulate to the greatest extent (13). For that reason, and to simplify the analytical procedures involved, three of these most frequently reported congeners were chosen as model compounds for this work. Congener numbers 18 (2,2',5), 77 (3,3',4,4') and 118 (2,2', 4,4',5,5') were chosen to make up the model mixture. The three structures are given in Figure 3.2. Each of these congeners has been shown to be either degraded aerobically or dechlorinated anaerobically (Rhee, Sokol et al. 1993; Williams 1994; Pagano, Scrudato et al. 1995) and to occur frequently in the environment.

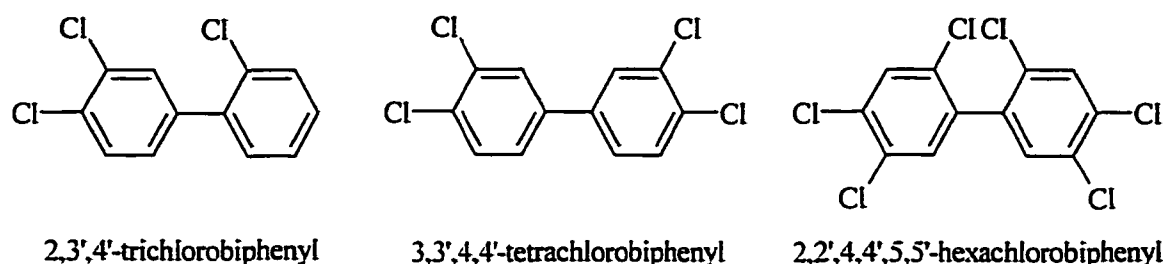


Figure 3.2 Structure of the three polychlorinated biphenyls used in this study

In addition, two other hexachlorobiphenyls were used for preliminary dechlorination testing: 2,2',3,3',4,4'- and 2,3',4,4',5,5'-hexachlorobiphenyl (Figure 3.3). The water aqueous solubilities of all congeners used are given in Table 3.2. PCBs are relatively insoluble in water; the higher the number of chlorine substituents, the less soluble the congener. Individual PCB congeners used for the biodegradation experiments were obtained from Ultra Scientific. All solvents used in substrate, lipid and DNA extractions were of the highest grade possible.

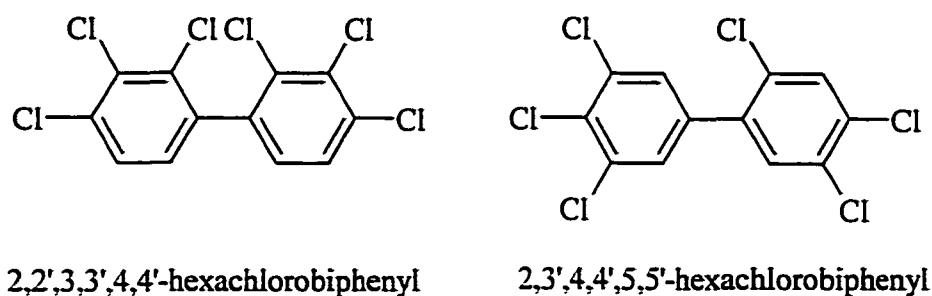


Figure 3.3 Structure of hexachlorobiphenyls used in preliminary dechlorination testing.

A mineral medium that provided no carbon source for growth and that provided the organisms with sufficient quantities of nitrogen, phosphorus, and trace quantities of

metal cations was used for all microcosm experiments (10). This medium was used by Klasson et al. in a study on the sequential treatment of PCBs in soil microcosms and was chosen by the authors to provide adequate nutrients for both the anaerobic and aerobic microorganisms (10). The compositions of all media and solutions used are given in Appendix I.

Table 3.2 Aqueous solubility of selected PCB congeners in water

PCB Congener	Solubility (mg/m ³)
2,2',5' – trichlorobiphenyl	500
2,3',4' – trichlorobiphenyl	291
3,3',4,4' – tetrachlorobiphenyl	25
2,2',4,4',5,5' – hexachlorobiphenyl	2.8
2,2',3,3',4,4' – hexachlorobiphenyl	9

3.C. Soil and Microorganisms

Soil samples were obtained from two sites previously contaminated with PCBs but with low current levels of PCBs. Previously contaminated soil was used since it has been shown that organisms able to use a certain substrate are more likely to be found in an environment contaminated by that substrate. Therefore, use of these soils increased the probability that PCB degraders were present while allowing us the advantage of studying a less complicated system for easier delineation of important engineering and

microbial parameters. Two soils were obtained. The first soil was a loamy sand from the Savannah River Site in South Carolina. It consisted of 85% sand, 9% silt and 6% clay with a pH of 5.0 and an organic matter content of 0.3%. Values for the following minerals and metals were determined by the Soil and Water Testing Laboratory at Colorado State University: 4.7 ppm NO₃-N, 9.1 ppm P, 19.2 ppm K, 1.9 ppm Zn, 14.5 ppm Fe, 1.03 ppm Mn and 6.72 ppm Cu. The soil was a distinct orange/red color due to iron hydroxides/oxide in the soil. The soil utilized for this work was a composite of samples from 4 soil cores dug at the Savannah River Site (Table 3.3).

Table 3.3 Characteristics of Soil Cores

Core	Depth	Date
1	132'-144'	April 98
2	132'-144'	November 97
3	138'-145'	February 97
4	137'-144'	February 97

In addition to PCB contamination, sample concentrations of TCE and PCE indicated the presence of dense, non-aqueous phase liquids (DNAPL). However, these soil cores were exposed to the atmosphere during storage and no residual levels of TCE or PCE were found by the time the soil was received in our laboratory.

The second soil was obtained from a Consolidated Packaging printing site. In contrast to the first soil, this soil was very high in organic matter and contained co-contamination with transformer oil and petroleum hydrocarbons. Levels of tri-, tetra-, and pentachlorobiphenyls were on the order of 50 ppm. All microorganisms to be used in this study were indigenous to the soil samples obtained.

Research activities using PCBs or PCB contaminated soil is regulated by the EPA under CFR Section 761.30(j). These regulations were followed in all aspects of this work. Any person may conduct research on PCB disposal methods without prior written approval from EPA if the following conditions are met:

- i) File a notification and obtain an EPA identification number
- ii) Notify in writing the EPA Regional Administrator, the State EPA, and local EPA at least 30 d prior to the commencement of any research and disposal activity
- iii) The amount of material containing PCBs treated annually does not exceed 500 gal or 70 ft³ of liquid and does not exceed a maximum concentration of 10,000 ppm PCBs
- iv) No more than 1 kg total of pure PCBs is used in research
- v) Each research activity under this section last no more than 1 calendar year
- vi) Store all PCB wastes in compliance with Sec. 761.65(b) and dispose of them according to the initial PCB concentration
- vii) Package and ship all PCB wastes pursuant to DOT requirements

Wastes and solids contaminated with PCBs were disposed of through the Colorado State University Environmental Health Services department according to the appropriate rules and regulations set forth in the Hazardous Waste Disposal Handbook.

3.D. Analytical Methods

3.D.1. Substrate Concentration

3.D.1.a. PCB and Dechlorination Products – GC-MS Analysis

The concentrations of the PCB congeners and dechlorination products were quantified using a Hewlett-Packard 5890 gas chromatograph equipped with an HP model 5971 mass selective detector (MSD). Samples were injected onto a Hewlett Packard HP-5 (cross linked 5% phenyl methyl silicone) capillary column with an inner diameter of 0.2 mm and a film thickness of 0.33 μm using an HP model 7673 autosampler. Samples were prepared for injection through the extraction procedure described in Section 3.G.2. An injection volume of 1 μL was used with the HP autosampler. Helium was used as a carrier gas (99.998% purity). The samples and standards were run using the method “PCB”, which is given in Appendix II. This method had a detection limit of 500 ppb for each of the congeners tested. The relative error for multiple injections of the same sample was tested and found to be less than 3%.

Octachloronaphthalene served as an internal standard for quantification of the results from the gas chromatograph. Congener concentrations were determined by comparison with standards from Ultra Scientific. Samples were checked for all possible dechlorination products, again determining concentrations with standards. A series of standard solutions with known concentrations of the PCB congeners was made up and analyzed on the GC-MSD. The concentrations of the standards ranged from 10 to 100 ppm. A calibration curve was made to correlate octachloronaphthalene peak areas/PCB peak areas to PCB concentration. A linear fit was used for all correlations. Examples of

these calibration curves are also given in Appendix II. Fresh standards were prepared from stock solutions every time samples were run. The stock solutions were purchased from Ultra Scientific and kept at -20°C in screw-cap vials for up to 6 months. Standards were run with every set of samples. Hexane-only blanks were run once every other sample run to ensure there was no sample carryover from run to run.

3.D.1.b Electron Donor Concentration – GC-FID Analysis

Methanol and butyric acid were identified and quantified using an HP 5890 II gas chromatograph and a flame ionization detector. A 900- μL sample was drawn from the aqueous phase of each soil slurry microcosm before any other samples were taken. These samples were added to 100 μL of an aqueous 10 g/L *iso*-butyric acid internal standard solution in a 2-mL glass screw-top GC vial. The samples were acidified by adding 10 μL 6N HCl to each vial. The samples were mixed, and 1 μL was injected onto a Chromosorb 101 glass column (100/120 mesh, 2 m X 3 mm). The detector temperature was maintained at 280°C and the injector at 250°C . The oven temperature was run isothermally at 162°C for 8 min. Three gases were used for analysis. Airflow was set to 400 mL/min, N_2 to 25 mL/min and H_2 to 30 mL/min. The purities of the gas were 99.9%, 99.98%, and 99.998% for air, N_2 , and H_2 , respectively. The nitrogen served as both the carrier and make-up gas. Flow rates were set using a bubble flow meter placed at the detector outlet port. The air and hydrogen flame produced a base signal of 50-60 pA. To light the FID, the airflow was reduced to 200 mL/min. After ignition, the FID was allowed to equilibrate for 1 hr before samples were injected for analysis. Peaks were identified by comparison with standards, and peak areas integrated using an HP 3396

integrator or HP Chemstation software. A typical chromatogram is shown in Appendix II. Retention times were 0.71 min, 3.51 min and 6.33 min for methanol, isobutyrate and butyrate, respectively.

Standards were analyzed with every sample set and were stored at -20°C between runs. Fresh standards were prepared every 2 months. A water blank was analyzed at the beginning and end of each sample set to determine if carryover between samples was occurring. Because manual injection was used, the relative error between multiple injections (5 injections) was larger than for the automatic injector. Relative errors of 10% for methanol and 7% for butyrate were determined. The detection limit for both compounds was determined to 1 ppm.

3.D.2. Metabolite Concentration – HPLC Analysis

During the aerobic degradation of less chlorinated polychlorinated biphenyls, chlorobenzoic acids are typically produced as a metabolic by-product. These chlorobenzoic acids frequently build up in the medium and inhibit further PCB degradation (8). Thus, their detection and quantification were of interest. To do this, a high-pressure liquid chromatography (HPLC) method was used. The aqueous fraction was removed from the soil slurry microcosms and extracted with an equal amount of ethyl acetate in a glass bottle. Because chlorobenzoic acids are soluble in water (4000 ppm), insignificant amounts were expected to be associated with the soil fraction. Controls were performed to test this assumption that showed nearly 100% recovery with extraction of the aqueous phase only. To perform the extraction, the bottle was shaken vigorously, and then the phases were allowed to separate. The upper ethyl acetate layer

was transferred to a test tube with a Pasteur pipette and the extraction was repeated with an equivalent amount of ethyl acetate. The extracts were combined and evaporated to dryness using a RapidVap nitrogen evaporation system. The residue was redissolved in 1 mL NaOH, filtered with a 0.2 μ m Acrodisc syringe filter and stored in a 250- μ L glass vial insert in a 2-mL HPLC vial at -20°C . To perform the HPLC analysis, the vials were removed from the freezer and placed in a Waters 700 Satellite Wisp autosampler.

A Bio-Sil C₁₈ HL column was used with a C₁₈ guard column. A flow rate of 2 mL/min of a 30%:70% acetonitrile: 0.05 M sodium acetate buffer (pH 4.5) was used for the experiments. The chlorobenzoic acids were detected with a Waters 486 Tunable Absorbance UV detector set at 280 nm. Retention times were compared to the retention times of standards for identification. Once a positive identification had been made, a calibration curve was prepared from authentic standards from Ultra Scientific.

3.D.3 Chloride Detection

A colorimetric assay from Hach (method 8113) was tested for determination of the amount of chlorine released by dechlorination during the soil slurry treatability and microcosm studies. This method can analyze chloride concentrations in a range from 0 to 20 ppm chloride. Since the chloride concentrations produced during the anaerobic dechlorination of the PCBs were expected to be significantly higher than this detection range, the samples were diluted to bring them within the range of the method. Because the calibration curve was linear, it was possible to scale up the results. Assumptions of this method are that no chloride is adsorbed by the soil and that the chloride is not volatile.

This method consisted of developing a standard curve with known amounts of NaCl and using this curve to calculate the chloride concentration in the samples. To begin, standards with concentrations of 0, 0.2, 0.5, 1 and 2 ppm chloride were prepared using dilutions of a 1000 ppm stock solution of NaCl (1.6488 g NaCl in 1 L deionized water). Fresh standards were made every time the analysis was performed. To each 1 mL standard or sample solution, 80 μ L mercuric thiocyanate solution (Hach #22121-29) and 40 μ L ferric ion solution (Hach #22122-42) were added and mixed. After 5 min, the optical density at 455 nm was measured with the 0 ppm standard being utilized as a blank. This method produced sporadic results and its use was discontinued. The presence of significant amounts of iron in the system was believed to be interfering with the method.

A second method, ion chromatography, was then investigated. A Waters ion chromatograph was utilized with standards of 9.5, 14.3, and 28.6 ppm NaCl in deionized, distilled H₂O. Using this method, it was determined that the background level of chloride in the soil samples was roughly 1000 ppm. The expected chlorine production from complete dechlorination of the dosed hexachlorobiphenyl to biphenyl was roughly 10 ppm. This represents a total change in chloride concentration of 1%. This change is too small to be detected by any method as it is within the experimental error of the method. This ion chromatographic method was also used to determine that background levels of both NO₃⁻ and SO₄²⁻ were below 10 ppm.

3.D.4. Ferrous Iron Analysis

The presence of compounds competing with the polychlorinated biphenyls as electron acceptors was of interest. The SRS soil was rich in total iron, so it seemed reasonable that iron reduction might occur in the anaerobic microcosms. To test for the reduction of ferric iron to ferrous iron ($\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$), a spectrophotometric method using 1,10-phenanthroline at pH 3.2 to 3.3 was used. With this method, three molecules of phenanthroline chelate one atom of ferrous iron to form an orange-red compound that can be measured at an optical density of 510 nm (2). For the analysis, 1 mL of aqueous sample from each microcosm was added to a 2 mL glass vial with 20 μL of concentrated H_2SO_4 . The acidic conditions prevent the ferrous iron in the sample from oxidizing to ferric iron in the presence of oxygen. These acidified samples were stored at 4 °C for less than 24 h before analysis.

The 1 mL samples were transferred to 10 mL volumetric flasks to which 200 μL ammonium acetate solution (125 g $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ in 75 mL deionized, distilled water (ddH_2O), and 350 mL glacial acetic acid) were added. The volume in the flask was brought to roughly 7.5 mL with ddH_2O and then 1 mL 1,10-phenanthroline solution (100 mg 1,10-phenanthroline monohydrate in 100 mL ddH_2O) was added to each volumetric flask. Finally, the volume was brought to 10 mL with ddH_2O and the contents mixed by inversion. After 10 min incubation at room temperature, the absorbance at 510 nm was measured.

To construct a calibration curve, standards were prepared using a stock iron solution of 100 ppm. To prepare this stock solution, 20 mL H_2SO_4 was added very

slowly to 50 mL ddH₂O. Then, 0.7022 g Fe(NH₄)₂(SO₄)₂·6H₂O was dissolved and the solution was diluted to 1 L and thoroughly mixed. To prepare the standards, amounts of stock solution of 0, 200, 400, 600 and 1000 µL for corresponding concentrations of 0, 2, 4, 6, and 10 ppm, respectively, were added to 10 mL volumetric flasks. After this, 100 µL of hydroxylamine solution (10 g NH₂OH·HCl in 100 mL ddH₂O) and 100 µL of sodium acetate buffer (100 g NaC₂H₃O₂·3H₂O in 400 mL ddH₂O) were added to each flask. As with the samples, the volume was brought to roughly 7.5 mL with ddH₂O, 1 mL of 1,10-phenanthroline was added, and the volume was raised to 10 mL with ddH₂O. After 10 min incubation, the absorbance at 510 nm was measured and a calibration curve prepared from the standards.

The detection limit of the method was determined to be 50 ppb. Solutions were prepared in glass-stoppered bottles with deionized, distilled water. All glassware for the analysis was acid-washed prior to use by adding 3-4 drops of concentrated H₂SO₄ wash solution (36.4 g Nochromix/L sulfuric acid), allowing the acid wash solution to remain in the glassware for 1-2 min and then rinsing with deionized H₂O. This acid washing removed any iron oxide deposits on the glassware.

3.E. Safety

Most of the chemicals used in this project are hazardous; therefore, all work was performed carefully and according to safety training. When using solvents for lipid or PCB extractions, all manipulations were carried out in a fume hood. In addition, appropriate safety clothing including latex gloves and a lab coat were worn. Particular care was taken when working with PCB-contaminated soil as PCBs are known to be mutagenic, estrogenic and

carcinogenic. All materials containing PCBs were double contained. This occurred by putting all soil microcosms, standard solutions and waste soil in a secondary containment vessel, usually a plastic container to ward against spills.

3.F. Quantitative Microbial Community Analysis

Changes in the microbial community composition and functional gene quantities were determined for use in decision-making on bioremediation strategies. To follow microbial community composition and degradation potential, techniques beyond differential plate counting were required. A combination of three techniques was used for microbial community analysis. Total community phospholipid ester-linked fatty acid (PLFA) patterns provided broad-based quantitative data on shifts in community composition. In conjunction with the community lipid analysis, a quantitative polymerase chain reaction (QPCR) method was utilized to track specific microbial genera and functional genes to better describe the effect of system perturbations on community dynamics. In order to drive primer choices for QPCR, denaturing gradient gel electrophoresis, a non-quantitative method, was used. This method was used to determine if changes in population composition were occurring and to then pinpoint what those changes were based on sequence analysis of different bands. Changes in microbial community composition were used in turn to understand and correlate changes in PCB bioremediation.

3.F.1. Plate Counting Methods

Traditional plate-counting techniques were used for enumeration of the total concentration of cells in aerobic-only microcosms. Two different media were used for plate counting. The first, ½ trypticase soy agar (TSA), is an undefined medium that should allow for growth of most types of microorganisms. The second medium was used to enumerate only those organisms able to degrade biphenyl and consisted of a mineral salts based agar (PAS) with biphenyl crystals in the lid as the sole carbon and energy source.

The spread plate method consisted of pipetting a sample onto the surface of the solidified agar medium in a Petrie dish and distributing the cells with a sterilized glass rod. After incubation for 1 wk, the colonies were counted and by using the dilution of the original sample the number of cells per g of original sample could be determined. To begin the method, 1 g of soil was added to 10 mL of sterile medium, either the TSA or the PAS medium. The test tube was then vortexed for 1 min to dislodge the cells. One mL of the supernatant was diluted ten-fold in sterile media and 0.1 mL of each dilution was pipetted onto agar plates. This diluted the sample again by 10. Final plate dilutions ranged from 10^1 – 10^3 .

3.F.2. Phospholipid Fatty Acid Analysis

The signature lipid biomarker analysis as described by White and Ringelberg was utilized for this work (17). This method does not depend on morphology or culturability; rather, it utilizes characteristic lipid biomarkers that are universally distributed in all cells. The method relies on the extraction, purification and concentration of “signature” lipid biomarkers from the cell membranes and walls of microorganisms (17).

In all steps of the signature lipid biomarker analysis, the organic solvents used must be HPLC grade, and water utilized must be nanopure-filtered organic-free, deionized and chloroform-extracted. In addition, all glassware used must be ion- and organic-free. Meticulous cleaning of all glassware is necessary beginning with use of phosphate-free detergent and followed by rinsing 5 times each with tap water and deionized water. Following cleaning, all glassware was heated at 450 °C for 4 h. Disposable glassware such as pipettes and silicic acid columns were not washed, but were heated to 450 °C for 4 h. Any items that could not be heated to this temperature (e.g., teflon bottles) were rinsed with methanol and then chloroform and allowed to dry. Organic materials such as finger lipids and hair are potential contaminants, so powder-free gloves were worn at all times during the analysis.

To begin the method, a modified Bligh and Dyer total lipid extraction was performed (5). A 20-g portion of each soil sample was weighed and transferred to a 250-mL teflon centrifuge bottle. Solvents were added in the ratio of 2:1:0.8 methanol:chloroform:phosphate buffer with a ratio of 1 g soil to 1 mL chloroform (e.g., 40 mL methanol:20 mL chloroform:16 mL 50 mM phosphate buffer) per 20 g soil being utilized. Each centrifuge bottle was then shaken and vented several times and then sonicated for 90 sec. The samples were extracted for 6-8 h. Once the first phase extraction was complete, the bottles were centrifuged for 30 min at 2000 rpm to separate the soil from the solvent. The resulting supernatant was then withdrawn with a Pasteur pipette and transferred either into a 250-mL separation funnel or a 250-mL Teflon centrifuge bottle. A second portion of chloroform (20 mL) was added to the centrifuge

bottles, which were then shaken, vented, and re-centrifuged for 30 min at 2000 rpm. The supernatant was added to the original supernatant in the separation funnel or centrifuge bottle. At this point, 20 mL of chloroform-extracted water was added to each separation funnel to split the phases. This provided a final solvent volume ratio of 1:1:0.9 for chloroform:methanol:water/buffer. The separation funnel was shaken vigorously with ventings and the phases were allowed to separate overnight or until the aqueous phase was no longer cloudy. If centrifuge bottles were utilized for this separation step, they were centrifuged for 15 min at 2000 rpm to separate the phases.

At this point, the lower organic phase was drained from the separation funnel into a 40-mL glass centrifuge tube. If centrifuge bottles were used, the bottom phase was removed using a baked 10-mL pipette. The solvents were removed with N₂ using the RapidVap evaporation system. The extraction was evaporated to approximately 2 mL, and transferred to a 10 mL test tube. The remaining extract was then removed using N₂. The total dried lipid was stored under N₂ atmosphere at -20 °C until lipid class separation was performed.

Lipid class separation was performed using silicic acid chromatography. This method separates the total lipid extract into neutral, glycolipid and polar lipid classes. The silicic acid interacts with the polar groups of the lipid classes allowing them to be selectively eluted using solvents of increasing polarity. The test tubes containing the total lipid extract were warmed to room temperature before the caps were opened. Silicic acid columns were prepared using large bore dispo-pipets. Before baking the pipettes, a small plug of glass wool was inserted into the bottom of the pipette. To construct the columns,

5 mL of a silicic acid slurry (0.5 g Unisil silicic acid to 5 mL 20 mM ammonium acetate in methanol) was added by Pasteur pipette to the baked large bore pipette. Silicic acid is easily hydrated and therefore was dehydrated at 100 °C for at least 1 h prior to use. Care was taken to ensure that there were no air bubbles in the bed. The cartridge was first wetted with 5 mL of acetone and then 5 mL chloroform, being sure that it did not dry out during the following procedure. The total lipid was resuspended in three portions of 200 µL and transferred to the silicic acid column with a Pasteur pipette. Once the silicic acid slurry had been loaded, 5 mL each of chloroform and acetone were used to elute neutral lipids and glycolipids, respectively. Finally, 10 mL of methanol was used to elute the phospholipid fraction. The phospholipid fraction was collected into a separate 10-mL test tube. Once this fraction had been collected, the solvent was removed from the phospholipid with N₂. The dried phospholipid fractions were stored under nitrogen at –20 °C until further analysis was performed.

Fatty acid methyl esters were created using a mild alkaline methanolysis to cleave the fatty acids from the phospholipid glycerol backbone and replace the glycerol bonds with methyl groups, creating fatty acid methyl esters (FAME). The dried phospholipid fraction was first redissolved in 1 mL of a 1:1 (v/v) chloroform:methanol solution and 1 mL of 0.2 M methanolic potassium hydroxide. The tubes were vortexed and incubated at 60 °C for 30 min. The samples were cooled to room temperature, and then 2 mL hexane was added and the sample was mixed. The sample was then neutralized with 200 µL of 1 N acetic acid. Neutralization is important not only because the methanolysis is incomplete at higher pH, but also because FAMEs have a higher affinity for water at

higher pH. The pH was checked with litmus paper by drawing a small amount of the lower phase into a Pasteur pipet and spotting the sample onto the paper to be sure the pH was equal to 7.0. To the neutralized solution, 2 mL nanopure water was added to separate phases. The tubes were vortexed for 30 sec and then centrifuged for 5 min at 2000 rpm. The upper phase was transferred to a new test tube. The lower phase was then re-extracted twice more with 2 mL hexane. These tubes were vortexed and centrifuged as before, with the upper phases being pooled with the first extract. The solvent was removed with N₂ and the dried FAMES were stored at -20 °C under a nitrogen atmosphere until separation and quantification were carried out.

Each sample was dissolved in a 50 pmol/μL C19:0 internal standard solution in 1 L iso-octane. In all cases, the sample was dissolved in 50 μL of the internal standard solution depending on the sample concentration. Analysis was done using an HP 5890 gas chromatograph equipped with an HP model 5971 mass selective detector. Samples were injected onto a Hewlett Packard HP-5 (cross linked 5% phenyl methyl silicone) capillary column with an inner diameter of 0.2 mm and a film thickness of 0.33 μm with an HP model 7673 autosampler. The temperature program used was as follows: 60 °C for 2 min, 10 °C/min to 150 °C, and finally 3 °C/min to 312 °C for a run time of 65 min. The injector temperature was set at 270 °C and the detector temperature at 290 °C (Appendix II). Quantification was based on a comparison to the internal injection standard C19:0. Equi-molar responses can generally be assumed with the range of microbial FAME concentrations typically observed (17). To determine the concentration of compound X (moles/volume) the following equation was used:

$$C_x = \left(\frac{A_x}{A_{ISTD}} \right) * C_{ISTD} * D \quad \text{Equation 3.1}$$

where A_x is the GC area of compound X, A_{ISTD} is the GC area of the internal standard as determined by the GC data system, C_{ISTD} is the concentration of the internal injection standard (C19:0) and D is the appropriate dilution factor. Identification of FAMES was achieved by comparison with several standard FAME solutions.

The fatty acid nomenclature is of the form 'A:B ω C' where 'A' designates the total number of carbon atoms, 'B' the number of double bonds, and 'C' the distance of the closest unsaturation from the aliphatic end of the molecule. In addition, a fatty acid's name can be modified with the suffixes 'c' for *cis* and 't' for *trans* (for the geometric isomers), the prefix cy for cyclopropyl FAMES, and the prefixes 'i' and 'a' for *iso* and *anteiso* methyl-branching, respectively. The term 'Me' is also used to designate methyl-branching that occurs at positions beside the final two carbons. Several FAMES in particular were followed as indicators of particular microbial groups. Anaerobic organisms are distinguished by large quantities of cy17:0 and cy19:0, while the FAMES 16:1 ω 7, 16: ω 7t and 18:1 ω 7 are characteristic of aerobic microorganisms (Figure 3.4). Comparing the concentrations of these two groups can provide the relative proportion of aerobic to anaerobic microorganisms at each sampling point. Sulfate-reducing bacteria were quantified by following the FAMES i17:1 ω 7, 10Me16:0, and 17:1 ω 6. Principal component analysis and multiple comparisons of means were used to compare lipid analysis results from one sampling point to another (15).

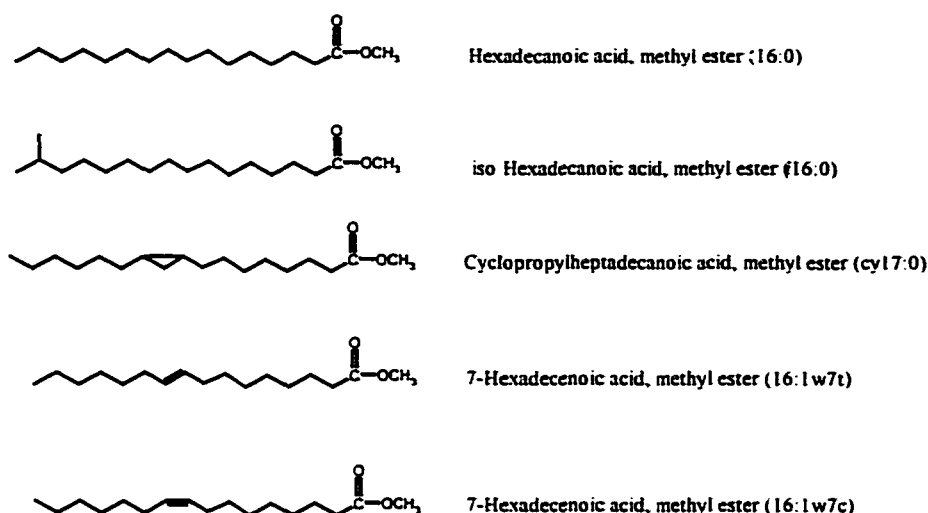


Figure 3.4 Structures of common phospholipids found in soil microorganisms

3.F.3. Molecular Based Methods

The two molecular methods used in this work were quantitative PCR (qPCR) and denaturing gradient gel electrophoresis (DGGE). Both methods require as a first step the extraction of total microbial DNA from soil samples.

3.F.3.a. Extraction of microbial DNA

The extraction method used employs a direct cell lysis and DNA extraction from soil without prior separation of the microbial cells from the soil particles. To begin the DNA extraction, 5 g soil was added to an autoclaved, 40-mL polycarbonate centrifuge tube. Lysozyme (75 mg) and 7.5 mL 200 mM phosphate buffer (pH 8.0) were added to each tube. Each tube was then vortexed for 30 s and heated for 90 min at 37 °C. After

heating, 450 μ L of 20% sodium dodecyl-sulfate (SDS) was added to each tube and the tubes vortexed for 30 s. The centrifuge tubes were then placed in a -80°C freezer for 30 min to begin the freeze-thaw lysis cycle. After 30 min, the tubes were placed in a 65°C water bath for 20 min to thaw. This freeze-thaw cycle was repeated twicemore (3 freeze-thaw cycles total) to ensure complete lysis of the cells. This combination of detergent and freeze-thaw cycles has been shown to extract DNA of large fragment size, which is important for PCR-based detection using 16S rDNA as a target sequence. After the final thaw, the tubes were centrifuged at $10,000 \times g$ for 15 min. The supernatant, which contained the cell lysate, was then transferred to a new, labeled centrifuge tube on ice. The soil pellet was then resuspended in 5 mL phosphate buffer by vortexing for 15 s.

At this point, the centrifuge tubes were heated for an additional 10 min at 65°C and centrifuged again for 15 min at $10,000 \times g$. The supernatant was again recovered and pooled with the supernatant from the previous step. If any soil particles or debris were still apparent in the supernatant, the pooled supernatants were centrifuged once more for 15 min at $10,000 \times g$. The soil from each centrifuge tube was rinsed with water into a tared weigh boat and dried for 48 h at 100°C to obtain an actual dry soil weight for each sample. To each tube, 15 mL of Tris-buffered liquid phenol (pH 8.0) was added and mixed by inversion. Extraction with phenol has been shown to remove contaminating compounds in the soil and to protect the DNA from soil nucleases (16). The tubes were then centrifuged at 10°C and $5,500 \times g$ for 10 min. The upper aqueous phase was carefully transferred into a new tube, and 15 mL of a 24:1 (v/v) chloroform:iso-amyl alcohol mixture was added. The liquids were again mixed by gentle inversion and

centrifuged at 10 °C and 5,500 x g for 5 min. The upper aqueous phase was again recovered into a new, labeled 40-mL centrifuge tube. Potassium acetate (2.8 mL, 8 M) was added and the entire tube placed in an ice bath for 10 min. The centrifuge tubes were then centrifuged for 15 min at 10,000 x g. The supernatant was recovered into a new tube into which 30 mL of 95% ethanol was added. The tubes were kept at –20 °C overnight to precipitate the DNA. After precipitation, the samples were centrifuged for 25 min at 10,000 x g. The supernatant was discarded, and the pellet was allowed to dry for 8-16 h at room temperature. The DNA pellet was resuspended at 4 °C in 0.75 mL TE overnight, transferred to a 2 mL microcentrifuge tube and stored at 4 °C until used in a PCR reaction.

3.F.3.b. Denaturing Gradient Gel Electrophoresis

3.F.3.b.1. Polymerase Chain Reaction for DGGE

To prepare samples for DGGE, a double amplification PCR protocol was set up. DNA extracts were first amplified with primers 16S-63f and 16S-1392r (11, 12). One μ L of the product from this first amplification was then used to perform PCR with primer set 16S-341f and 16S-534r (14). Both primer sets were developed for environmental organisms and amplify a 1330 bp and a 193 bp fragment, respectively. The primer sequences are given in Appendix III. Following PCR, the amplification products were run on a 1.5% agarose gel to confirm that the PCR was successful. This double amplification method was required as the starting DNA concentration in the extracts was not high enough to produce visualizable product after a single round of PCR. The first

primer set (16S-63f and 16S-1387r) was developed specifically for amplification of environmental organisms, but the PCR product generated is too large to be used for DGGE. The primer set of 16S-341f and 16S-534r are interior to the first primer set and produce a fragment small enough to be used in DGGE methods.

To set up the PCR reactions, 24 μL of 1X PCR buffer (Appendix I) containing 1 pM/ μL each forward primer and reverse primer was added to PCR tubes and overlaid with oil. The tubes were then treated with UV light for 10 min to cross-link any contaminating DNA. One μL of sample DNA was added under the oil of one tube and the tubes were placed in a PTC-100 thermal cycler (MJ Research, Inc.). All temperature programs used a hot start protocol, which consisted of raising the temperature to 95 °C for 5 min to denature the DNA strands and then lowering the temperature and holding at 80 °C while 1.5 units *Taq* polymerase were added to each tube. This protocol decreases non-specific annealing of the *Taq* polymerase to non-denatured strands. The thermal cycler then carried out the temperature programs described in Appendix III.

3.F.3.b.2. DGGE

To begin the DGGE, the glass plate for gel adhesion to was first prepared. The plate must be scrupulously cleaned by washing with a plate cleaning solution (10 M NaOH, 0.2 M SDS) using a scrubbing pad and then rinsed with H_2O and dH_2O . The plate was dried with Kim Wipes, re-cleaned with 95% ethanol and again wiped dry with Kim Wipes. To promote gel adherence, the plate was next treated with a bind-silane solution (Appendix I). This solution was applied with a Kim Wipe using even, circular, vertical and horizontal motions to ensure complete coverage of the plates. After application of

the bind-silane solution, the plate was left to dry for 5 min before being sprayed with 95% ethanol and wiped clean. This ethanol wash was then repeated 3 more times. At this point, gloves were changed and the opposite plate was prepared. The opposite plate was washed with a soft sponge and grease-cutting dish detergent. The plate was then rinsed once with ddH₂O and once with 70% ethanol before being treated twice with 400 µL of Sigma Cote on a cotton gauze pad.

The gel plates were then assembled per the Bio-Rad DCODE manual. Plastic, 0.4 mm spacers were placed on each side of the non-gel plate, and the two plates placed with treated sides toward each other. The plates were then clamped together being careful that the bottoms were flush to ensure that leakage did not occur.

Once the plates had been cleaned, 100 mL of three different gel solutions (0%, 50%, and 80% denaturing) were prepared (Appendix I). Before pouring the gels themselves, a plug was prepared by adding 1 µL of a solution of 2 mL 0% denaturing solution, 2.5 µL APS solution and 2.5 µL TEMED to each gel set (Appendix I). This plug was allowed to harden for 30 min before the full gel was poured. The gel solutions were prepared by adding 10 mL of 0% denaturing solution, 10 µL APS solution, and 10 µL TEMED solution to a sterile 15 mL tube and inverting the tube several times to mix. This process was repeated for the 50% and 80% denaturing solutions. The 50% and 80% solutions were loaded into the gradient maker using the provided tubing until 7.5 mL of each had been drawn up (Figure 3.5). The tubing was then brought together using a forked connector with a syringe needle on the end. The needle was placed in between the gel plates and the gradient maker used to load the gel. The gel was loaded slowly and at a constant rate to avoid developing air bubbles. At this point, the needle was placed in a

beaker of water and water drawn up into the tubing to prevent any residual gel from polymerizing on the tubes. A small amount of the 0% denaturing solution was then added to the top of the gel to promote polymerization. Sharkstooth combs were added to the top of the gel and clamped with the flat side inserted into the gel. Because of the presence of formamide in the denaturing solutions, the gel shrinks as it polymerizes. To counteract this effect, additional 0% denaturing solution was added as needed. The gel was allowed to polymerize for roughly 2 h before being loaded.

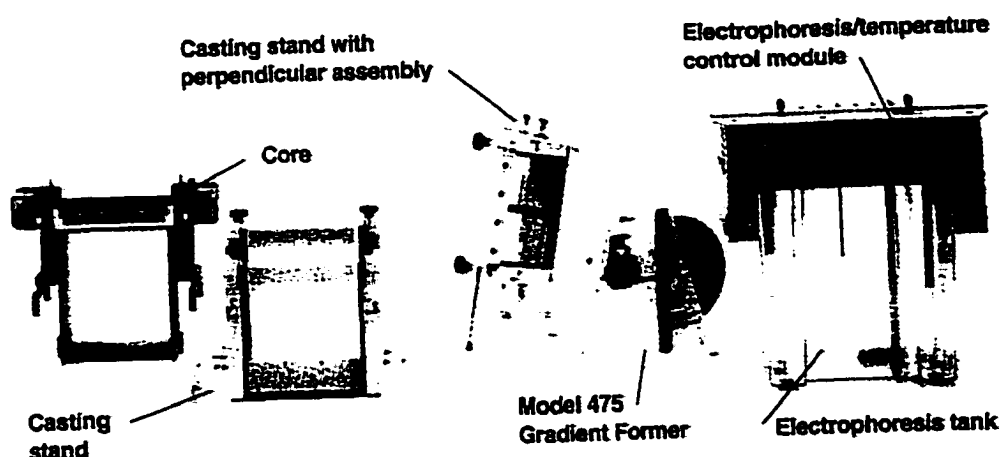


Figure 3.5 DCODE DGGE apparatus (1)

The DGGE gels were run at a temperature of 60 °C. A water bath was brought to 60 °C and connected to the gel apparatus at both ends with a pump to circulate the water. The gel was allowed to warm for 30 min before loading samples. During this time, 6 L of ½ TAE buffer was prepared and warmed to 60 °C in a microwave. The buffer was

added to the gel chamber until the aquarium and electrode areas were full. The sharktooth comb was removed, the well area rinsed with buffer, and a new sharktooth comb inserted with the teeth into the gel. Previously prepared PCR samples (1 μ L) were added to 7 μ L DGGE buffer (Appendix I) in 0.5 mL microcentrifuge tubes. These samples were loaded onto the gel in the wells formed by the comb. The power supply was set to 200V, and the gel was run for approximately 6 h or until the second dye line was an inch from the bottom.

After electrophoresis, the gel was stained to visualize the separated DNA bands. The gel plate was removed from the apparatus and placed into a plastic tub. The gel was then completely covered in fixer solution (40 mL glacial acetic acid, 360 mL ddH₂O) and put on shaker for 20 min to overnight. After fixing, the fixer solution was drained and saved for later use. The gel was washed in ddH₂O 4 times, with shaking for 2 min each time. The developer (12 g sodium carbonate in 400 mL ddH₂O) was then prepared and placed in the refrigerator immediately. The silver stain was also prepared by adding 0.3 g AgNO₃ to 400 μ L of ddH₂O. Formaldehyde (0.75 mL) was then added immediately prior to use. After the water washes, the silver stain was poured into the tub, being sure to not pour directly onto the gel. The silver stain and gel were placed on the shaker for 20 min. In the last few minutes of this step, 0.75 mL formaldehyde and 20 μ L sodium thiosulfate solution (0.455 g sodium thiosulfate in 1 mL ddH₂O) were added to the developer solution in the refrigerator. The silver solution was poured out and 200 mL ddH₂O was added to the tub. The tub was gently rocked by hand a few times before the ddH₂O was poured out. At this point, the developer was added to the tub and the tub rocked gently by hand until the gel was developed. Once the gel had developed sufficiently, the saved

fixer was added directly to the gel in the tub. The tub was then placed on the shaker for 10 min. The developer/fixer solution was poured out and 400 mL ddH₂O added to the tub that was then placed on the shaker for 20 min. The water was then removed and the gel plate allowed to dry. After drying, gels were scanned into Adobe Photoshop, or a negative taken of the gel for lab recording.

3.F.3.c. Cloning and Sequencing of Bands Differing Between Samples

When differences in banding patterns were observed between samples, those bands not observed in all samples were chosen for sequencing. To do this, each band chosen was labeled with a specific number using a permanent marker. A sterile, 0.5 mL micro-centrifuge tube was labeled for each band with the date, band number and sample ID. To remove a band of interest from the gel plate, 25 µL of sterile TE was pipetted onto the band and allowed to sit for 20-30 sec to rehydrate that portion of the gel. A sterile pipette tip was then used to scrape up the rehydrated portion of the gel containing the band of interest. The gel section was deposited into the labeled 0.5 mL tube. Following the addition of each band to its respective tube, an additional 25 µL of TE was added to each tube. The tubes were stored at 4 °C overnight to allow the DNA to diffuse from the gel to the TE. The next day, 1 µL of each band solution was used in a PCR reaction using the 16s-63f and 16s-534r primer set as described earlier.

To determine more specifically what changes in the microbial community were occurring, the PCR products from each chosen DGGE band were cloned and their nucleotide sequence determined. Before a product could be cloned, it was purified using a QIAquick PCR purification kit from QIAGEN. To begin this process 5 volumes (100

μL) of buffer PB was added to 1 volume (20 μL) of PCR reaction and mixed in a 1.5 mL microcentrifuge tube. Using a sterile pipette tip, the sample was then loaded onto a QIAquick spin column and placed in a 2 mL collection tube. The column was then centrifuged for 30 sec in a microcentrifuge; after this step, the DNA is bound to the column. The eluant was discarded, and the QIAquick column was placed back in the same tube. The DNA was then washed by adding 0.75 mL of buffer PE to the QIAquick column and centrifuging for 30 sec. The eluant was then discarded again and the QIAquick column placed back in the centrifuge tube. Centrifuging for 1 min at the maximum speed of the microcentrifuge then dried the column. The DNA was eluted by placing the QIAquick column into a clean 1.5 mL microfuge tube, adding 50 μL buffer EB (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane and centrifuging the column for 1 min. The DNA was then dissolved in the supernatant and was ready to be cloned.

An Invitrogen TA cloning kit was used for all cloning reactions. This kit uses the pCR®2.1 vector and relies on the *Taq* polymerase addition of a single deoxyadenosine (A) to the 3' ends of PCR products. The pCR®2.1 vector has single 3' deoxythymidine (T) residues, which allows the PCR inserts to ligate efficiently with the vector. The system relies on blue-white screening from bacterial expression of the *lac Z α* fragment that encodes the first 146 amino acids of β -galactosidase. The competent INV α F' *E. coli* cells used for transformation have the F' *endA1recA1hsdR17(rk⁻,mk⁻)supE44thi-1gyrA96relA1 ϕ 80lacZ Δ M15 Δ (lacZYA-*argF*)U169 λ -genotype.*

To begin the cloning procedure, the amount of DNA in each sample was determined spectrophotometrically at an absorbance of 260 nm. The amount of DNA to be used in the ligation reaction was determined with the following equation:

$$x(\text{ng PCR product}) = \frac{y(\text{bp PCR product})(50 \text{ ng vector})}{(\text{size in bp of vector})} \quad \text{Equation 3.2}$$

where for primer set 16S-341f and 16S-534r, $y=100$ and the size of the vector is 3900 bp. Once the correct amount of DNA had been determined, a ligation reaction for each PCR product was set up in a 0.2 mL thin-walled PCR tube. Each ligation reaction had a total volume of 10 μL , with the following amounts of reaction components: 1 μL 10X ligation buffer, 2 μL PCR vector, 1 μL T4 ligase, $x \mu\text{L}$ of PCR product (determined from above), and $(6-x) \mu\text{L}$ sterile water. The tubes were then incubated in the PCR thermal cycler at 14 °C for 16–20 h. For efficient ligation, PCR products were used within 2 days of amplification, and all ligation solutions were thawed on ice and kept on ice throughout the ligation reaction set-up. The ligation reactions could be stored at –20 °C for several days to a week before being used in the transformation step.

Once the PCR products had been ligated into the vector, the plasmid was then transformed into *E. coli*. In preparation for this step, Luria Broth agar plates with 50 $\mu\text{g/mL}$ of ampicillin (LB-amp50) were prepared. The preparation of these agar plates is described in Appendix I. One h before the transformation step, a water bath was set to 42 °C and allowed to heat to temperature. Also at this time, two LB-amp50 plates per ligation reaction were spread with 40 μL of a 40 mg/mL x-galactosidase solution and then

put in an incubator at 37 °C to warm for 30 min. The instructions for transformation in the TA cloning kit were followed. To begin, the vials containing the ligation reactions were thawed on ice, centrifuged for 20 s and then again placed on ice. One 50 µL vial of frozen One Shot™ competent cells per ligation reaction, the vial of SOC medium, and the vial of 0.5 M β-mercaptoethanol were thawed on ice. Then, 2 µL of 0.5 M β-mercaptoethanol was pipetted into each vial of the competent cells and mixed by stirring gently with the pipette tip. It is very important throughout the transformation procedure to not mix by pipetting up and down. Next, 2 µL of each ligation reaction was pipetted directly into one vial of competent cells and mixed by stirring gently with the pipette tip. The vials were incubated on ice for 30 min and the remaining ligation reaction stored at –20 °C. After the 30 min incubation period, the vials containing the competent cells were heat shocked for exactly 30 s in the 42 °C water bath. The vials were then placed on ice for 2 min, at which point 250 µL SOC medium (Appendix I) was added to each tube. The LB agar plates were removed from the incubator and labeled with the sample name. Then 50 µL and 200 µL from each transformation were spread on separate LB agar plates using a glass rod sterilized with ethanol. Once the liquid had been absorbed, the plates were incubated, inverted, at 37 °C for at least 18 h. The plates were then shifted to a 4 °C refrigerator for 2-3 h to allow for proper color development of the colonies.

Colonies that have a white color indicate successful insertion of the PCR product into the vector. From each plate containing between 50-200 colonies, up to 24 white colonies were transferred to a new LB-amp50 plate. This was done using sterile toothpicks and a grid underneath the agar plates to ensure correct placement of the colonies. These new agar plates were then incubated overnight at 37 °C to ensure that the

chosen colonies were indeed successful vector insertions, as evidenced by the growth of white colonies on the second plates. After overnight growth of the colonies, toothpick PCR was performed to check that the correct product was inserted into the vector. To perform toothpick PCR, the TA cloning kit primers were used (M13 and T7), and PCR IX buffer as normal used. To add the DNA, at least 8 colonies per transformation reaction were chosen. A sterile toothpick was used to scrape a small amount of each colony and transfer it to the appropriate PCR tube. Careful note was made which clone number was added to which PCR reaction so that those PCR reactions that worked could be paired with the correct clone. The Hot Fang thermal cycler program (Appendix III) was used to amplify the DNA from the toothpick PCR. The PCR products were run on an agarose gel to visualize successful amplification.

Once successful amplification was confirmed, single strand conformation polymorphism (SSCP) was performed on each clone. This type of analysis shows whether or not a DGGE band consists of only a single species or multiple species. The method is based on the electrophoretic mobility of single stranded DNA; the mobility can vary considerably as a result of only small changes in nucleotide sequence. In this work, single-stranded DNA was generated by denaturing the double-stranded PCR products. The mobility of each of the two complementary strands was then observed on silver stained electrophoretic gels. If only a single species was present, then the banding pattern of each clone from a single DGGE band would be the same. If a mixture of microorganisms is present, then each clone may have its own banding pattern. When multiple banding patterns were present, more than one clone was sent to be sequenced. The SSCP analysis was performed by a research associate in the Microbiology

Department. Briefly, the method consists of adding 1 μL of PCR product to 9 μL of DLM (Appendix I). The samples were then heated to 95 °C in a thermal cycler for 7 min, after which they were immediately placed into ice. These samples were loaded onto polyacrylamide gels prepared in a similar manner as the DGGE gels. However, no urea is added to the SSCP gels as no denaturing gradient is required. The gels were run at 5 °C and stained as described for the DGGE gels.

Each clone to be sequenced was used to inoculate a 5 mL solution of LB-amp50; these were shaken overnight at 37 °C. Plasmid DNA was isolated from each of these overnight cultures to be sent for sequencing. The Promega Wizard Plus Minipreps DNA Purification system was used to isolate plasmid DNA. Following to the manufacturer's manual, the culture tubes containing the overnight growth of each chosen clone were centrifuged for 15 min at 10,000 x g. The supernatant was poured out, and the tube blotted upside down on paper towels to remove excess medium. The cell pellets were then completely resuspended in 300 μL of Promega Cell Resuspension Solution and transferred to a sterile, labeled 1.5 mL microcentrifuge tube. To each microcentrifuge tube, 300 μL of Promega cell Lysis Solution was added and mixed by inverting 4 times. After addition of the Promega Cell Lysis Solution, the solution in the tubes should clear immediately. At this point, 300 μL of Promega Neutralization Solution was added and mixed by inverting several times. The lysate in the centrifuge tubes was then centrifuged at 10,000 x g for 5 min. For each sample, one 3 mL sterile, disposable syringe was labeled, the plunger removed, and a syringe barrel attached to the luer-lock extension of the syringe. One mL of resuspended Promega resin was added to each sterile syringe with a sterile pipette tip. The resin was resuspended by swirling between each addition.

All of the cleared lysate from each microcentrifuge tube was transferred using a pipette tip to the barrel of the minicolumn-syringe assembly containing the resin. The syringe plunger was carefully inserted, and the slurry gently pushed into the minicolumn. The syringe was detached from the minicolumn, the plunger removed from the syringe barrel, and the minicolumn reattached to the syringe. Two mL of Promega Column Wash Solution was added to the barrel of the minicolumn-syringe assembly. The plunger was again inserted into the syringe, and the wash solution gently pushed through the minicolumn. At this point, the syringe was removed from the minicolumn and the minicolumn transferred to a 1.5-mL microcentrifuge tube. The minicolumn-centrifuge tube combination was centrifuged at 10,000 x g for 2 min to dry the resin. Finally, the minicolumn was transferred to a new 1.5-mL microcentrifuge tube, and 50 μ L ddH₂O added to the top of the minicolumn. The minicolumn-microcentrifuge tube combinations were centrifuged at 10,000 x g for 20 s to elute the DNA. These eluted samples were then sent to be sequenced by Davis Sequencing at the University of California, Davis. Each sample was sequenced in the forward (T7) and reverse (M13r) directions.

Once sequences had been returned from Davis Sequencing, they were aligned with the sequence alignment program Seqman by DNASTar. The consensus sequences obtained were compared to known sequences using the National Center for Biotechnology Informatics World Wide Web browser that maintains and distributes the Gen Bank database.

3.F.3.d. PCR

The polymerase chain reaction is a commonly used technique for amplification of small amounts of nucleic acids. The PCR has recently been utilized for studies of the diversity and composition of natural microbial communities (Zheng, Alm et al. 1996; El Fantroussi, Mahillon et al. 1997; Heuer, Krsek et al. 1997). The use of qPCR to provide quantitative numbers in microbial ecology is promising. However, there are some inherent disadvantages of PCR that make qPCR difficult. Because of the exponential nature of the amplification process, minute differences in any parameter can have a large effect on the end concentration of PCR product. For this reason, it is usually easier to follow and detect changes in the amount of PCR product than to determine actual numbers of target sequences present. For this work, qPCR was not used, but it was expected that this work would allow for further use of qPCR using the primer sequences generated.

Published primer sequences were used to determine the presence or absence for several genera and functional genes of interest. Primers for methanogens, hypothesized to play a role in reductive dechlorination, and for sulfate reducers were tested as indicators of the effect of engineered perturbations on the anaerobic community. The primers used to detect the presence of methanogens were developed to amplify a 0.75 kb portion of the α -subunit gene for methyl co-enzyme M reductase (MCR) (9). This enzyme catalyzes the final step in methanogenesis and appears to be well conserved in all methanogens. To detect the presence of sulfate-reducing bacteria, primers specific to a region of the 16S rRNA common to known sulfate reducers, including *Desulfovibrio desulfuricans* and *Desulfobacter hydrogenophilus*, were used (3). The *bph* functional gene (aerobic PCB degraders) was also followed in the microcosm studies. Primers for

the enzyme 4',chloro-2,3-dihydroxybiphenyl dioxygenase (*bphC*) were developed by Erb and co-workers (7). This enzyme is responsible for a key step in the aerobic degradation of PCBs. The primer set used to test for the presence of biphenyl degraders was tested by Erb et al. and shown to be able to amplify the *bphC* gene from nearly all of the cultured biphenyl degraders. The primers were found to amplify a 758 bp fragment and to be more specific for those sequences similar to *Pseudomonas* species Q1 than to sequences similar to that found in *Burkholderia* sp. LB400.

For the published primer sequences, positive control tests were performed with DNA isolated from pure cultures representative of that genus. Negative controls with DNA isolated from species representatives of non-target genera were run with each primer set. Temperature programs used for each primer set are given in Appendix III.

Table 3.4 Published primer sequences for genera and functional genes of interest

genus/functional gene	Reference
Sulfate-reducing bacteria	(Ovreas, Forney et al. 1997)
Methanogens	(Hales, Edwards et al. 1996)
<i>bph</i> gene	(Erb and Wagner-Dobler 1993; Mondello, Turcich et al. 1997)
dioxygenase genes	(Cigolini et al. 1997)

3.G. Microcosms for PCB Bioremediation

3.G.1. Microcosm Set-up and Sampling

Soil samples taken from the Savannah River Site field site were stored at room temperature in a 10-gallon cooler until ready for use in the microcosms. Before use in the

microcosm(s), soil was sieved with a 4-mesh sieve to remove large particles and homogenize the soil.

Soil slurry experiments were conducted to determine if PCB-dechlorinating and PCB-degrading microorganisms were present in the soil samples, as well as the effects of the addition of various electron acceptors/donors on the degradation process. For the soil slurry experiments, 15% v/v soil slurries were made up with soil and mineral medium in 100 mL serum bottles. Microcosms were spiked with the congener mixture and operated in either a strictly anaerobic, strictly aerobic or sequential anaerobic/aerobic regime. The effects of addition of methanol or butyrate to the anaerobic serum bottles and biphenyl to the aerobic serum bottles on degradation of the PCB congeners were also determined, with duplicate flasks being sacrificed per time point. Serum bottles were monitored for the original congener concentration, dechlorination and degradation products, total microbial biomass, chlorine production, and PCB degraders.

The aerobic and anaerobic serum bottles were set up in a single day, with the serum bottles to be used for sequential treatment set up the following week, also in a single day. To begin the procedure, all serum bottles, caps and media were autoclaved for at 121 °C for 30 min. To each serum bottle, 25 g of sieved, homogenized soil was added, and the exact amount of soil added recorded. Bottles were chosen randomly from the box to be filled to ensure there were no time-dependent artifacts caused by sampling. Stock solutions of each PCB congener in hexane were added using a glass syringe. The correct amount of each congener to give a final concentration of 50 mg/kg each of 2,2',5- and 3,3',4,4'-PCB and 100 mg/kg 2,2', 4,4',5,5'-PCB was added (313 µL of 4,000 mg/L 2,2',5-PCB, 1.25 mL of 3,3',4,4'-PCB, and 625 µL of 2,2',4,4',5,5'-PCB). The bottles

were then left unopened in the fume hood for 1 hr to allow the hexane to volatilize. At this point, 30 mL of autoclaved mineral medium was added to each bottle using a sterile pipette. Formalin (1.5 mL) was added to the sterile control treatment serum bottles (Treatments #3, 6, 8, 14, 16) to ensure sterile conditions. Treatments 2, 3, 10, 13, 14, 15, and 16 received 125 μ L of biphenyl stock solution (50 g/L in hexane); treatments 5, 6, 11, 13, and 14 received 6.5 μ L butyric acid; and treatments 7, 8, 12, 15, and 16 received 8.5 μ L of methanol (Table 3.1). The bottles were then sealed with Teflon-lined rubber septa and aluminum crimp caps. All bottles were shaken at 120 rpm in the dark to ensure adequate mixing and to minimize photodegradation. The bottles were incubated throughout the experiment at room temperature, which varied between 23 °C and 26 °C.

Microcosms were sampled at 0, 1, 2, 3, 5, and 7 wk for the aerobic serum bottles and at 0, 4, 12, 16, 22, 30, 40, 50 and 60 wk for the anaerobic serum bottles. The sequential serum bottles were sampled anaerobically at 0, 4, 12, 16, 22, 30, 40 and 60 wk before being switched to aerobic conditions at which point they were sampled at 0, 2, 5, and 7 wk.

3.G.1.a. Sampling Procedure

To begin the sampling procedure, each unopened serum bottle was placed in a 250-mL centrifuge bottle, which was capped and then centrifuged for 20 min at 3000 rpm. This separated all the soil particulates from the aqueous phase to facilitate sampling. For each microcosm, the redox potential and pH were measured and the presence or absence of gas production as determined by pressure buildup in the bottles was recorded. The aqueous phase was then decanted into a labeled 50 mL test tube.

From the aqueous phase, 1 mL samples were taken for chloride analysis (Section 3.D.3) and ferrous iron analysis (Section 3.D.4.). Another sample (0.9 mL) was added to a 2 mL glass vial to be used for analysis of the electron donor if added (Section 3.D.2.). The remaining aqueous phase was then used in the extraction for analysis of PCBs and degradation intermediates. To finish the sampling procedure, the soil from each microcosm was split into two fractions. Roughly 20 g of the 25 g original soil was measured into an autoclaved, labeled 25 mL sample vial. These vials were immediately placed in a -80 °C freezer to be used later for lipid and DNA extractions (Sections 3.F.1 and 3.F.2.a).

3.G.2. Substrate, Intermediate and Product Extraction

To begin sampling of the serum bottles, the bottle was removed from the shaker and allowed to sit overnight to facilitate settling of the suspended soil particles. Decachlorobiphenyl stock solution (125 µL of a 400 mg/L solution) was added to each sample with a gas-tight syringe. The decachlorobiphenyl was used as a surrogate compound throughout the extraction procedure. The aqueous fraction was transferred to a separate 40-mL test tube equipped with a Teflon-lined cap. Fifteen mL acetone was added to the soil fraction, the bottle resealed with a Teflon-lined rubber septa and an aluminum crimp cap, and the contents shaken vigorously for 5 min. Once the soil particles had again settled, the acetone was transferred into a 150 mL serum bottle with a glass Pasteur pipette. The soil was then re-extracted with an additional 15 mL acetone following the procedure described above. The liquid was pooled with the original 15 mL acetone. The soil fraction was then extracted in an identical manner with two 15 mL

samples of hexane; the extracts were added to the acetone extracts in the serum bottle. The aqueous layer that had been portioned off in the sample portioning was then extracted with 10 mL hexane, which was added and vigorously shaken. The upper layer was pipetted off and added to the 60 mL of extract already in the 100 mL tube. This step was repeated with an additional 10 mL hexane. Next, the samples were concentrated from the approximately 80 mL to 1 mL using a RapidVap nitrogen evaporation system. To each 1 mL sample, 30 μ L of octachloronaphthalene stock solution (800 mg/L) was added to serve as an internal standard. The extracts were then run on the GC-MS using the temperature program described above.

3.G.3. Ortho Dechlorination Enrichment Setup

Ortho dechlorination of one of the hexachlorobiphenyls was observed in the preliminary dechlorination studies. The observed dechlorination of 2,2',3,3',4,4'-hexachlorobiphenyl to 3,3',4,4'-tetrachlorobiphenyl was unusual because ortho dechlorination is not often reported in the literature and no pentachlorobiphenyl intermediate was detected. Because of these interesting and unexpected results, further studies were initiated to investigate the phenomenon. Microcosms were set up in 10-mL vials with glucose as an electron donor. Other than the size of the microcosms, all other conditions remained consistent with the original experiment. To prepare the microcosms, 5 g soil was added to each vial. In the fume hood, 250 μ L of a 2000 mg/L 2,2',3,3',4,4'-hexachlorobiphenyl stock solution in hexane was added to each vial. The vials were allowed to sit open for 30 min to allow the hexane to evaporate. The vials were then

capped and transferred to the anaerobic chamber where 15 mL of anaerobic mineral medium and 5 mL of glucose solution (0.1 g/mL) were added to each vial. The vials were then stored in the dark, without shaking, until being sampled. Duplicate vials were sampled every 8-10 wk. The entire contents of a single vial was extracted as described above (Section 3.F.1.a) to determine dechlorination products and the extent of dechlorination.

3.G.4. Vitamin B₁₂ Experiments

Research has shown that reduced forms of vitamin B₁₂ and other corrinoids can carry out the dechlorination of highly chlorinated compounds, including polychlorinated biphenyls (4, 6, 18). In addition, the production and subsequent release of vitamin B₁₂ into aqueous media by anaerobic microorganisms is well documented. Therefore, tests were performed to determine if the observed PCB dechlorination was due to a non-biological, microbially mediated process such as reduction by vitamin B₁₂. Soil slurry microcosms similar to those originally set up were used for these experiments so that comparisons could be made between the experiments. The microcosms were scaled down, but the same liquid medium/soil ratio was maintained. Four treatments were tested: 1) a negative control that contained sterile soil and anaerobic medium only; 2) a positive control that contained sterile soil, medium, and vitamin B₁₂; 3) a treatment with sterile soil and filtrate from microcosms displaying dechlorination that had no electron donor addition (treatment #4); and 4) sterile soil and filtrate from microcosms displaying dechlorination with butyrate added as an electron donor (treatment #7). Treatment 2 provided information on whether or not vitamin B₁₂ was able to catalyze dechlorination

under the conditions observed in the original microcosms. Treatments 3 and 4 determined if there were any molecules, such as vitamin B₁₂, in the actively dechlorinating microcosms that were able to carry out reductive dechlorination of the PCBs. The soil for each treatment was sterilized by autoclaving for 30 min at 121 °C on three consecutive days. Because these experiments involved sterile soil, another deviation from the original microcosms was required to set the redox potential at levels corresponding to those of the original experiments. In the absence of microorganisms, the vials would not become anaerobic without external control of the redox potential. For this reasons, sodium thioglycollate was used as it poised the microcosms at -130 mV.

Each microcosm consisted of a 10-mL glass vial with Teflon-lined screw cap. Five g soil was added to each vial, with 8 vials being set up per treatment. The vials were then autoclaved for 30 min on 3 consecutive days. All subsequent manipulations were carried out in the anaerobic chamber under an atmosphere of 10% H₂:5% CO₂:85% N₂. In the chamber, 100 mg/kg (250 µL of a 2000 mg/L solution in hexane) of 2,2',4,4',5,5'-hexachlorobiphenyl was added to each vial using a glass syringe. To treatments 1 and 2 (positive and negative controls), 6 mL of anaerobic (0.05% sodium thioglycollate), sterile mineral medium was added. To the positive controls, only 0.5 mmol/L vitamin B₁₂ was added (300 µL of a 14 g/L solution in H₂O). For treatments 3 and 4, which required supernatant from actively dechlorinating microcosms, two microcosm bottles per treatment (from the original experiment) were sacrificed. Because of the large percentage of fines in the soil, the supernatants required centrifugation before filtering. To accomplish this, the microcosms were opened in the anaerobic chamber and decanted into centrifuge bottles which were then sealed. The centrifuge bottles were removed from the

anaerobic chamber, centrifuged at 10,000 x g for 15 min and placed back in the anaerobic chamber. The supernatant from the centrifugation was then filtered through a 0.2- μ m syringe filter and added to each of the corresponding vials from treatments 3 and 4. The vials were then capped and removed from the anaerobic chamber. They were shaken in the dark on the same shaker as the original microcosms at the same rpm. The bottles were sampled every 10 wk and the entire contents of one vial extracted to determine if dechlorination was occurring. The samples were run on the GC-MS as described in Section 3.D.1.a

3.G.5. Initiation of Aerobic Conditions Experiment

To carry out a sequential anaerobic/aerobic treatment process, a method for initiating aerobic conditions was needed. The method chosen has a direct impact on the microbial population and its ability to carry out degradation of the less-chlorinated congeners. Two methods were tested for initiation of aerobic conditions, 1) sparging with air and 2) addition of hydrogen peroxide (H_2O_2). These methods were tested in soil slurry microcosms set up to match those originally used for the investigation of aerobic and anaerobic treatments alone. The microcosms were run anaerobically for 1 wk to simulate the conditions of the sequential bottles.

To set up the experiment, 60 mL glass serum bottles were autoclaved and labeled. Twenty-five g soil was added to each bottle and 50 mg/kg 2,2',5'-PCB in hexane added using a glass syringe. The bottles were left in the fume hood for 1 hr to allow the hexane to evaporate. To each bottle, 30 mL anaerobic mineral medium was added. The medium was prepared by boiling under N_2 atmosphere for 20 min, autoclaving while stoppered,

and dispensing in the anaerobic chamber. Finally, each bottle was sparged for 5 min with a 85% N₂: 10% CO₂: 5% H₂ gas mixture using a gas flow rate of 0.6 L/min. The sparging caused the redox indicator resazurin to change from purple to pink, indicating slightly reduced conditions; this is consistent with conditions found in the anaerobic microcosms. The bottles were then capped with Teflon-lined rubber septa and aluminum crimp caps and placed on their sides at room temperature on a tabletop shaker. The bottles were covered in foil to prevent any photooxidation of the PCBs. Two bottles were sampled at this point for PCB concentration and samples were frozen at -80 °C for DNA and lipid analysis. The dissolved oxygen levels in each microcosm were measured before sampling using an Ingold DO probe. The DO probe was calibrated using air saturated water for one calibration point and with 2.5% (w/v) sodium metabisulfite solution for the zero calibration point. Values for the DO concentration were taken after 3-4 minutes, once the readings had reached a steady value.

After 1 wk under anaerobic conditions, the bottles were removed from the shaker for initiation of aerobic conditions. Two bottles were removed at this point for determination of changes in PCB concentration and microbial community composition. For the air-sparged treatment, the bottles were opened and sparged with air for 20 min and recapped. A Pasteur pipette (diameter of 2 mm) was used to deliver the air at a volumetric flow rate of 0.2 L/min, as measured by a bubble flow meter. This flow rate provided 300% of the mass of air required to fully saturate the medium. DO probe measurements indicated that at the end of the 20-min sparge period, the DO concentration in the microcosms ranged from 6.5-6.8 ppm. For the hydrogen peroxide treatments, 100, 250, or 500 µL of 30% H₂O₂ corresponding to a final concentration 0.1%, 0.25%, or

0.5%, was added to each of 8 serum bottles. Since one part H_2O_2 supplies 0.47 parts O_2 , these values correspond to theoretical oxygen concentrations of 470, 1175, and 2350 mg/L. However, H_2O_2 can decompose because of soil-catalyzed reactions that decrease the amount of available H_2O_2 . Soils containing high concentrations of natural organic carbon, iron, and copper can experience a decrease in the available H_2O_2 concentration from a theoretical 500 ppm to roughly 100 ppm. These bottles were then recapped and placed back on the shaker until sampling occurred. Two bottles per treatment were sampled immediately after treatment with either air or H_2O_2 and also at 2, 5, and 7 wk. Samples were stored for DNA and lipid analysis. The aqueous portion was extracted for chlorobenzoates (the aerobic degradation products of PCBs), and the remaining soil fraction was extracted for determination of PCB concentration.

Control microcosms were prepared to determine if either air sparging or hydrogen peroxide treatment effected any change in the PCBs themselves. Microcosms were prepared in a manner identical to those described above, with two exceptions. First, the soil was sterilized in the microcosms by autoclaving on 3 successive days before addition of the sterile medium and PCB. Second, instead of 60 mL bottles, 15 mL vials were used with 5 g soil and 6 mL sterile medium. The PCB added was scaled down appropriately to provide a final concentration of 50 mg/kg as in the non-sterile treatments. The bottles were incubated under anaerobic conditions for 1 wk and then switched either by air sparging or by addition of hydrogen peroxide (0.1%, 0.25% or 0.5%). Samples were then taken at 0, 2, 5, and 7 wk and extracted for PCB concentration.

3.H. References

1. 1996. The DCode Universal Mutation Detection System. Bio-Rad Laboratory.
2. 1980. Standard Methods for the Examination of Water and Wastewater, vol. 1. American Water Works Association.
3. **Amman, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria on multispecies biofilms. *Applied and Environmental Microbiology*. **58**:614-623.
4. **Assaf-Anid, N., L. Nies, and T. M. Vogel.** 1992. Reductive dechlorination of a polychlorinated biphenyl congener and hexachlorobenzene by Vitamin B₁₂. *Applied and Environmental Microbiology*. **58**:1057-1060.
5. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*. **37**:911-917.
6. **Burris, D. R., C. A. Delcomyn, M. H. Smith, and A. L. Roberts.** 1996. Reductive dechlorination of tetrachloroethylene and trichloroethylene catalyzed by vitamin B₁₂ in homogeneous and heterogeneous systems. *Environmental Science and Technology*. **30**:3047-3052.
7. **Erb, R. W., and I. Wagner-Dobler.** 1993. Detection of polychlorinated biphenyl degradation genes in polluted sediments by direct DNA extraction and polymerase chain reaction. *Applied and Environmental Microbiology*. **59**:4065-4073.
8. **Guilbeault, B., M. Sondossi, D. Ahmad, and M. Sylvestre.** 1994. Factors affecting the enhancement of PCB degradative ability of soil microbial populations. *International Biodeterioration and Biodegradation*. **33**:73-91.

9. **Hales, B. A., C. Edwards, D. A. Ritchie, G. Hall, R. W. Pickup, and J. R. Saunders.** 1996. Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Applied and Environmental Microbiology*. **62**:668-675.
10. **Klasson, K. T., J. W. Barton, B. S. Evans, and M. E. Reeves.** 1996. Reductive microbial dechlorination of indigenous polychlorinated biphenyls in soil using a sediment-free inoculum. *Biotechnology Progress*. **12**:310-315.
11. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. John Wiley and Sons, Chicester, UK.
12. **Marchesi, J. R., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, and W. G. Wade.** 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology*. **64**:795-799.
13. **McFarland, V. A., and J. U. Clarke.** 1989. Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: considerations for a congener-specific analysis. *Environmental Health Perspectives*. **81**:225-239.
14. **Muyzer, G., E. C. d. Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polmerase chain reaction-amplified genes encoding for 16S rRNA. *Applied and Environmental Microbiology*. **59**:695-700.

15. **Ott, R. L.** 1992. An introduction to statistical methods and data analysis, 4 ed.
Duxberry Press, Belmont, CA.
16. **Van Elsas, J. D., and K. Smalla.** 1995. Extraction of microbial community DNA
from soils, p. 1.3.3: 1-11, Molecular Microbial Ecology Manual. Kluwer
Academic Publishers.
17. **White, D. C., and D. B. Ringelberg.** 1998. Signature lipid biomarker analysis, p.
255-272. *In* R. S. Burlage, R. Atlas, d. Stahl, G. Geesey, and G. Sayler (ed.),
Techniques in microbial ecology, vol. 1. Oxford University Press, New York.
18. **Woods, S. L., and D. J. Trobaugh.** 1999. Polychlorinated biphenyl reductive
dechlorination by Vitamin B₁₂s: Thermodynamics and regiospecificity.
Environmental Science and Technology. **33**:857-863.

CHAPTER 4

PRELIMINARY RESULTS

4.A. Preliminary Soil Screening

The degradation of polychlorinated biphenyls in soil can be very slow, with substrate degradation requiring weeks or even many months to be completed. Because full scale treatability testing could possibly take six months to a year, preliminary experiments were carried out to determine whether the candidate soils were appropriate for use in the full-scale study. These preliminary experiments were used to determine three things: 1) if organisms able to anaerobically dechlorinate PCBs were present in the soil, 2) if organisms able to aerobically degrade PCBs were present in the soil, and 3) what the basic microbial community structure was. Information from these experiments was then utilized to decide which soil would be used for the remainder of the work.

4.A.1. Soil Acquisition

To ensure the soil chosen for experimentation was suitable, several candidate soils were sought out to use for the experiments. Ideal qualities for the soil were: 1) prior contamination with PCBs, but with low current levels, 2) abundant microorganisms, 3) little co-contamination from other chemicals, and 4) microorganisms with PCB-

degrading capabilities. Soil was obtained from two sites previously contaminated with PCBs. While some metabolic activities (e.g., the ability to utilize glucose) are present in virtually all microbial populations, other activities (such as the ability to degrade chlorinated compounds) appear to be present in a much more limited number of populations. It has been reported that microbial populations exposed to a certain chemical are more often able to degrade that chemical than microorganisms that have never been exposed (6).

The first of these soils was obtained from a Consolidated Packaging (CP) site in Michigan where printing activities took place. The soil obtained from this site was highly organic (40-50%) primarily contaminated with low levels (40 mg/kg soil) of tri-, tetra-, and pentachlorobiphenyls (Aroclor 1254 and 1260) (7). Co-contamination with transformer oil, benzopyrene, benzo(a)fluoranthene, and petroleum hydrocarbons was also present. The second soil was obtained from the Savannah River Site (SRS), a 310-square-mile site located in southwest South Carolina. The Savannah River Site was constructed during the 1950's to produce plutonium-239 and tritium in the production of nuclear weapons; much of the site is contaminated with the radionuclides. In addition, contamination by organics such as solvents (trichloroethylene, perchloroethylene), polychlorinated biphenyls, and hydrocarbons exists throughout the site. The soil obtained from this site was sampled from a depth ranging from 132-145 feet (5). This soil is a sandy loam with less than 1 mg PCBs/kg soil. The soil has high levels of iron and low levels of organic matter. The specific characteristics of the SRS soil are given in Section 3.C.

4.A.2 Microbial Characterization

Before any PCB degradation tests were carried out, a combination of plate counts and a signature lipid biomarker analysis was utilized to characterize the indigenous populations of both soils. Both methods indicated a higher biomass with more microbial diversity for soil from Consolidated Packaging than for the soil from the Savannah River Site. Initial total cell counts were determined for each soil using plating on $\frac{1}{2}$ TSA, and were 2.68×10^6 and 5.09×10^4 colony forming units/g for the CP and SRS soil, respectively. In addition to enumerating total microbial concentration, the estimation of the concentration of microorganisms able to degrade biphenyl was of interest because the aerobic degradation of PCBs occurs through the action of an enzyme responsible for the oxidation of the biphenyl molecule. Thus, the concentration of biphenyl-degrading microorganisms is an indicator of the potential for degradation of less-chlorinated PCBs in a specific sample. Using a plate counting technique with biphenyl as the sole carbon source, concentrations of biphenyl-degrading microorganisms of 5×10^5 and 3.5×10^5 colony forming units/g were determined for the CP and SRS soils, respectively. While the results for the SRS soil appear to be contradictory (biphenyl plate counts higher than total plate counts), this could just be an indication that the microbial population is better able to grow on the biphenyl plates than the relatively rich TSA medium.

Because plate counts rarely give an accurate picture of environmental microbial populations, phospholipid fatty acid (PLFA) analysis was also utilized to characterize the indigenous microbial populations. PLFA analysis can be used to determine both a total biomass content of soil samples and also give a general description of the diversity and types of

microorganisms present in a specific sample (10). Profiles from each soil indicated the presence of both anaerobic and aerobic microorganisms, as evidenced by the presence of specific FAMES, e.g. *i*15:0 and *i*17:0 (anaerobic) and 16:1 ω 7c,t and 18:1 ω 7c (aerobic). Despite these similarities, the pattern of FAMES was distinctly different for the two soils. Figures 3.1 and 3.2 show an example of the gas chromatograph-mass spectrometer (GC-MS) FAME profile for the CP and SRS soil, respectively. Each peak on the GC-MS profile indicates a specific fatty acid methyl ester. The higher number of different FAMES in the CP soil indicates a greater diversity of microorganisms than in the SRS soil. In addition, the presence of lipid biomarkers belonging to sulfate-reducing bacteria were present in the CP soil while those markers were absent in the SRS soil. Sulfate-reducing bacteria have been implicated in the dechlorination of highly chlorinated PCB congeners possibly indicating the potential of dechlorination by CP microorganisms (13).

Using total pmol of phospholipid detected, total microbial cell counts were approximated from the PLFA analysis. A conversion factor of 2.5×10^4 cells/pmol was used to estimate total cell counts of approximately 2.3×10^8 cells/g for the CP soil and 2×10^6 cells/g for the SRS soil. This conversion factor was utilized by Rajendran et al. to estimate the microbial biomass in surface sediment samples from Osaka Bay, which is heavily contaminated with metals and pesticides (8). The calculated cell concentrations are orders of magnitude higher than those obtained using traditional cell counts for total bacterial numbers, confirming the frequently reported phenomenon of plate counts being insufficient as a method for enumeration of environmental microorganisms.

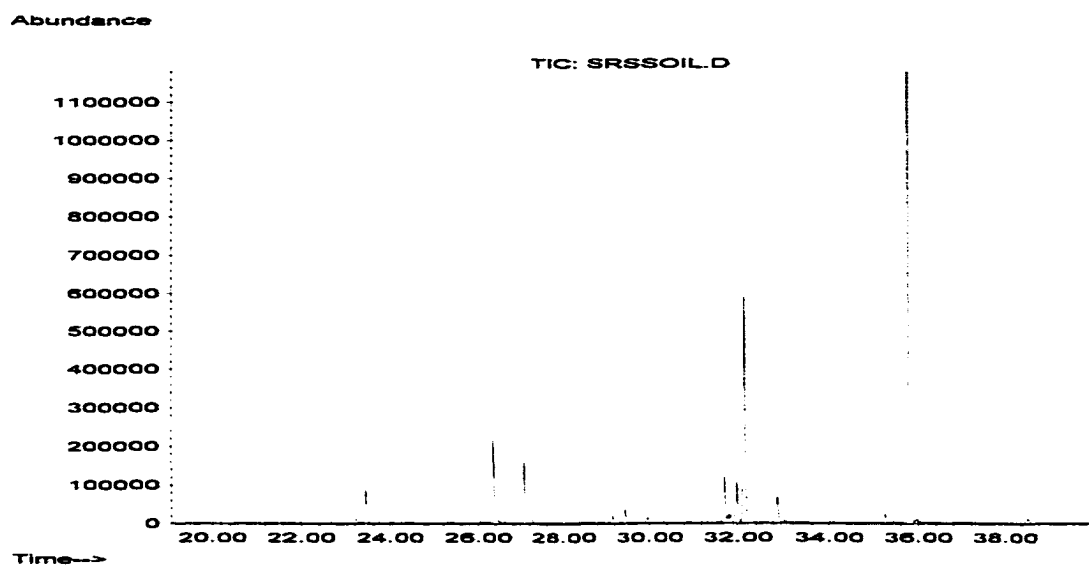


Figure 4.1 Savannah River Site Soil – PLFA Analysis GC Trace

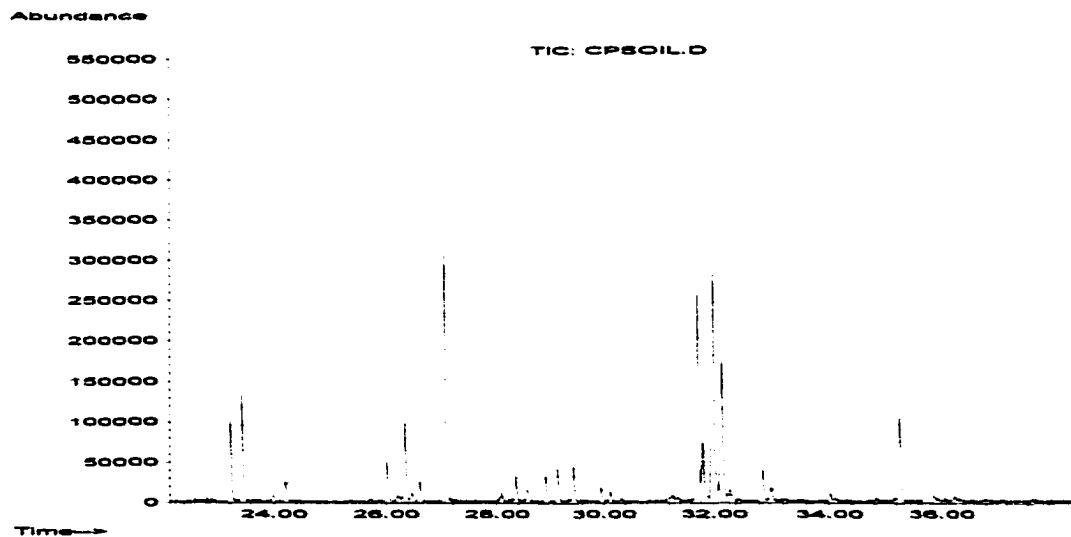


Figure 4.2 Consolidated Packaging Soil – PLFA Analysis GC Trace

4.A.3. Ability of Indigenous Organisms to Degrade PCBs

The first step in the successful implementation of a sequential anaerobic-aerobic treatment process with indigenous organisms was the confirmation of both metabolic activities at a single site. Thus, soil slurry experiments were conducted to determine if PCB-dechlorinating and PCB-degrading microorganisms were present in the soil samples.

4.A.3.a. Anaerobic Dechlorination

An initial slurry microcosm study of the two soils was performed to test the capability of the indigenous microorganisms to dechlorinate PCBs. Three hexachlorobiphenyls were chosen: 2,2',3,3',4,4'-, 2,2',4,4',5,5'-, and 2,3',4,4',5,5'-hexachlorobiphenyl. All three are present in several Aroclor mixtures and would thus be expected to be present in many contaminated matrices. In addition, differences in their chlorination patterns allowed us to develop a profile of the dechlorination ability of the microbial community. The soil slurry microcosms for the SRS soil were amended with glucose to provide an electron donor source for the dechlorination. No electron donor amendment was required for the CP soil; it was assumed that sufficient organic matter was present to act as an electron donor. Samples were taken every 4 wk for 22 wk for each soil and tested for PCB dechlorination as indicated by the presence of less chlorinated degradation products. A sterile control was also sampled for each soil. The redox indicator resazurin was used to indicate whether reducing conditions were achieved in each microcosm. Under reducing conditions, resazurin turns first pink, and then clear as conditions become more reduced.

Dechlorination in both soils demonstrated the presence of indigenous organisms capable of carrying out the reductive dechlorination of PCBs. However, in each case,

dechlorination only reached 2-5% of the original hexachlorobiphenyl during the 22-wk preliminary study. This indicates that dechlorination in these soils was relatively slow. Dechlorination was first observed at week 4 and week 8 for the CP and SRS soils, respectively. No dechlorination was observed in any of the sterile control samples. After 1 wk, the CP soil became reduced, as indicated by a change in color of the redox indicator to clear. SRS soil microcosms amended with glucose also became reduced after 3 wk; those microcosms not amended with glucose did not achieve reducing conditions sufficient to cause a change of color in the redox indicator resazurin. In both the CP and SRS soils, a lag time between the onset of reducing conditions as indicated by a color change and the first evidence of dechlorination occurred.

Different dechlorination patterns were observed for the two soils and were congener specific. Results of the dechlorination study are summarized in Table 4.1. The SRS soil appeared to have a wider range of dechlorination than the CP soil. The pattern observed in the CP soil fits that of the described pattern P (2). Process P dechlorination is restricted to *para* dechlorination, primarily of tetra- through heptachlorobiphenyls containing 34-, 234-, 245-, and 2345-chlorophenyl groups (2). This activity was observed in Woods Pond sediments by Bedard et al. and removed only those *para* substituents with at least one adjacent chlorine. No dechlorination pattern described to date correlates with dechlorination of 2,2',4,4',5,5'-PCB at both the *meta* and *para* positions as was observed in SRS soil; thus, two different microbial groups may be responsible for the dechlorination observed in these preliminary studies. In addition, the *ortho* dechlorination observed in the SRS soil of 2,2',3,3',4,4'-PCB was very unusual. Reports of *ortho* dechlorination in soil are rare and the absence of formation of any pentachlorobiphenyls is also unusual (12). The presence of this rare activity also indicates the

presence of a unique microbial population not identified by previous studies. Finally, no dechlorination was observed with 2,2',3,4',5,5'-PCB. This congener has the same chlorination pattern on the reacting ring as 2,2',4,4',5,5'-PCB which was dechlorinated at the *meta* and *para* positions. This result revealed that the chlorination pattern on the non-reacting ring appeared to affect the pattern of dechlorination on the reacting ring.

Table 4.1 Reductive dechlorination patterns observed in soil slurries

Congener	CP soil	SRS soil
2,2',3,3',4,4'	<i>para</i> to 2,2',3,3',4	<i>ortho</i> to 3,3',4,4'
2,2',4,4',5,5'	<i>para</i> to 2,2',4, 5,5'	<i>meta</i> to 2,2',4,4',5 and <i>para</i> to 2,2',4,5,5'
2,3',4,4',5,5'	no dechlorination	no dechlorination

4.A.3.b Aerobic Degradation

In addition to testing for anaerobic dechlorinating activity, an experiment was performed to test the ability of the indigenous organisms in each soil to aerobically degrade less-chlorinated PCB congeners. Three congeners were tested: 2,3',4'-trichlorobiphenyl, 2,2',5,5'-, and 3,3',4,4'-tetrachlorobiphenyl. Each slurry microcosm was dosed with all three congeners at a concentration of roughly 100 mg/kg soil each. Additional microcosms were set up to investigate the role of biphenyl in inducing PCB degradation. PCB and biphenyl concentrations were monitored for each microcosm over a period of 2.5 wk. No aerobic degradation of the PCBs or the biphenyl was observed in microcosms of the Consolidated Packaging soil. The reason for this is unknown but was

hypothesized to be due to the presence of inhibitory substances in the soil such as sulfur or dyes.

In the Savannah River Site soil, both the amended and unamended microcosms showed 100% degradation of 2,3',4'- and 2,2',5,5'-PCB as well as biphenyl in the amended microcosms (Figure 4.3 – 4.4). Insignificant extents of 3,3',4,4'-PCB degradation were observed (Figure 4.5). In a study by Bedard and co-workers that investigated the ability of a pure strain of *Corneibacterium* sp. MB1 (reclassified as *Rhodococcus gloverulus*) and a mixed culture from a biphenyl enrichment, both were able to degrade 2,3',4'-PCB and the mixed culture was able to degrade 2,2',5,5'-PCB (3). Most researchers have found that aerobic PCB degradation begins with attack of a 2,3-dioxygenase (1, 4). This enzyme should be able to degrade those congeners with free 2,3 sites (e.g. 2, 3',4', and 3,3',4,4') but not those compounds with blocked 2,3 sites (e.g. 2,2',5,5'). However, in this work both degradation of 2,3',4'- and 2,2',5,5'-PCB was observed. This would indicate the presence of a dioxygenase that preferentially cleaves the biphenyl ring at a free 3,4 site in addition to the commonly found 2,3-dioxygenase. Unterman et al. used two complementary strains, one that primarily utilized a 2,3-dioxygenase and the other that utilized a 3,4-dioxygenase, to effect more extensive PCB degradation than observed with either strain alone, demonstrating the utility of a mixed culture approach to PCB degradation (9).

The congener 3,3',4,4' also has a free 2,3 site but not a free 3,4 site. Based on these results, the 2,3-dioxygenase should also degrade this compound. However, the resistance of particular congeners (3,3',4,4') to aerobic degradation cannot be explained solely based on the presence or absence of free 2,3 or 3,4 sites (2). A study by Williams

et al. also observed degradation of 2,2',5,5'-PCB by several PCB-degrading strains but no degradation of 3,3',4,4'-PCB (11).

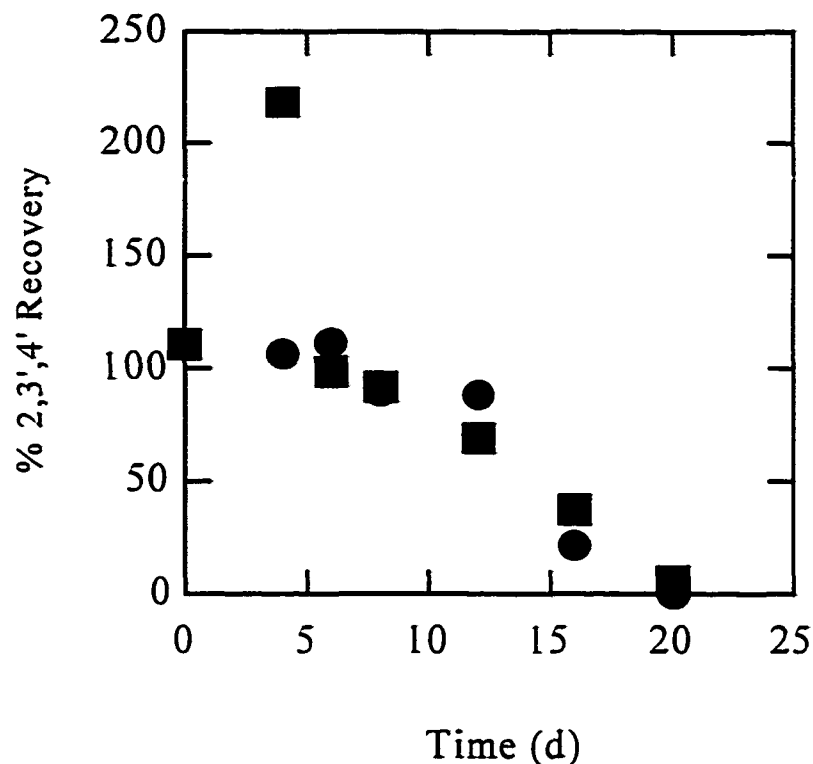


Figure 4.3 Degradation of 2,3',4'-PCB in SRS soil. $\square$ - with biphenyl amendment, $\bullet$ - without biphenyl amendment. The concentrations of the biphenyl amended and unamended microcosms had nearly the same initial recovery and thus appear on the graph as a single point. The value for the unamended treatment at wk 5 is an obvious outlier and was not taken into consideration for analysis.

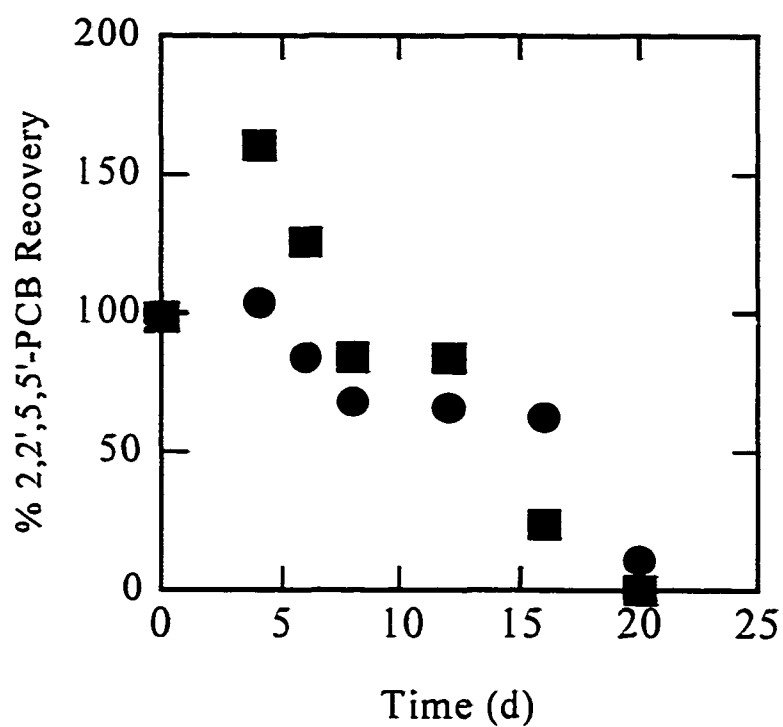


Figure 4.4 Degradation of 2,2',5,5'-PCB in SRS soil. $\square$ - with biphenyl amendment, $\bullet$ - without biphenyl amendment. The value for the unamended microcosm treatment at wk 5 is again an outlier, suggesting a problem with sampling at wk 5. This value was not used for analysis.

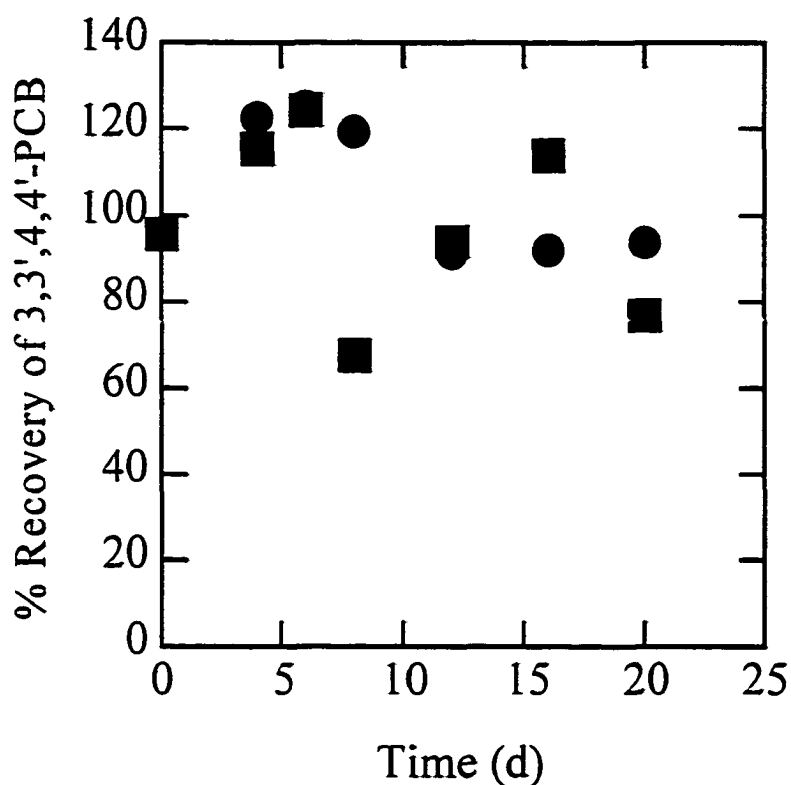


Figure 4.5 Degradation of 3,3',4,4'-PCB in SRS soil. □ - with biphenyl amendment, ● - without biphenyl amendment.

No significant differences in degradation rate or extent were observed between biphenyl-amended and unamended microcosms. If the degradation of the less chlorinated congeners was indeed cometabolically dependent on biphenyl, microcosms amended with biphenyl should have displayed increased rates or extents of degradation. The implications of this result are explored further in Chapter 5.

4.A.4. Conclusions of Preliminary Soil Screening

Two major conclusions were derived from the preliminary experimental results. First, dechlorination of PCBs could be achieved in both soils investigated. Second, the Savannah River Site soil was more desirable to work with based on its proven ability to degrade less-chlorinated congeners under aerobic conditions and the inability of the CP microorganisms to aerobically degrade these congeners. Based on these conclusions, all other work on this project was investigated using soil obtained from the Savannah River Site.

4.B. References

1. **Ahmed, M., and D. D. Focht.** 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. *Canadian Journal of Microbiology*. **19**:47-52.
2. **Bedard, D. L., and J. F. Quensen III.** 1995. Microbial reductive dechlorination of polychlorinated biphenyls, p. 127-216. *In* L. Y. Young and C. E. Cerniglia (ed.), *Microbial Transformation and Degradation of Toxic Organic Chemicals*. Wiley-Liss.
3. **Bedard, D. L., R. Unterman, L. H. Bopp, M. J. Brennan, M. L. Haberl, and C. Johnson.** 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. *Applied and Environmental Microbiology*. **51**:761-768.
4. **Furukawa, K., N. Tomizuka, and A. Kamibayashi.** 1979. Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. *Applied and Environmental Microbiology*. **38**:301-310.

5. **Jerome, K.** 1998. Personal Communication.
6. **Madsen, E. L.** 1991. Determining in situ biodegradation: Facts and challenges. *Environmental Science and Technology*. **25**:1663-1673.
7. **Michel, F.** 1998. Personal Communication.
8. **Rajendran, N., O. Matsuda, Y. Urushigawa, and U. Simidu.** 1994. Characterization of microbial community structure in the surface sediment of Osaka Bay, Japan, by phospholipid fatty acid analysis. *Applied and Environmental Microbiology*. **60**:248-257.
9. **Unterman, R., M. J. Brennan, R. E. Brooks, D. P. Mobley, J. B. McDermott, and C. C. Schwartz.** 1987. Presented at the American Chemical Society.
10. **White, D. C., and D. B. Ringelberg.** 1998. Signature lipid biomarker analysis, p. 255-272. *In* R. S. Burlage, R. Atlas, d. Stahl, G. Geesey, and G. Sayler (ed.), *Techniques in microbial ecology*, vol. 1. Oxford University Press, New York.
11. **Williams, W. A., J. H. Lobos, and W. E. Cheetham.** 1997. A phylogenetic analysis of aerobic polychlorinated biphenyl-degrading bacteria. *International Journal of Systematic Bacteriology*. **47**:207-210.
12. **Wu, Q., K. R. Sowers, and H. D. May.** 1998. Microbial reductive dechlorination of Aroclor 1260 in anaerobic slurries of estuarine sediments. *Applied and Environmental Microbiology*. **64**:1052-1058.
13. **Ye, D. Y., J. F. Quensen, and S. A. Boyd.** 1992. Anaerobic dechlorination of polychlorobiphenyls (Aroclor 1242) by pasteurized and ethanol-treated microorganisms from sediments. *Applied and Environmental Microbiology*. **58**:1110-1114.

CHAPTER 5

ANAEROBIC DECHLORINATION

5.A. Reductive PCB Dechlorination

Once a soil had been chosen and the indigenous microbial population shown to be able to dechlorinate PCBs, a more extensive dechlorination study was carried out. The purpose of this part of experimentation was to determine the effect of electron donor amendments on PCB dechlorination and microbial community composition. Because the SRS soil chosen was initially aerobic and had little natural organic matter, it was hypothesized that some organic amendment was necessary, not only to serve as an electron donor but also to initiate anaerobic conditions. To test this hypothesis, a control with no organic amendment was also investigated. The two electron donors chosen for the anaerobic dechlorination study were methanol and butyrate. Both had been shown to be useful for initiating conditions amenable for dechlorination of chlorinated compounds, including PCBs, PCE and TCE (11, 17, 29). The microcosms were set up with anaerobic mineral media without addition of a reducing agent and were sampled over a 60 wk period.

5.A.1. PCB Concentration

Reductive dechlorination of PCBs typically occurs with those congeners with greater than 4 chlorines per biphenyl molecule. To test the specificity of dechlorination and determine the extent dechlorination in a particular sample, each microcosm was extracted to determine the concentration of each dosed PCB and of each dechlorination product. The anaerobic microcosms were sampled at wk 0, 4, 12, 22, 30, 40, 50, and 60. Figure 5.1 shows the change in concentration of the 2,2',4,4',5,5'-hexachlorobiphenyl in the soil microcosms. Unamended microcosms dechlorinated this congener faster than butyrate or methanol fed microcosms. This was an unexpected result as the reductive dechlorination process cannot occur without an electron donor. Reductive dechlorination usually occurs through transfer of electrons from an organic compound to PCBs through the action of the microbial population. Thus, in the unamended treatments, either the natural organic matter is being utilized for dechlorination or an inorganic compound is being utilized. However, the natural organic matter is very low in this soil and could reasonably have been expected to be depleted long before the 60-wk incubation period ended. Thus, it is possible that an inorganic compound was serving as the electron donor. However, there have been no reports of use of an inorganic electron donor for PCB dechlorination.

Dechlorination of the hexachlorobiphenyl in unamended microcosms was first observed at wk 4 with a final dechlorination of approximately 20% at wk 60. Although the microcosms were prepared with anaerobic mineral media, no reducing agent was added to help eliminate all traces of oxygen in the microcosms. At wk 4, the conditions in the microcosms were probably not very reducing. This would be particularly true

since there was no electron donor to be used with the remaining oxygen to drive the change to more reducing conditions. In fact, conditions in these microcosms likely were not significantly reduced even by wk 60. The dechlorination observed under the unamended conditions is thus even more unusual. One other case similar to this has been reported by Kastner (21). In his work, the dechlorination of TCE and PCE was dependent on the transition from aerobic to anaerobic conditions. It was hypothesized that aerobic or facultative anaerobic bacteria were involved in the reductive dechlorination of PCE and TCE by catalyzing the transformation when the redox potential dropped to non-physiological levels.

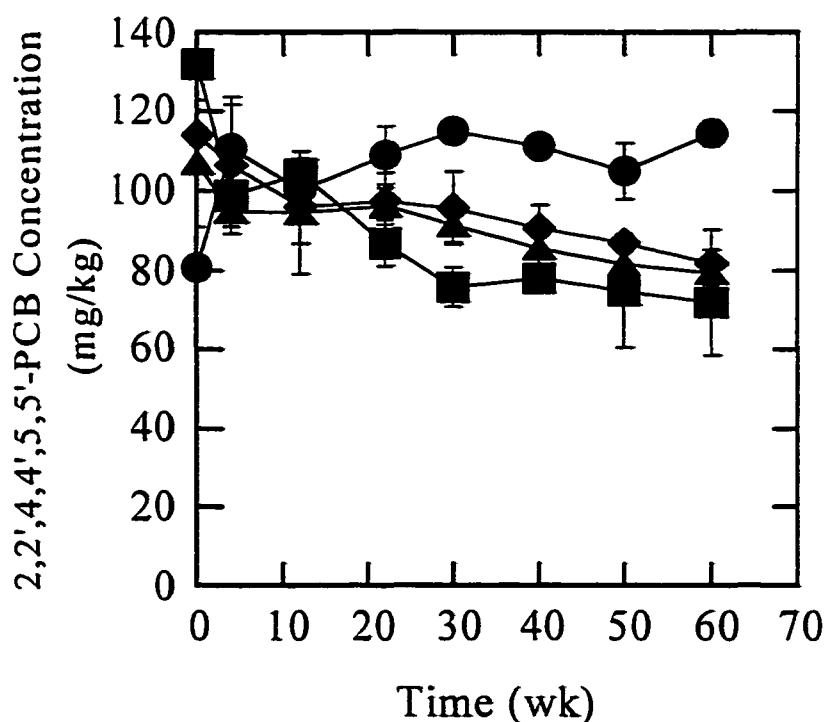


Figure 5.1 Dechlorination of 2,2',4,4',5,5'-PCB in soil microcosms. ● - sterile controls, ■ - no amendment, ◆ - butyrate-amended, ▲ - methanol-amended. Error bars represent the standard deviation between duplicate samples.

Methanol and butyrate-amended microcosms were similar in both initial and final rates and final extent of dechlorination. While the dechlorination rate appeared to have leveled off for the unamended microcosms by the end of the 60 wk period, in the methanol and butyrate-amended microcosms the rate of dechlorination did not appear to be slowing. A difference in dechlorination rate or extent was expected with different electron donor amendment. A deliberate choice of electron donor has the potential to influence which microbial population will be selected based on the rate at which H₂ is released by the fermentation of the electron donor. Butyrate fermentation was expected to provide a slow, steady addition of H₂ to the microorganisms in the microcosms while methanol was expected to provide a more rapid burst of H₂ (17). Dechlorinating organisms have been shown to compete better for low levels of H₂ than high, so butyrate was expected to select for a community better able to dechlorinate PCBs (17). While these differences in production of H₂ may have occurred, they did not appear to have an impact on the rate or extent of dechlorination and thus probably had little impact on the microbial community composition. In contrast, Nies and Vogel observed different rates and extents of PCB dechlorination with different electron donor amendment (methanol, glucose, acetone, and acetate) (29). In their work, methanol-fed microcosms demonstrated the greatest rate and extent of dechlorination of 2,3,5,6-tetrachlorobiphenyl while acetate-amended microcosms had the least. Also in contrast to this work, they observed no significant dechlorination in unamended microcosms.

Similar to the preliminary study, both *meta* and *para* dechlorination to 2,2',4,4',5- and 2,2',4,5,5'-pentachlorobiphenyl was observed. The primary product was 2,2',4,5,5'-PCB with less than 25% of the total dechlorination proceeding to 2,2',4,4',5-PCB.

Figure 5.2 shows the production of this primary dechlorination product. Further dechlorination to 2,2',5,5'-PCB in small amounts was also observed (Figure 5.3). No difference in dechlorination pattern was observed between treatments. Nies and Vogel also observed no difference in dechlorination pattern with different electron donor treatments.

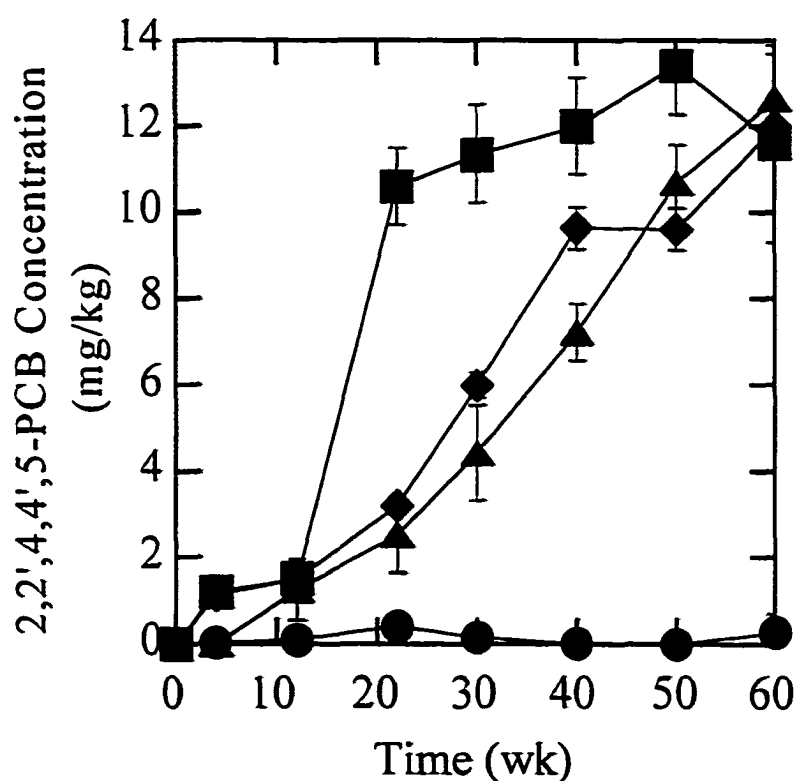


Figure 5.2 Production of 2,2',4,4',5-PCB from dechlorination of 2,2',4,4',5,5'-PCB in soil microcosms. ● - sterile controls, ■ - no amendment, ◆ - butyrate-amended, ▲ - methanol-amended. Error bars represent the standard deviation between duplicate samples.

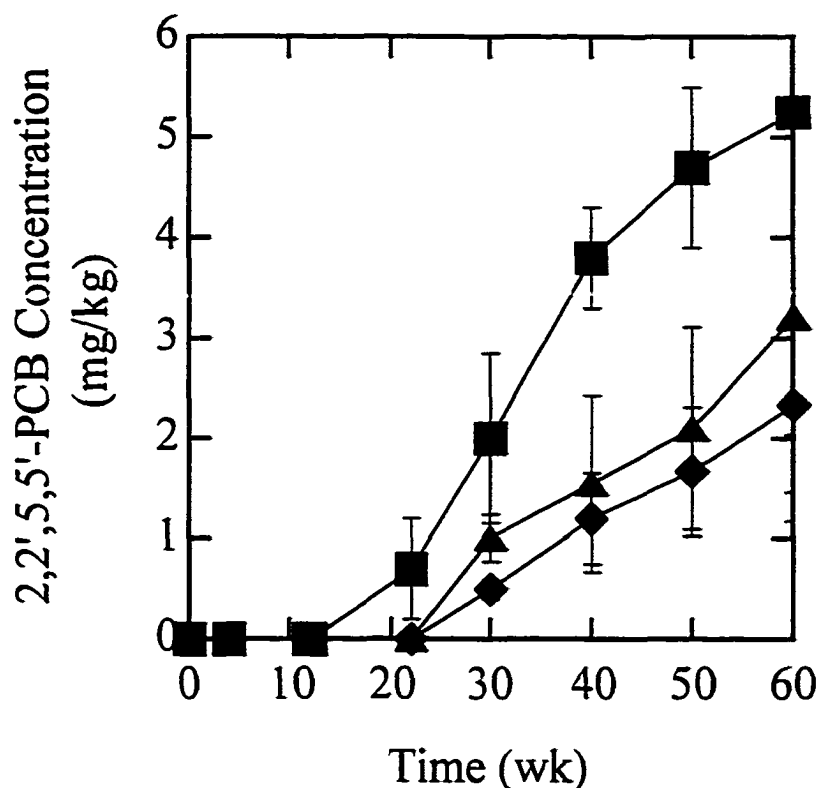


Figure 5.3 Production of 2,2',5,5'-PCB in soil microcosms. ■ - no amendment, ♦ - butyrate-amended, ▲ - methanol-amended. Error bars represent the standard deviation between duplicate samples.

The rate of dechlorination was slow. However, other researchers have observed similarly low rates. Rhee et al. observed 30% dechlorination in 5 months with only an additional 8% dechlorination by the end of the 24 month period (32). Sokol and co-workers observed 40% dechlorination of 2,2',3,4,4',5'-PCB in 7 months of incubation (36). Faster rates have also been observed. For example, Deweerd and Bedard observed 50% dechlorination of hexa- through nonachlorobiphenyls in 20 wk in Housatonic River sediments (5, 10).

Degradation of 2,3',4'-PCB was observed in the first 22 weeks of the anaerobic treatment. Since the initiation of anaerobic conditions was dependent on the microbial utilization of the electron donor, highly reducing conditions were never achieved in any of the microcosms. The time course of this degradation is shown in Figure 5.4.

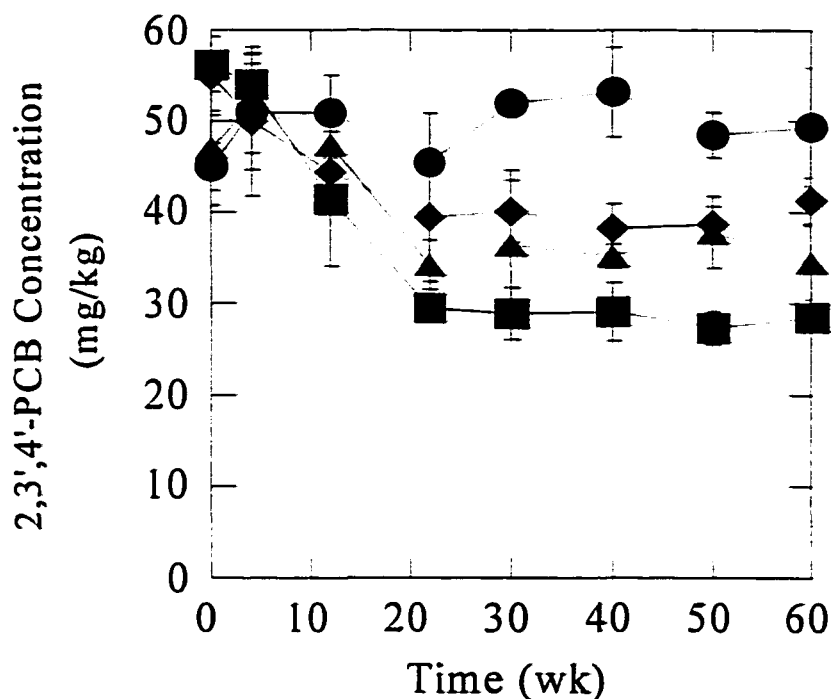


Figure 5.4 Degradation of 2,3',4'-PCB in anaerobic-phase microcosms. ● - sterile controls, ■ - unamended, ◆ - butyrate-amended, ▲ - methanol-amended. Error bars represent the standard deviation between duplicate samples.

As observed in the preliminary experiments, 3,3',4,4'-PCB was not degraded at all under the less reducing conditions that persisted in the first 22wk (Figure 5.5).

However, some dechlorination of 3,3',4,4'-PCB occurred in later samples. This dechlorination was not consistent within treatments and accounted for only 2-3% of the

3,3',4,4'-PCB dosed. The dechlorination pattern consisted of removal of chlorine substituents from both the *meta* and *para* positions to 3,3',4' and 3,4,4'-PCB.

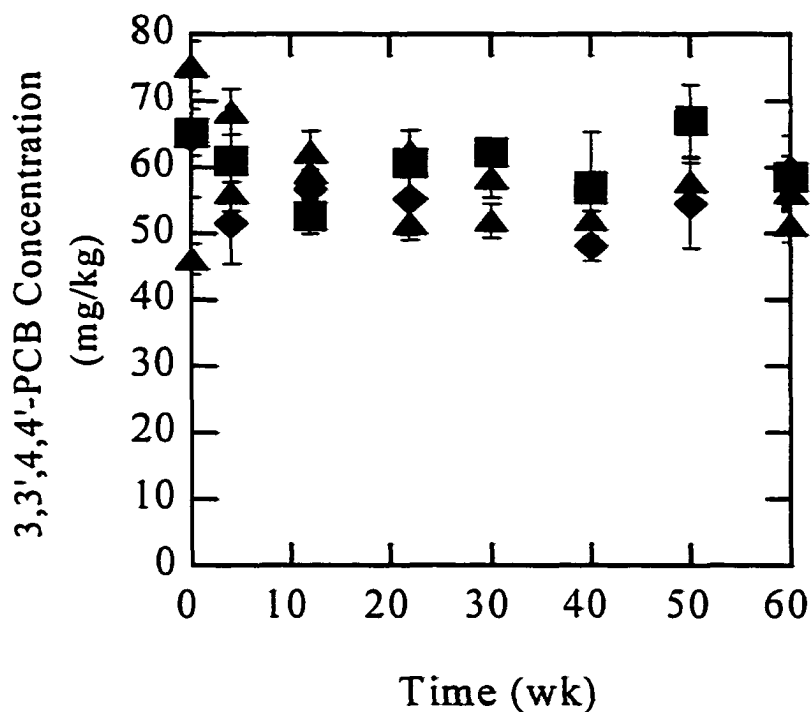


Figure 5.5 Degradation of 3,3',4,4'-PCB in anaerobic microcosms. ● - sterile controls, ■ - unamended, ◆ - butyrate-amended, ▲ - methanol-amended. Error bars represent the standard deviation between duplicate samples.

Table 5.1 shows the final concentration of each dechlorination product observed and a mole balance based on the observed dechlorination of the hexachlorobiphenyl dosed. Most of the balances were not significantly different from 100%. No double check of the mass balance could be made. The natural chloride concentration was very high (~1000 ppm) and interfered with detection of chloride produced by dechlorination. If all of the hexachlorobiphenyl was dechlorinated to tetrachlorobiphenyl, 8.4 ppm

chloride would be produced. This amount of chloride is less than 1% of the natural chloride concentration and could not be detected by available methods.

Table 5.1 Degradation of PCBs in anaerobic microcosms (mmol/kg)

Congener	Unamended	Butyrate-amended	Methanol-amended	Sterile
2,2',4,4',5,5'	0.20 ± 0.037	0.223 ± 0.024	0.221 ± 0.009	0.318 ± 0.009
2,2',4,5,5'	0.036 ± 0.007	0.039 ± 0.003	0.037 ± 0.016	0.001 ± 0.001
2,2',4,4',5	0.01 ± 0.003	0.011 ± 0.003	0.008 ± 0.004	ND
2,2',5,5'	0.018 ± 0.011	0.008 ± 0.003	0.011 ± 0.007	ND
Total	0.264 ± 0.058	0.281 ± 0.033	0.277 ± 0.036	0.319 ± 0.010
Initial Concentration	0.278 ± 0.051	0.318 ± 0.089	0.298 ± 0.046	0.307 ± 0.074

The reduction in the number of chlorines per biphenyl molecule is often used as an indication of the success of an anaerobic dechlorination treatment process. This value was plotted versus time for each treatment in Figure 5.6. The expected trend is not observed in the figure; in fact, the number of chlorines per biphenyl first increased, and then began to decrease as expected. This is due to the decrease in the trichlorobiphenyl concentration in the first 22 wk of treatment. This phenomenon skews the graph so that the reduction in the number of chlorines per biphenyl from the dechlorination cannot be observed. However, when this same parameter (# chlorine/biphenyl) is plotted versus time without the trichlorobiphenyl concentration included, a decrease is observed in all treatments but the sterile control (Figure 5.7). Kim and Rhee observed a decrease from 3.8 Cl-/biphenyl to 2.5 Cl-/biphenyl in 20 wk in sediments from Owasco Lake, NY (23). A decrease from 4.25 to 3.5 chlorine/biphenyl was observed by Klasson et al. in 19 wks in soil slurry bioreactors (24).

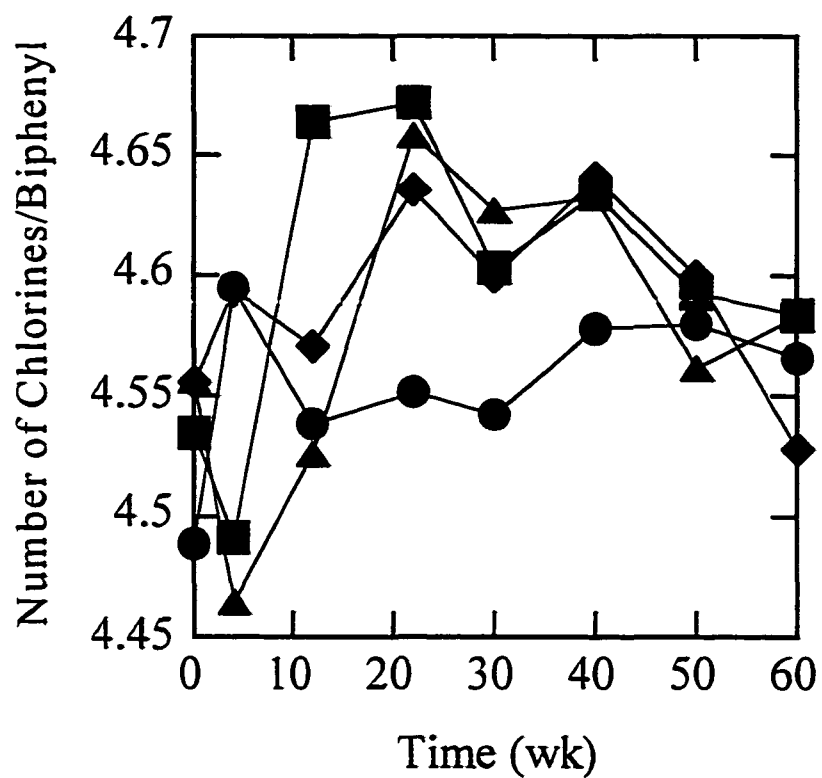


Figure 5.6 Change in the number of chlorines per biphenyl. ● - sterile controls, ■ - unamended, ◆ - butyrate-amended, ▲ - methanol-amended.

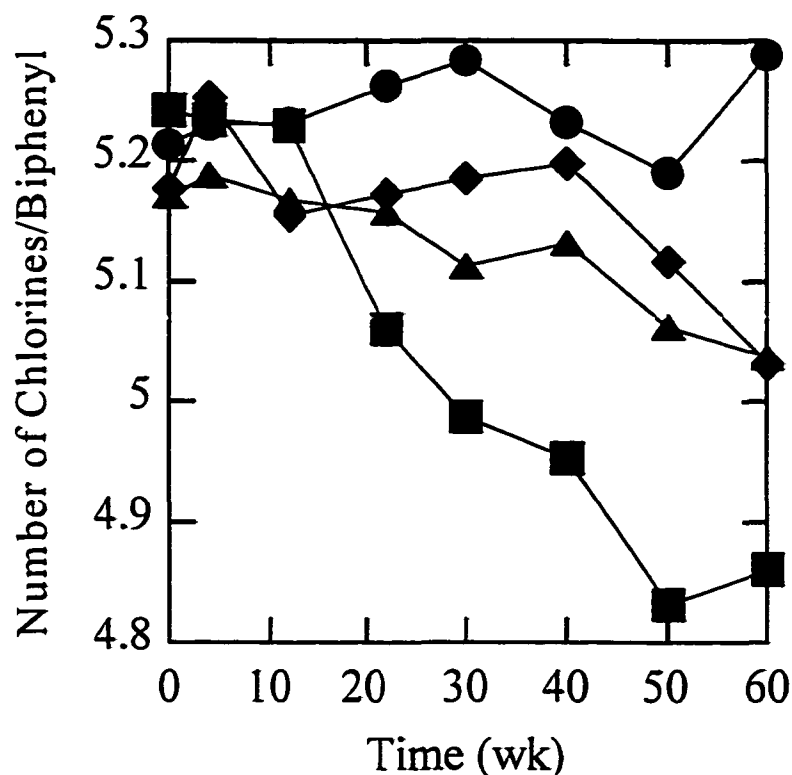


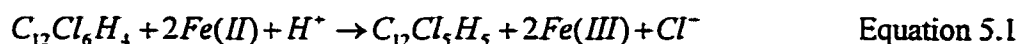
Figure 5.7 Change in the number of chlorines per biphenyl without the concentration of 2,3',4'-PCB included. ● - sterile controls, ■ - unamended, ◆ - butyrate-amended, ▲ - methanol-amended.

5.A.2. Ferrous Iron Concentration

An initial concern with achieving dechlorination was the high concentration of total iron in the system. The amount of energy provided by iron-reduction is greater than that provided from PCB dechlorination (Table 2.1); thus, use of iron as an electron acceptor by the microbial population could decrease or eliminate the use of the PCBs as an electron acceptor and thus inhibit the dechlorination of PCBs. If iron-reduction were occurring, an increase in the ferrous iron content of the microcosms would be expected

from the reduction of ferric iron to ferrous iron. A spectrophotometric method was used to determine ferrous iron concentrations. Unfortunately, some trouble was encountered in developing this method early in the experiment. Figure 5.8 shows the results of the ferrous iron assay for the final weeks of the anaerobic phase. No significant production of ferrous iron was observed during this time in any of the treatments. In fact, the trend appears to be a decrease in the ferrous iron over the course of the experiment. With the large error bars at wk 20 this trend is not significant. Although, the production of ferrous iron during the early portion of the anaerobic phase cannot be ruled out, it is unlikely because the concentration of ferrous iron was higher in the sterile control than any of the other treatments at each sampling point.

This decrease in ferrous iron suggests that iron is being oxidized in this system, not reduced. The possibility that iron was serving as the electron donor for dechlorination was investigated. To do this, the overall reaction was written for dechlorination of 2,2',4,4',5,5'-hexachlorobiphenyl to 2,2',4,5,5'-pentachlorobiphenyl with oxidation of ferrous iron to ferric iron (Equation 5.1). The ΔG°_{rxn} was determined to be 39.3 kcal/mol. Since the free energy of reaction is positive, this indicates that any ferrous iron oxidation observed is not coupled to dechlorination in our system.



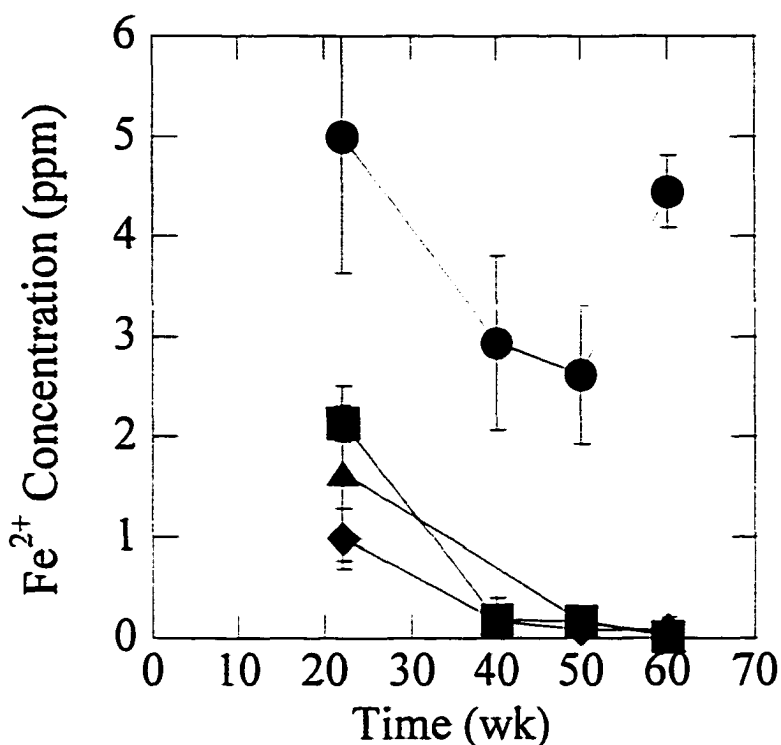


Figure 5.8 Concentration of ferrous iron in anaerobic microcosms. ● - sterile controls, ■ - no amendment, ◆ - butyrate-amended, ▲ - methanol-amended. Error bars represent the standard deviation between duplicate samples.

5.A.3. Electron Donor Concentration

The original experimental plan called for a single addition of electron donor at time zero at a concentration of 250 mg/kg. This concentration was calculated to be sufficient for complete dechlorination of all the added PCBs. However, this was modified as the electron donor was quickly depleted before significant dechlorination occurred. Electron donor concentrations were determined at each sampling point and additional electron donor added if concentrations in the sampled microcosms fell below

10% of the original concentration. Figures 5.9 and 5.10 show the concentrations of butyrate and methanol in the anaerobic soil microcosms over the 60 wk sampling time. Concentrations of both methanol and butyrate remained near zero throughout the sampling period.

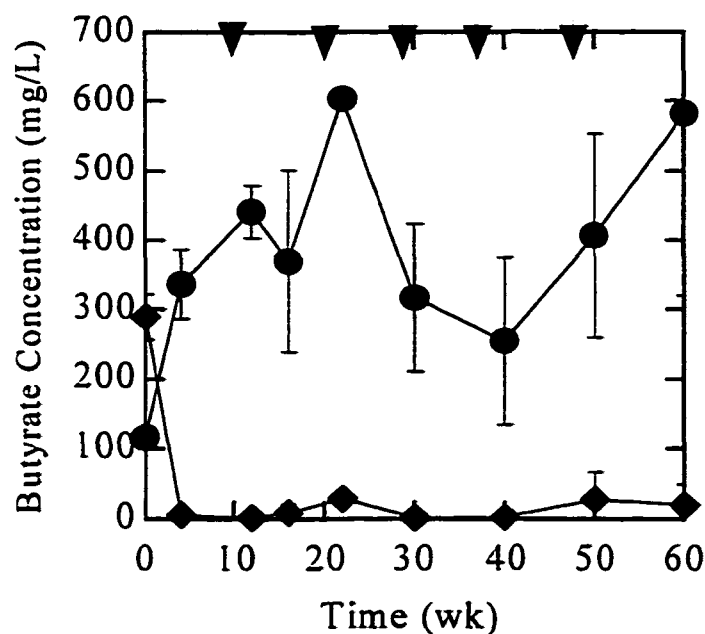


Figure 5.9 Degradation of butyrate in soil microcosms. ● - sterile controls, ◆ - butyrate-amended microcosms. Arrows indicate additions of butyrate to nonsterile microcosms only. Error bars represent the standard deviation between duplicate samples.

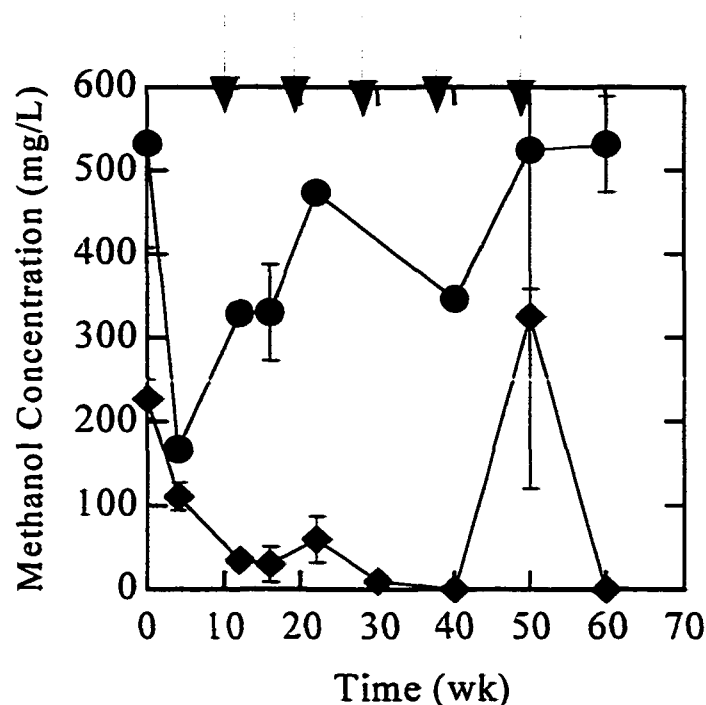


Figure 5.10 Degradation of methanol in soil microcosms. Figure 5.8 Degradation of butyrate in soil microcosms. ● - sterile controls, ◆ - methanol-amended microcosms. Arrows indicate additions of methanol to non-sterile microcosms only. Error bars represent the standard deviation between duplicate samples.

5.B. FAME analysis

As described in Section 5.A.2, the indigenous organisms were indeed capable of carrying out dechlorination in all three treatments investigated. However, the rate of dechlorination for unamended microcosms was significantly different than either butyrate- or methanol-amended microcosms, although the final extent of dechlorination was nearly the same. To help explain the observed differences in PCB dechlorination, several non-culture-based microbiological methods were used to characterize the microbial population. One of these methods, the phospholipid fatty acid method combined with fatty acid methyl ester

profile analysis, has been shown to be valuable for detecting changes in microbial communities in a variety of complex matrices (6, 18, 22). The PLFA/FAME method can be used to determine total biomass levels in each sample as well as to detect more specific changes in microbial population composition. The method is based on the fact that there are a large number of different fatty acids in the lipids of microorganisms and that different organisms have different combinations of these fatty acids. The specific fatty acid methyl esters observed in the lipid samples and used for analysis throughout this work are shown in Table 5.2.

Table 5.2 Specific fatty acid methyl esters used for analysis

<i>Monounsaturated cis</i>	<i>Saturated Even</i>	<i>Branched iso</i>	<i>Cyclo</i>
16:1 cis 7	14:0	14:0 iso	cy17:0
16:1 cis 9	16:0	15:0 iso	cy19:0
18:1 cis 7	18:0	16:0 iso	
18:1 cis 9		17:0 iso	<i>Polyunsaturated</i>
	<i>Saturated Odd</i>		18:2w6,9
<i>Monounsaturated trans</i>	15:0	<i>Branched anteiso</i>	
16:1 trans 7	17:0	15:0 anteiso	
18:1 trans 7		17:0 anteiso	

Using the PLFA/FAME method, a value for total biomass can be determined by summing the pmol of each specific FAME and then converting this number to an equivalent amount of phospholipid. Figure 5.11 shows the change in total pmol/g soil in the unamended, butyrate-amended and methanol-amended microcosms. To investigate the effect of the carrier solvent used in this study, controls to which only hexane was added were used. Values for this control treatment are also shown in this graph. For each of the three PCB added treatments, a large spike in the total pmol/g was observed at a time that corresponded to the

highest levels of dechlorination. This may indicate that dechlorination is linked to microbial growth in our system. In addition, levels of total biomass in unamended treatments decreased after 40 wk. This agrees with the observed decrease in dechlorination rate in the unamended microcosms after this time. This decrease in dechlorination could be due either to a decline in the concentration of microorganisms or simply a decrease in dechlorination rate due to the buildup of inhibitory by-products.

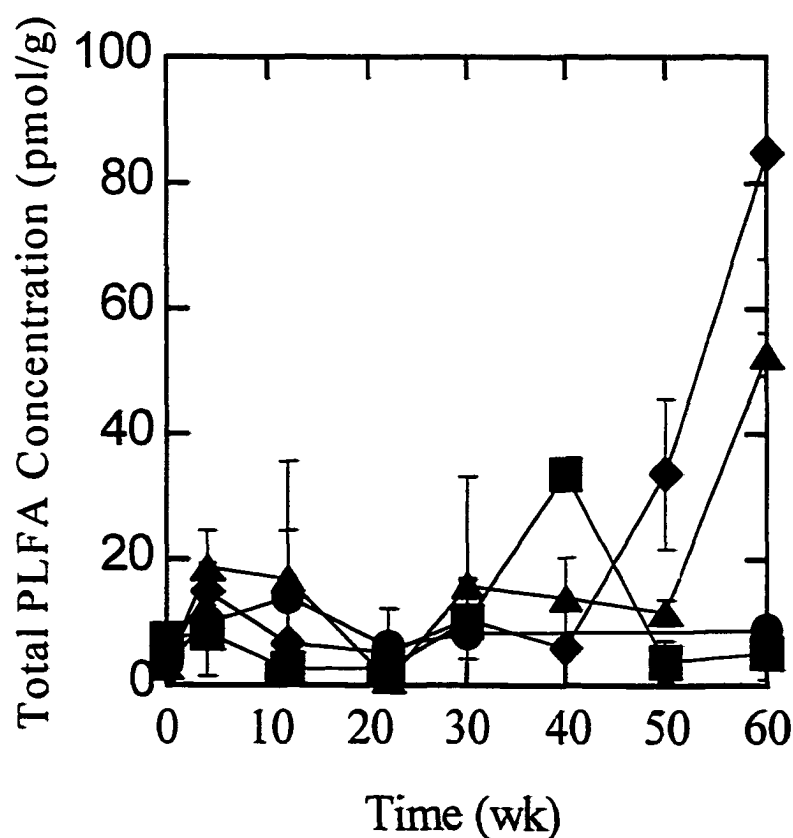


Figure 5.11 Total lipid concentration in anaerobic microcosms. ■ - unamended microcosms, ● - hexane addition only, ◆ - butyrate addition, and ▲ - methanol addition. Error bars represent the standard deviation between duplicate samples.

Not only did the total biomass change from time point to time point with each treatment method, the mole percent of specific fatty acids also changed. The mole percent of terminally branched fatty acids, in particular those with an *iso* configuration increased in each of the three PCB-amended treatments (Figure 5.12). No such increase was observed in the hexane-only additions. These specific fatty acids are typically found in prokaryotic, anaerobic microorganisms. This increase corresponds to the progress of dechlorination of the hexachlorobiphenyl in the butyrate- and methanol-amended treatments and indicates an increase in the anaerobic population. However, the dechlorination time doesn't match the anaerobic fatty acid increase for unamended microcosms. In the unamended microcosms, the dechlorination rate decreased after wk 30 but a continued increase in anaerobic biomarkers was observed. Therefore, increases in the dechlorinating population may not correspond to or be well described by increases in the anaerobic biomarkers (i14:0+i15:0). Interestingly, little decrease was observed in markers for aerobic microorganisms.

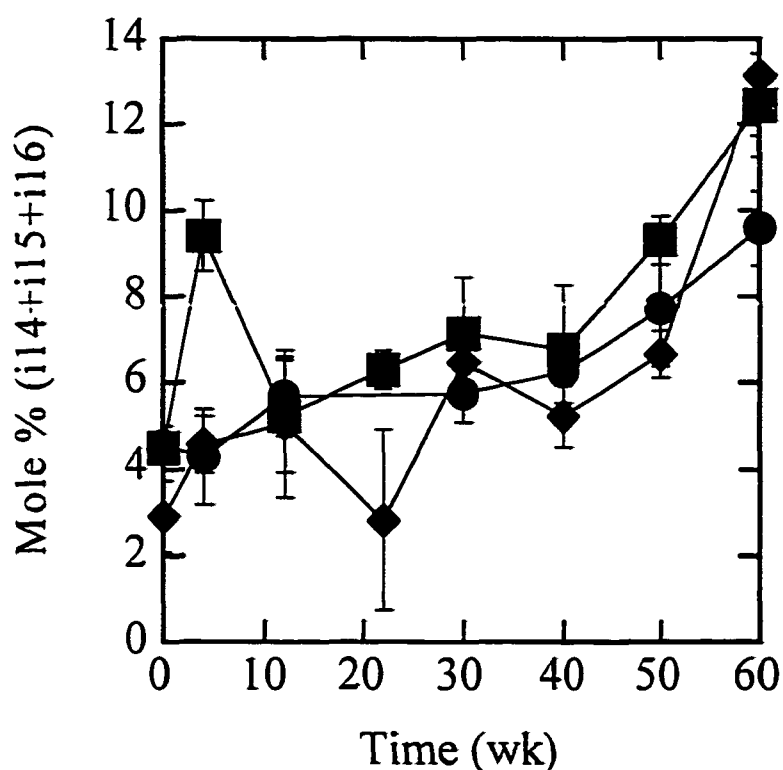


Figure 5.12 Change in mole percent of the FAMES i14:0, i15:0, and i16:0. ■ - unamended microcosms, ● - butyrate amendment, and, ♦ - methanol amendment. Error bars represent the standard deviation between duplicate samples.

In addition to comparing specific fatty acids, a multivariate statistical method, principal components analysis (PCA), was utilized to compare overall FAME profiles between samples. In PCA, the original variables (PLFAs, in this case) are orthogonally transformed into a new set of uncorrelated variables called principal components (PCs). The degree to which each principal component is influenced by each original variable is its eigenvector loading. Three PCs were needed to describe the differences between samples. PCs 1, 2, and 3 describe 71%, 9%, and 6% of the variation in the data, respectively. Figures 5.12-5.14 show 2-dimensional plots of the principal components. In Plots 5.13 and 5.14, the samples formed

several distinct clusters. One of these clusters includes those samples from the unamended microcosms at wk 40, the butyrate-amended microcosms at wk 60, and the methanol-amended microcosms at wk 60. These samples are those displaying the highest rate of dechlorination in Figures 5.1 and 5.2. This clustering shows that the same population is probably carrying out the dechlorination in each treatment. This hypothesis is consistent with the fact that the same pattern of dechlorination was observed for each treatment. These three samples are enriched in two specific FAMES, i16:0 and 18:0. The fatty acid i16:0 is indicative of either *Bacillus*-type organisms or anaerobic gram-negative bacteria. Fatty acid 18:0 is found in nearly all prokaryotes and is thus not a good biomarker for discriminatory purposes.

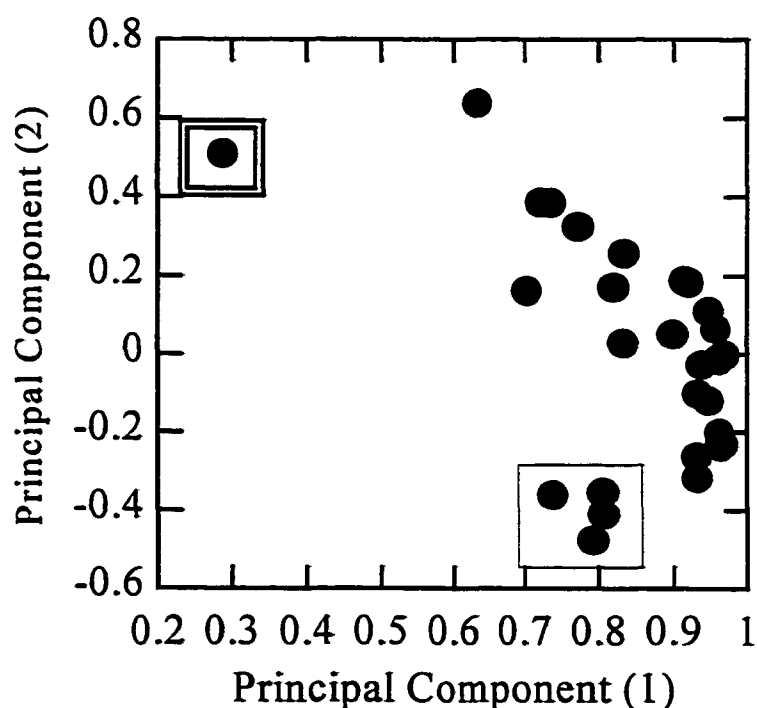


Figure 5.13 Principal component analysis of anaerobic microcosms PLFA/FAME profiles, Factor 1 by Factor 2. Samples in the single-lined rectangle are unamended treatment at wk 22, butyrate-amended treated at wk 22, and methanol-amended treatment at wks 0 and 22. The sample in the double-lined rectangle is hexane-only amendment at wk 60.

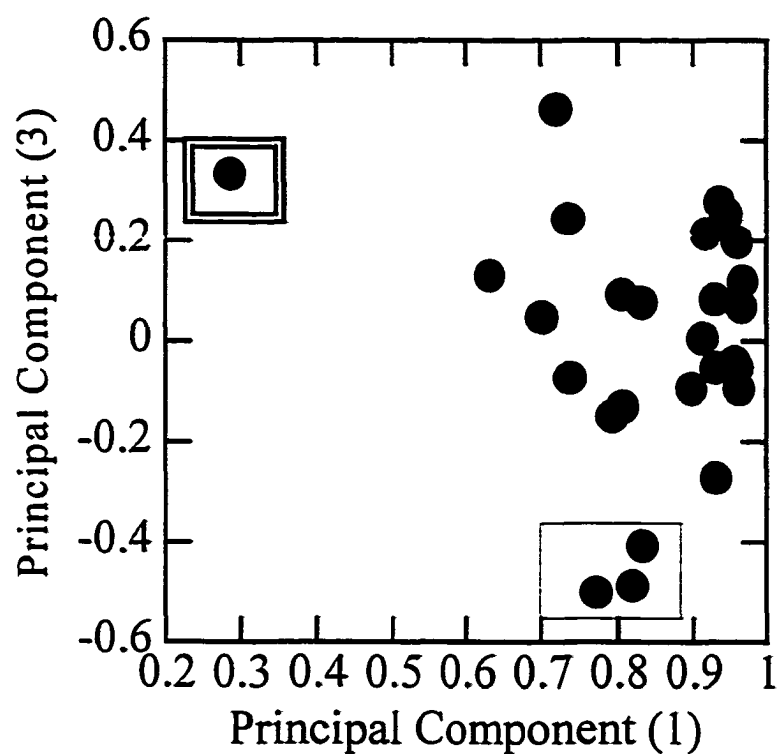


Figure 5.14 Principal component analysis of anaerobic microcosm PLFA/FAME profiles, Factor 1 by Factor 3. Samples in the single box are unamended treatment at wk 40, butyrate treatment at wk 60 and methanol treatment at wk 60. Sample in the double box is hexane only treatment at wk 60.

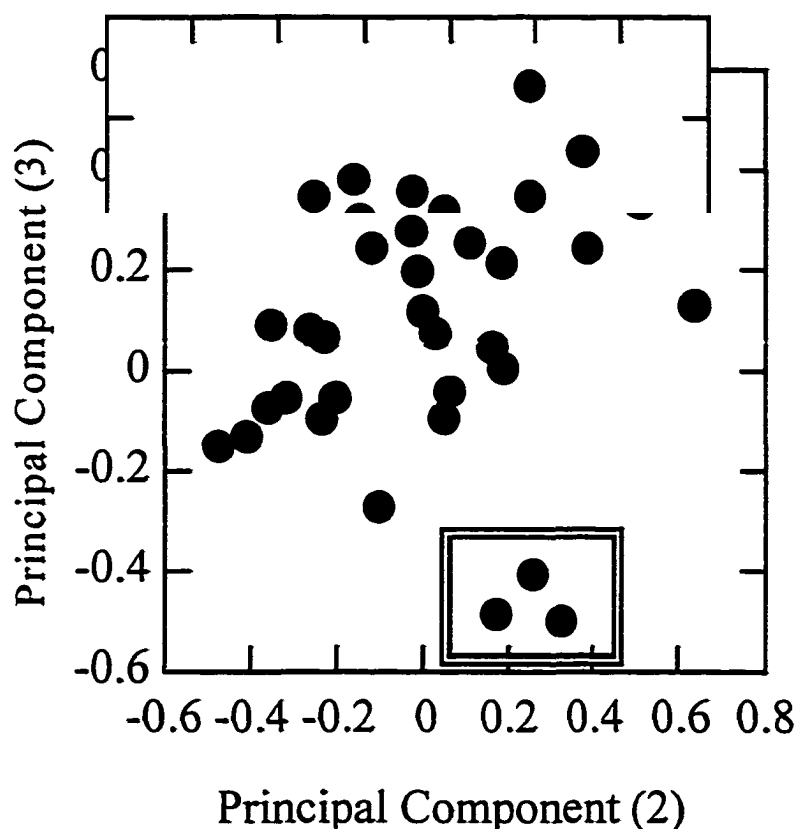


Figure 5.15 Principal component analysis of anaerobic microcosm PLFA/FAME analysis profiles, Factor 2 by Factor 3. Samples in the double box are unamended treatment at wk 40, butyrate treatment at wk 60 and methanol treatment at wk 60.

Also of interest is the hexane-only treatment at wk 60 that mapped by itself by in Figures 5.13 and 5.14. This could indicate that a significant population composition change occurred through the addition of hexane over the 60 wk. A few studies have observed an effect due to the carrier solvent for dosing on PCB dechlorination. Nies and Vogel reported that dechlorination was dependent on the amount of acetone used for dosing; however, their results were based on the use of inocula previously enriched on acetone (29). Also, in Figure 5.13 we see that samples from wk 22 form a small cluster. These

samples are all high in the FAMES 16:0 and 18:0 and possibly represent an error in the experimental method on this sampling day.

5.C. PCR with Specific Primers

While the PLFA/FAME analysis enhanced our understanding of the microbial community composition in each sample, more specific knowledge about the community composition was desired than was able to be derived from the FAME analysis alone. Two sets of PCR primers were chosen to detect the presence or absence of sulfate-reducers and methanogens in the anaerobic samples (3.F.2.D.) (1, 19). Both sulfate-reducers and methanogens have been shown to catalyze the reductive dechlorination of PCBs and were thus of interest in our work (23, 26). In addition, a primer set specific for the *bphC* gene was tested with the anaerobic samples (15). The biphenyl dioxygenase pathway is important for the aerobic degradation of PCBs and the survival of organisms with these degradative genes is important for the aerobic phase in sequential treatment.

Figure 5.16 shows the results of agarose gel electrophoresis of the products from PCR amplification of DNA extracted from selected anaerobic samples with these three primer sets. DNA was extracted from time zero samples and from unamended and butyrate-amended samples at wk 30. As observed in the figure, no methanogens or sulfate-reducers were detected in any of the anaerobic samples tested (Lanes 6-9, 10-13). This is interesting as it means that some other type of microorganism must be carrying out the observed dechlorination, possibly an organism able to use PCBs in a respiratory process.

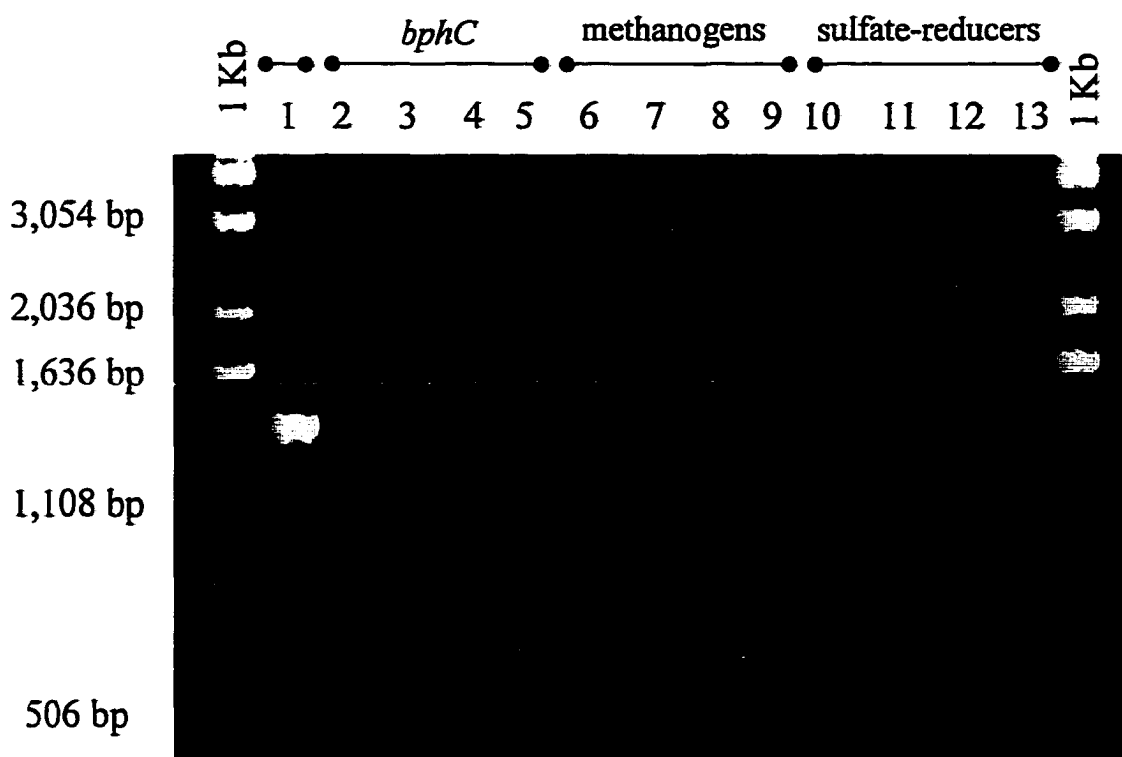


Figure 5.16 Agarose gel electrophoresis of PCR products from anaerobic microcosms. Lane 1 is a positive *E. coli* control amplified with universal 16S rDNA primers. Lanes 2-5 are a positive control (biphenyl enrichment culture), time zero, unamended, wk 30 and butyrate-amended wk 30 DNA run with the *bphC* primer set. Lanes 6-9 are a positive control (*M. jannaschii*), time zero, unamended wk 30 and butyrate-amended wk 30 DNA run with the methanogen specific primer set. Lanes 10-13 are a positive control (*Desulfovibrio* sp.), time zero, unamended wk 30 and butyrate-amended wk 30 DNA run with the sulfate-reducing bacteria specific primer set.

No amplification with the *bphC* gene specific primer set was observed in these wk 30 samples but was observed with both a positive control and the time zero sample. This might indicate a loss of the biphenyl-degrading aerobic population in the anaerobic samples. The same primer set was used to test samples at the beginning and end of a 7-wk aerobic treatment experiment for the presence of biphenyl-degrading

microorganisms. In the aerobic study, a loss of amplification ability in the final 7-wk samples in both biphenyl-amended and unamended treatments was observed, even though active degradation of the trichlorobiphenyl and biphenyl was occurring. Thus the inability of the *bphC* primer set to amplify from these samples could simply indicate a shift in the aerobic population to organisms with non-homologous genes that degrade PCBs.

5.D. DGGE

Performing PCR with sulfate-reducing bacteria and methanogen specific primers provided information about what organisms were not present in the anaerobic samples. However, no information about what organisms *were* present and carrying out the dechlorination could be determined from this method. To gain insight into the microbial community composition changes in populations and differences between samples, PCR followed by DGGE was utilized. DGGE allows DNA sequences from mixed microbial populations to be separated and visualized based on sequence differences. Primers specific to the 16S rRNA but universal enough to amplify a 200 bp fragment from all bacteria were used. DNA extracted from samples at wk 30 was used for DGGE. This time point was chosen because all three treatments (methanol, butyrate, or no amendment) were undergoing dechlorination but hadn't begun to level off at later times. When analyzing sequence data, two disadvantages of DGGE must be remembered. First, the excised band represents only a few hundred nucleotides of the target sequence. To use these sequence data for a full phylogenetic study would be unwise as this number of nucleotides is only a small fraction of the total genetic information in a full genome (37).

Second, it cannot be excluded that a particular band may contain more than one sequence; this can confuse the sequencing analysis.

Figure 5.17 shows the DGGE gel produced with the anaerobic samples. Each sample was run in duplicate lanes with a lane left blank in between samples to account for any discrepancies in the gel that can distort the banding patterns. Each band chosen for cloning and sequencing is labeled with a number. A sample from time zero soil was also included as a comparison. Few differences between the time zero soil and other samples existed, with the exception of the unamended soil microcosms.

To determine the types of organisms present in the different bands, the DNA from each band was cloned using the pCR®2.1 vector and sequenced by automated fluorescent sequencing at Davis Sequencing (University of CA, Davis). Sequences were analyzed using the Blastn program from the National Center for Biotechnology Information. In addition, 10 clones per band were run using single-strand conformation polymorphism (SSCP) polyacrylamide gels to determine if each band represented a single organism type or if multiple sequences were present in each band. A representative SSCP gel is shown in Figure 5.18. Very little replication of sequences between clones for a particular band was observed. The presence of multiple sequence types within a single DGGE band was confirmed when several clones per band were sequenced with different identities ascribed to each band. Table 5.3 shows the closest phylogenetic relative of each clone sequenced with the percent similarity between sequences. It is interesting to note that all but one of the 15 clones (7%) sequenced had greater than 90% similarity with previously described organisms. It is important to recognize, however, that the high percent relatedness observed could be caused by use of the relatively small DGGE fragment for sequence

comparisons. Dojka et al. surveyed the microbial communities associated with a jet-fuel contaminated aquifer and found that 10 of 94 (11%) bacterial sequences cloned had no phylogenetic association with known taxonomic divisions (12). Nogales et al. found that 3 of 133 (2%) sequences cloned were not affiliated to any described phylogenetic group (30). In addition, all but one of the sequences (i.e., 93%) in this work group within the *Proteobacteria* division with representatives from the α , β , and γ subclasses. In work by Nogales and co-workers, 74% of cloned sequence types from a PCB-contaminated soil clustered within the *Proteobacteria* (30).

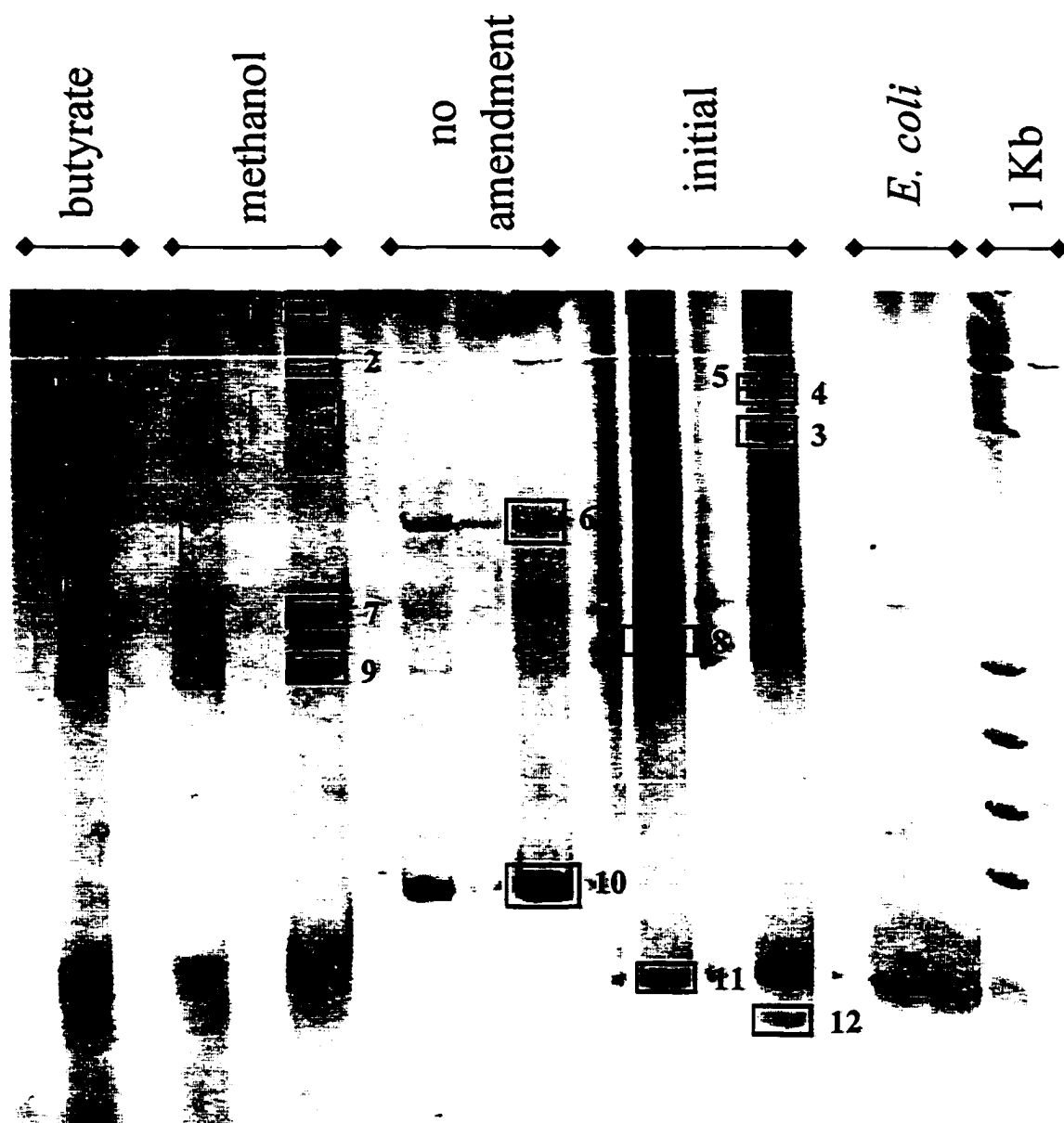


Figure 5.17 DGGE analysis of PCR-amplified DNA extracts from anaerobic microcosm samples. A denaturing gradient from 50%-80% was utilized with Urea acting as the denaturant. Lanes 1, 3, 5, 7, 9, 11, 13, and 15 were not loaded. Lane 2, butyrate-amended microcosm at wk 30. Lanes 4 and 6, methanol-amended microcosm at wk 30. Lanes 8 and 10, unamended microcosm at wk 30. Lanes 12 and 14, time zero microcosms. Lanes 16 and 17, *E. coli* (positive control). Lane 18, 1 Kb size marker.

Table 5.3 Phylogenetic relatedness of clone sequences from DGGE bands

Band (Clone)	Division	Closest Phylogenetic Relative	% Similarity to Closest Relative
1 (1-3)	<i>Proteobacteria</i> (β)	<i>Dechlorimonas</i> sp. NM	100% (8)
1 (1-4)	<i>Proteobacteria</i> (α)	<i>Sphingomonas</i> sp.	100% (30)
2 (2-1)	<i>Proteobacteria</i> (β)	<i>P. saccharophila</i>	100% (7)
3 (3-3)	<i>Proteobacteria</i> (β)	<i>Comamonas</i> <i>acidovorans</i> MC1	92% (27)
4 (4-3)	<i>Proteobacteria</i> (?)	Uncultured bacterium	100% (13)
5 (5-1)	<i>Proteobacteria</i> (γ)	Uncultured bacterium	100% (14)
6 (6-5)	<i>Proteobacteria</i> (α)	<i>Rhodococcus planei</i>	97%(38)
6 (6-6)	<i>Proteobacteria</i> (β)	<i>Comamonas</i> <i>testosteroni</i>	86% (20)
7 (7-2)	<i>Proteobacteria</i> (β)	<i>Comamonas</i> sp. D22	100%(28)
7 (7-6)	<i>Proteobacteria</i> (β)	<i>Acinetobacter junii</i>	100%(35)
8 (8-1)	<i>Proteobacteria</i> (β)	<i>E. coli</i>	100%
9 (9-6)	<i>Proteobacteria</i> (β)	<i>Acinetobacter</i> sp. P1L001	98% (33)
10 (10-5)	<i>Proteobacteria</i> (γ)	Uncultured bacterium	96%(34)
11 (11-3)	<i>Proteobacteria</i> (β)	<i>E. coli</i>	100%
12 (12-3)	<i>Acidobacterium</i>	Uncultured bacterium	96% (25)

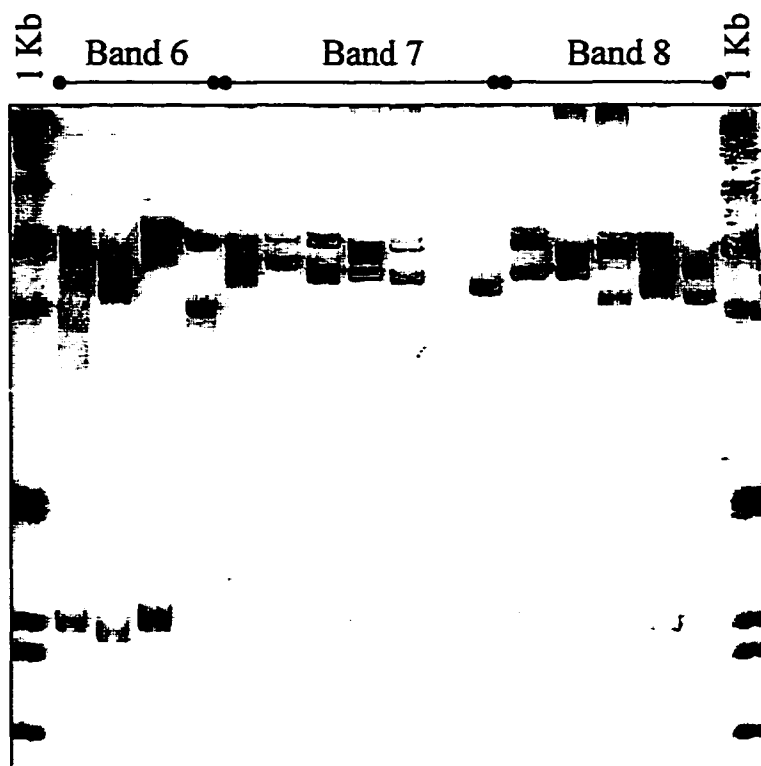


Figure 5.18 SSCP gel of representative clones from soil microcosms

Two very different types of organisms were sequenced from DGGE Band 1; SSCP analysis indicated the presence of at least five different sequence types in Band 1. The first of these (1-3) was 100% similar in the region amplified to a *Dechlorimonas* sp. whose closest relative is *Ferribacterium limneticum*, an iron-reducing species. The *Dechlorimonas* sp. is a gram-negative, non-fermenting, facultative anaerobe that anaerobically reduces perchlorate (8). While it is related to an iron-reducing species, this microorganism, and other similar perchlorate-respiring organisms, have been shown to be able to use both butyrate and Fe(II) as an electron donor. However, none were able to use methanol. This clone is the only anaerobic representative in the sequences cloned and thus may play a key role in the observed PCB dechlorination. The second clone

sequenced (1-4) from Band 1 groups within the α -subclass of *Proetobacteria* and based on this short sequence had 100% similarity with an uncultured bacterium sequenced from a PCB-polluted soil (30). This uncultured species was highly similar (99%) to several *Sphingomonas* sp., all of which are aerobic microorganisms known to degrade chlorophenols, phenanthrene, and carbofuran. One particular *Sphingomonas* species, *Sphingomonas paucimobilis*, was isolated from PCB-contaminated soil and is able to degrade halogenated biphenyls and benzoic acids (9).

Sequence types from several bands clustered within the *Comamonadaceae* family in the *Comamonas* and *Pseudomonas* subgroups. The clone sequence from Band 2 (2-1) had 100% similarity to *Pseudomonas saccharophila* strain DSM 654T. For Band 3, the clone sequenced (3-3) had 92% similarity to *Comamonas acidovorans* strain MC1 and one clone from Band 6 (6-6) had 86% similarity with a *Comamonas testosteroni* species developed as a recombinant bacteria to grow on both ortho- and para-substituted chlorobiphenyls by Hrywna and co-workers (20). Other *Comamonas testosteroni* species are commonly isolated soil heterotrophs, and *Comomonas testosteroni* strain B-356 was used by Barriault and Sylvestre to increase Aroclor 1242 degradation in soil microcosms (4). *Comamonas acidovorans* MC1 was isolated from herbicide-contaminated building rubble and is able to degrade the chiral herbicides, dichlorprop and mecoprop (27). In addition, one clone from Band 7 (7-2) had 100% similarity with *Comamonas* sp. D22 that was isolated from an observation well at a liquid radiation waste repository. While the soil used in this work has no known pollution by radionuclides, portions of the Savannah River Site do have this type of contamination. The multiple appearance of closely related *Comamonas*-like sequences in the DGGE fingerprint raised the question whether this

could be due to the double PCR amplification, in which one bacterial strain produces more than one signal on DGGE. This possibility cannot be discarded based on the current data. Nevertheless, the presence of these aerobic, xenobiotics-degrading organisms shows that degradative potential exists in the soil and that aerobic microorganisms are still present (if not active) in these wk 30 samples.

Two sequences were isolated that cluster within the *Acinetobacter* group of the β -proteobacteria. Clone 7-6 had 99% similarity with *Acinetobacter junii* and clone 9-6 with *Acinetobacter* sp. P1L001. Asturias et al. have characterized a related *Acinetobacter* species, strain P6, that is highly effective at aerobically degrading PCBs (3).

A high sequence similarity to several uncultured γ -proteobacterium was found with the clone sequence from Band 5 (5-1). One of these uncultured bacteria was found in a runoff stream (pH 2.5) directly below an acid mine drainage site at Iron Mountain, California. The clone in our study has high similarity to clone TRA5-3 from the Iron Mountain site, which clusters in the vicinity of two recently isolated organisms that are capable of iron oxidation and had 92% similarity with *Psycrobacter glacincola*.

The unamended treatment was enriched specifically in Bands 6 and 10, neither of which appeared to be present to a great extent in any other samples. In addition to the *Comamonas testosteroni* (clone 6-6), an additional clone sequence from band six (6-7) was most closely related to *Rhodococcus planei* with 97% similarity. *Rhodococcus* are typically aerobic organisms and several have been shown to carry out PCB degradation. The clone sequence from Band 10 (10-5) had 95% similarity to an uncultured γ -*Proteobacterium* and 93% similarity to *Burkholderia* sp. VUN10013, a known PAH-degrading microorganism from the β -proteobacterium subgroup. It also had 92%

sequence similarity with clone TRA5-3 described above that was isolated from an acidic, iron rich site in California. The three clones isolated from Bands 6 and 10 have similarities to aerobic microorganisms able to catalyze the degradation of ring structures. Although enrichment of these organisms can explain the increased rate of degradation of 2,3',4'-PCB in the first 30 wk of the anaerobic phase, neither group would be expected to catalyze the dechlorination of the hexachlorobiphenyls. In addition, the detection of these aerobic microorganisms at wk 30 indicates that the unamended microcosms were not highly reducing at this time point.

The Band 12 (12-3) clone sequence had 96% similarity to a soil clone from a metal-contaminated Superfund site. It also displayed 94% similarity with a soil clone that clustered in the *Acidobacterium* group from a grassland soil (16). *Acidobacterium* is a relatively recently recognized prokaryotic Kingdom with great phylogenetic and metabolic diversity. Nogales et al. found that 14% of the cloned sequence types in their PCB-contaminated soil clustered within the *Holophaga-Acidobacterium* phylum (30). The soil utilized in their study was similar to the SRS soil used in this work in that it was a sandy soil with a pH of 5-6.

Sequences from Bands 8 and 11 had 100% similarity with *E. coli*. Since *E. coli* is not expected to be present in large numbers in typical soil samples, the presence of this sequence is likely due to experimental contamination. This contamination could have been derived from a number of different steps, but probably occurred after running the DGGE. If the DNA in Band 8 was from *E. coli*, the fragment would be expected to migrate to the same position as the *E. coli* positive control. Since a different migration

was observed for Band 8, the contamination of this band must have occurred in the cloning, plasmid isolation, or sequencing steps.

No single PCB-dechlorinating organism has been isolated to date and attempts to do so have been hindered by the ability to maintain a dechlorinating culture in a defined medium. Few studies have looked at the composition of anaerobic, PCB-dechlorinating cultures. Pulliam Holoman and co-workers used sequence analysis of amplified 16S rDNA to describe a 2,3,5,6-tetrachlorobiphenyl ortho-dechlorinating microbial community (31). During active dechlorination, species in the δ -subgroup of *Proteobacteria*, the low G+C gram-positive subgroup, and the *Thermotogales* subgroup dominated. All three include sulfur-, sulfate-, and/or iron (III)-respiring bacterial species. In addition, one species had high sequence similarity to the obligate PCE-dechlorinating *Dehalococcoides*. Similar sequences were not detected in this work although only a fraction of the bands were sequenced. However, several clones were similar to organisms able to use ferrous iron as an electron donor. The use of ferrous iron as an electron donor in these samples would explain why no ferrous iron production was observed throughout the anaerobic phase.

5.E. Vitamin B₁₂ Experiments

The possibility that the PCB dechlorination observed was caused by a non-biological, microbially mediated process was investigated through the use of reduced vitamin B₁₂ experiments. Two different studies have shown that vitamin B₁₂ can catalyze the reductive dechlorination of PCBs (Woods et al., 1999; Assaf-Anid et al., 1992). In addition, the production and subsequent release of vitamin B₁₂ into aqueous media by

anaerobic microorganisms is well documented. While proving that vitamin B₁₂-mediated dechlorination of PCBs is possible, neither study attempted to show that vitamin B₁₂ released by anaerobic microorganisms was the cause of observed dechlorination in an actual soil microcosm study.

Four treatments were investigated: 1) a positive control with vitamin B₁₂ amendment, 2) a negative control with no vitamin B₁₂ and only mineral medium, 3) a treatment of filtered medium from butyrate-amended dechlorinating microcosms, and 4) filtered medium from methanol-amended actively dechlorinating microcosms. Because the goal of this experiment was to observe any non-biologically mediated processes, sterile soil and medium were utilized to ensure sterile conditions. In addition, a reducing agent was added to simulate the conditions found in the anaerobic microcosms at wk 30. Under these engineered reducing conditions, the medium changed from red to amber indicating that the vitamin B₁₂ had become partially reduced to B_{12r}. Further reduction to B_{12s} would have been indicated by a second color change to blue. Vitamin B_{12s} is a stronger electron donor than is vitamin B_{12r} and would be a better catalyst for reductive dechlorination. Woods et al. and Assaf-Anid et al. found that the amount of dechlorination observed was dependent on the redox state of the vitamin B₁₂. When dithiothreitol was used as a reducing agent, the vitamin B₁₂ was in the B_{12r} state and dechlorination of pentachlorobiphenyls on the order of 3.5% were obtained. When titanium citrate was used (providing much lower redox conditions) nearly 40% dechlorination of the same pentachlorobiphenyl was observed (2, 39).

Although vitamin B_{12s} would have provided a better reducing agent, the goal of this experiment was not to effect the greatest amount of dechlorination, but rather to

understand the conditions encountered in the anaerobic microcosms. No dechlorination was observed in any of the four treatments tested in 28 wks of incubation. Figure 5.19 shows the concentration of 2,2',4,4',5,5'-PCB in the experimental microcosms from the four treatments described above. No dechlorination was observed in any of the treatments. These results indicate that the observed dechlorination was in fact microbially mediated and not due to a co-factor such as the vitamin B₁₂. It is unusual that the vitamin B_{12r} did not enact any sort of dechlorination in this system. However, this could be due to other chemical conditions in the microcosms that make it unamenable to dechlorination by the vitamin B_{12r}.

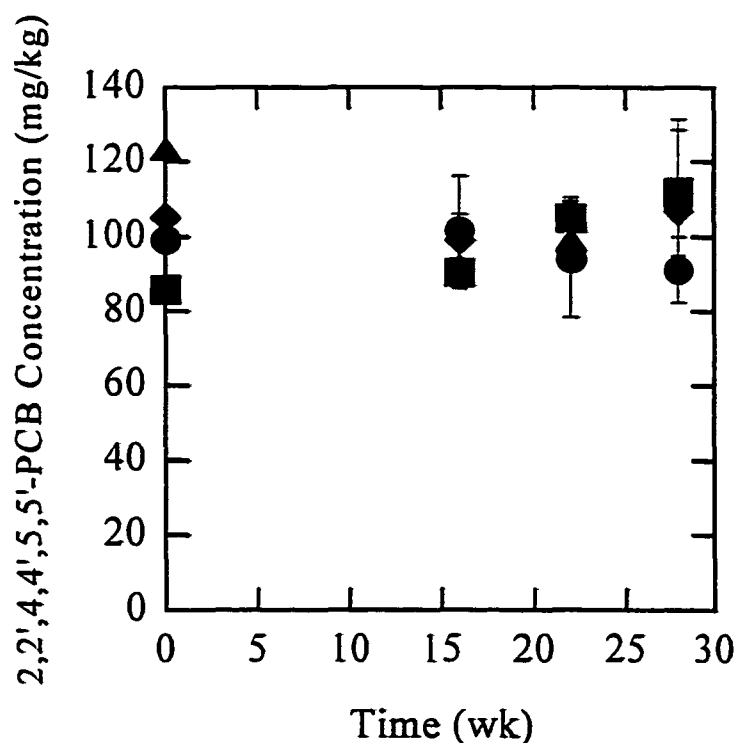


Figure 5.19 Investigation of non-microbially mediated dechlorination of 2,2',4,4',5,5'-hexachlorobiphenyl in anaerobic soil microcosms. ■ - positive controls (vitamin B₁₂ addition), ◆ - negative controls, ● - butyrate-amended microcosm filtered medium, ▲ - methanol-amended microcosm filtered medium. Error bars represent the standard deviation between duplicate samples.

5.F. Conclusions

In the anaerobic microcosms, significant dechlorination occurred in all 3 treatments. Dechlorination of the hexachlorobiphenyl was first observed between weeks 4 and 22 for the various treatments even though conditions were not highly reducing at any time point. The most rapid dechlorination occurred in unamended microcosms, while the butyrate and methanol-amended microcosms dechlorinated at a slower rate but reached nearly the same extent of dechlorination. Dechlorination at the *meta* and *para* positions were observed with a further *meta* dechlorination to produce a tetrachlorobiphenyl. Non-microbially mediated dechlorination was ruled out by means of sterile control experiments with microcosm filtrate. Results from the PLFA/FAME analysis corresponded with the PCB concentration results; at times of high dechlorination rates, an increase in total biomass was observed. In addition, those samples undergoing the most extensive dechlorination clustered together indicating similar microbial communities.

Most of the cloned sequence types were related to the 16S rRNA sequences of aerobic species, and several to acidiphilic bacteria, which is consistent with the physicochemical characteristics of the soil, i.e. acidic pH and sandy texture. Although the combination of DGGE and sequencing is not quantitative, the predominance of these sequence types in the SRS soil suggests that the microorganisms represented by these sequences may play important roles in the microbial activity within this soil. The similarity to the facultative anaerobe *Dechlorimonas* of one of the soil clones may indicate that this is a key organism in the dechlorination process. However, it cannot be excluded that the cell lysis procedure and primers utilized missed some important,

unknown prokaryotes in the soil that are responsible for the dechlorination observed. In addition, only 15 clones were sequenced, therefore the organisms carrying out the dechlorination might have been successfully extracted from the DNA, but may not yet be sequenced. Few differences in DGGE banding patterns were observed between butyrate- and methanol-amended treatments and those from time zero, in agreement with the PLFA/FAME analysis.

The results from the chemical and microbial analysis combined suggest that 1) highly reducing conditions are not necessary for PCB dechlorination, 2) iron-oxidation is likely occurring but may not play a key role in the dechlorination processes in this soil, and 3) aerobic microorganisms survived under the conditions needed for the dechlorination.

5.G. References

1. Amman, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl. 1992. Molecular and microscopic identification of sulfate-reducing bacteria on multispecies biofilms. *Applied and Environmental Microbiology*. 58:614-623.
2. Assaf-Anid, N., L. Nies, and T. M. Vogel. 1992. Reductive dechlorination of a polychlorinated biphenyl congener and hexachlorobenzene by vitamin B₁₂. *Applied and Environmental Microbiology*. 58:1057-1060.
3. Asturias, J. A., E. Moore, M. Yakmov, S. Klatte, and K. N. Timmis. 1994. Reclassification of the polychlorinated biphenyl-degraders *Acinetobacter* sp. strain P6 and *Corynebacterium* sp. strain MB1 as *Rhodococcus gloverulus*. *Systemic Applied Microbiology*. 17:226-231.

4. **Barriault, D., and M. Sylvestre.** 1993. Factors affecting PCB degradation by an implanted bacterial strain in soil microcosms. *Canadian Journal of Microbiology*. **39**:594-602.
5. **Bedard, D. L., and H. M. Van Dort.** 1998. Complete reductive dehalogenation of brominated biphenyls by anaerobic microorganisms in sediment. *Applied and Environmental Microbiology*. **64**:940-947.
6. **Bobbie, R. J., and D. C. White.** 1980. Characterization of benthic microbial community structure by high-resolution gas chromatography of fatty acid methyl esters. *Applied and Environmental Microbiology*. **39**:1212-1222.
7. **Chen, P., W. Zhou, and L. L. Tavlarides.** 1997. Remediation of polychlorinated biphenyl contaminated soils/sediments by supercritical fluid extraction. *Environmental Progress*. **16**:227-235.
8. **Coates, J. D., U. Michaelidou, R. A. Bruce, S. M. O'Connor, J. N. Crespi, and L. A. Achenbach.** 1999. Ubiquity and diversity of dissimilatory (Per)chlorate-reducing bacteria. *Applied and Environmental Microbiology*. **65**:5234-5241.
9. **Davison, A. D., D. R. Jardin, and P. Karuso.** 1999. 5-chloropicolinic acid is produced by specific degradation of 4-chlorobenzoic acid by *Sphingomonas paucimobilis* BPSI-3. *Journal of Industrial Microbiology and Biotechnology*. **23**:347-352.
10. **Deweerd, K. A., and D. L. Bedard.** 1999. Use of halogenated benzoates and other halogenated aromatic compounds to stimulate the microbial dechlorination of PCBs. *Environmental Science and Technology*. **33**:2057-2063.

11. **DiStefano, T. D., J. M. Gossett, and S. H. Ziner.** 1991. Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. *Applied and Environmental Microbiology*. **57**:2287-2292.
12. **Dojka, M. A., P. Hugenholtz, S. K. Haack, and N. R. Pace.** 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Applied and Environmental Microbiology*. **64**:3869-3877.
13. **Dunbar, J., S. Takala, S. M. Barns, J. A. Davis, and C. R. Kuske.** 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Applied and Environmental Microbiology*. **65**:1662-1669.
14. **Edwards, E. A., and E. E. Cox.** 1997. Field and laboratory studies of sequential anaerobic-aerobic chlorinated solvent biodegradation, p. 261-265. *In* B. C. Alleman and A. Leeson (ed.), *In Situ and On-Site Bioremediation*, vol. 3. Battelle Press, Columbus.
15. **Erb, R. W., and I. Wagner-Dobler.** 1993. Detection of polychlorinated biphenyl degradation genes in polluted sediments by direct DNA extraction and polymerase chain reaction. *Applied and Environmental Microbiology*. **59**:4065-4073.
16. **Felske, A., A. Wolterink, R. V. Lis, and A. D. L. Akkermans.** 1998. Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). *Applied and Environmental Microbiology*. **64**:871-879.

17. **Fennell, D. E., M. A. Stover, S. H. Zinder, and J. M. Gossett.** 1995. Bioremediation of chlorinated solvents, p. 9-16. *In* R. E. Hinchee, A. Leeson, and L. Semprini (ed.), *Bioremediation of Chlorinated Solvents*. Battelle Press, Columbus, OH.
18. **Frostegard, A., S. O. Petersen, E. Baath, and T. H. Nielsen.** 1997. Dynamics of a microbial community associated with manure hot spots as revealed by phospholipid fatty acid analyses. *Applied and Environmental Microbiology*. **63**:2224-2231.
19. **Hales, B. A., C. Edwards, D. A. Ritchie, G. Hall, R. W. Pickup, and J. R. Saunders.** 1996. Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Applied and Environmental Microbiology*. **62**:668-675.
20. **Hrywna, Y., T. V. Tsoi, O. V. Maltseva, J. F. Q. III, and J. M. Tiedje.** 1999. Construction and characterization of two recombinant bacteria that grow on *ortho*- and *para*-substituted chlorobiphenyls. *Applied and Environmental Microbiology*. **65**:2163-2169.
21. **Kastner, M.** 1991. Reductive dechlorination of tri- and tetrachloroethylenes depends on transition from aerobic to anaerobic conditions. *Applied and Environmental Microbiology*. **57**:2039-2046.
22. **Kieft, T. L., D. B. Ringelberg, and D. C. White.** 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium. *Applied and Environmental Microbiology*. **60**:3292-3299.

23. **Kim, J., and G.-Y. Rhee.** 1999. Reductive dechlorination of polychlorinated biphenyls: Interactions of dechlorinating microorganisms with methanogens and sulfate reducers. *Environmental Toxicology and Chemistry*. 18:2696-2702.
24. **Klasson, K. T., J. W. Barton, B. S. Evans, and M. E. Reeves.** 1996. Reductive microbial dechlorination of indigenous polychlorinated biphenyls in soil using a sediment-free inoculum. *Biotechnology Progress*. 12:310-315.
25. **Marsh, T. L., R. L. Kurzhals, M. A. Dojka, G. Icopini, D. T. Long, and N. R. Pace.** 1999. Molecular diversity of a soil bacterial community from a metal contaminated Superfund site. GenBank. AF145877.
26. **May, H. D., A. W. Boyle, W. A. Price II, and C. K. Blake.** 1992. Subculturing of a polychlorinated biphenyl-dechlorinating anaerobic enrichment on solid media. *Applied and Environmental Microbiology*. 58:4051-4054.
27. **Muller, R. H., S. Jorks, S. Kleinsteuber, and W. Babel.** 1999. *Comamonas acidovorans* strain MC1: a new isolate capable of degrading the chiral herbicides dichlorprop and mecoprop and the herbicides 2,4-D and MCPA. *Microbiological Research*. 154:241-246.
28. **Nazina, T. N., I. M. Kosareva, A. S. Davidov, T. P. Tourova, E. V. Novikova, R. R. Hafisov, and A. B. Poltarau.** 2000. Physico-chemical and microbiological characteristics of subsurface water from observation wells of liquid radiation waste repository. *Microbiologia*. 69:1-10.
29. **Nies, L., and T. M. Vogel.** 1990. Effects of organic substrates on dechlorination of Aroclor 1242 in anaerobic sediments. *Applied and Environmental Microbiology*. 56:2612-2617.

30. **Nogales, B., E. R. B. Moore, W. R. Abraham, and K. N. Timmis.** 1999. Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. *Environmental Microbiology*. 1:199-212.
31. **Pulliam Holoman, T. R., M. A. Elbersen, L. A. Cutter, H. D. May, and K. R. Sowers.** 1998. Characterization of a defined 2,3,5,6-tetrachlorobiphenyl-*ortho*-dechlorinating microbial community by comparative sequence analysis of genes coding for 16S rRNA. *Applied and Environmental Microbiology*. 64:3359-3367.
32. **Rhee, G.-Y., R. C. Sokol, C. M. Bethoney, and B. Bush.** 1993. A long-term study of anaerobic dechlorination of PCB congeners by sediment microorganisms: pathways and mass balance. *Environmental Toxicology and Chemistry*. 12:1829-1834.
33. **Schwieger, F., and C. C. Tebbe.** 1999. Effect of field inoculation with *Sinorhizobium meliloti* L33 on the composition of bacterial communities in rhizospheres of a target and a non-target plant - Linking 16S rRNA gene based SSCP-community profiles and diversity of cultivated isolates. GenBank. AF214138.
34. **Shikano, S., and H. Mitsui.** 1998. GenBank. AB011750.
35. **Snaidr, J., R. Amann, I. Huber, W. Ludwig, and K. H. Schleifer.** 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Applied and Environmental Microbiology*. 63:2884-2896.
36. **Sokol, R. C., O.-S. Kwon, C. M. Bethoney, and G.-Y. Rhee.** 1994. Reductive dechlorination of polychlorinated biphenyls in St. Lawrence River sediments and

- variations in dechlorination characteristics. *Environmental Science and Technology*. **28**:2054-2064.
37. **Teske, A., P. Sigalevich, Y. Cohen, and G. Muyzer.** 1996. Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Applied and Environmental Microbiology*. **62**:4210-4215.
38. **Triplett, E. W., J. A. Jansen, and J. Borneman.** 1998. Contrasting cultured and uncultured microorganisms from Amazonian soils. GenBank. **AF083604**.
39. **Woods, S. L., and D. J. Trobaugh.** 1999. Polychlorinated biphenyl reductive dechlorination by vitamin B₁₂: Thermodynamics and regiospecificity. *Environmental Science and Technology*. **33**:857-863.

CHAPTER 6

AEROBIC DEGRADATION STUDY

6.A. Introduction

Aerobic microbial degradation of polychlorinated biphenyls has been well documented both in laboratory and field settings (1, 3-5, 7). Numerous bacterial species have been isolated that are able to carry out the degradation of PCB congeners with four or fewer chlorine substituents. A smaller number of isolates have the ability to degrade a wider substrate range that includes highly chlorinated congeners with up to six chlorine substituents (10, 15). In addition, researchers have developed enrichment cultures capable of the same aerobic degradation. Chapter 4 described preliminary work in which the ability of the indigenous organisms to degrade less-chlorinated PCB congeners was tested. For the full-scale treatability study, an aerobic degradation experiment was undertaken with one of those less-chlorinated congeners, 2,3',4'-PCB.

For each phase of the treatability study (aerobic, anaerobic, or sequential) soil slurry microcosms were dosed with the three congener mixture, consisting of 2,3',4'-, 3,3',4,4'- and 2,2',4,4',5,5'-PCB. For the aerobic phase of experimentation, the engineering parameter to be tested was the addition of biphenyl and the goals were to determine its effect of PCB degradation and microbial community composition. The requirement of biphenyl to initiate the

cometabolic degradation of PCBs has been reported in soil slurries and microcosms. For this reason, it was interesting to evaluate the effect of biphenyl amendment on the enrichment of microorganisms able to degrade PCBs more rapidly or to a greater extent. Although only the trichlorobiphenyl was expected to be degraded aerobically, all three congeners were dosed to keep experimental conditions constant for all phases of treatment. To carry out the sequential treatment, it was important to first understand the aerobic degradation characteristics so that changes in population composition or degradation ability or efficiency due to the prolonged anaerobic phase could be ascertained.

6.B. Polychlorinated Biphenyl Degradation

The trichlorobiphenyl, 2,3',4'-PCB, was added to the soil microcosms at time zero at a concentration of 50 mg/kg as described in the Materials and Methods section. Three treatments were investigated: 1) microcosms with the congener mixture addition only, 2) microcosms with the congener mixture addition plus biphenyl amendment, and 3) a sterile control with both the congener mixture and biphenyl amendment. Samples were extracted at 0, 1, 2, 3, 5, and 7 wk to determine PCB and biphenyl concentrations.

The degradation of 2,3',4'-PCB in each of the three treatments is shown in Figure 6.1. Error bars represent the standard deviation between two replicate microcosms sampled for each treatment. No degradation was observed in the sterile control. There was however, complete degradation of the 2,3',4'-PCB in both biphenyl-amended and unamended microcosms. In addition, biphenyl was completely degraded in the amended microcosms, but not at all in the sterile control (Figure 6.2). The time course of degradation of biphenyl matched that of the 2,3',4'-PCB, with both compounds being completely degraded by wk 5. This is in contrast to

observations by Focht and Brunner where the mineralization of PCBs was delayed in all cases until after the degradation of biphenyl had begun (7). They attributed this to the need for the induction of the biphenyl dioxygenase enzymes carrying out the PCB degradation.

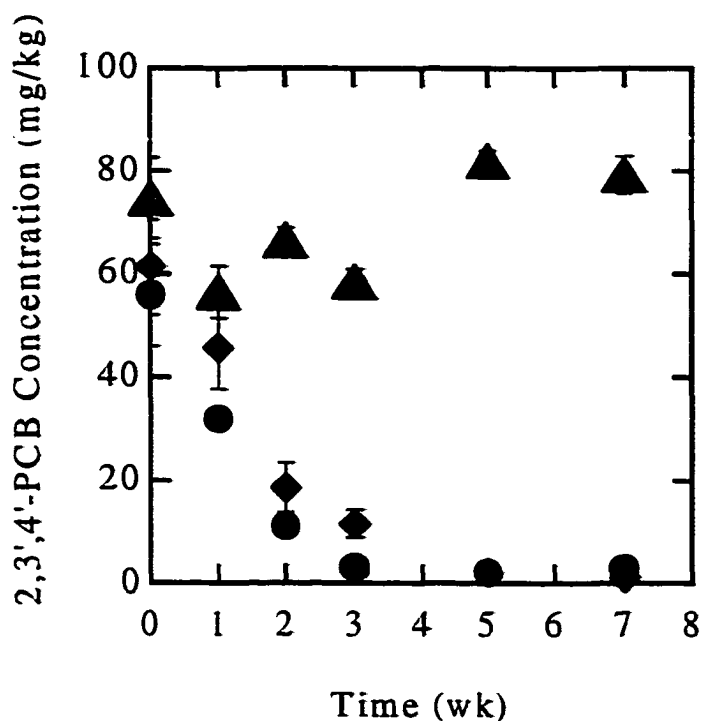


Figure 6.1 Degradation of 2,3',4'-PCB in aerobic slurry microcosms. ● - biphenyl-amended microcosms, ◆- unamended microcosms, and ▲ - sterile control. Error bars represent the standard deviation between duplicate samples.

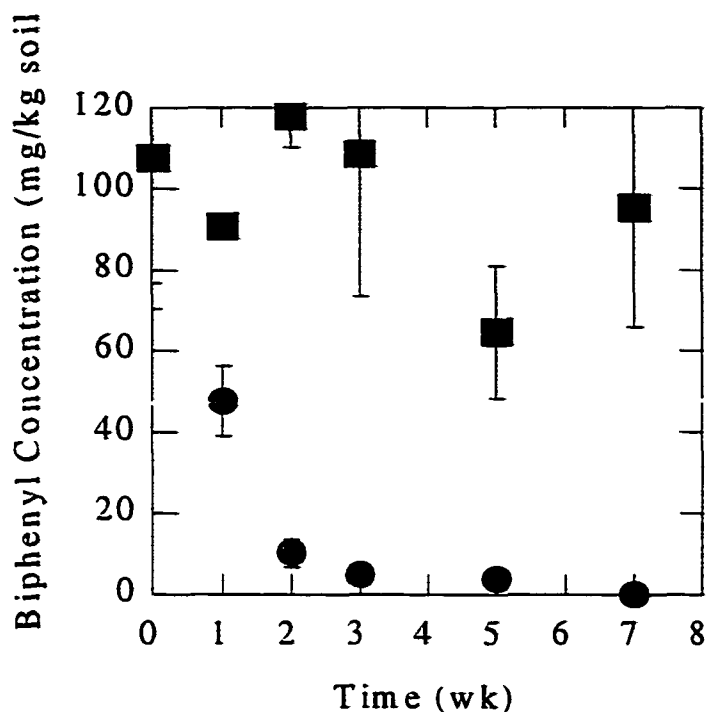


Figure 6.2 Biphenyl degradation in amended aerobic microcosms. ● - Biphenyl-amended microcosms, ■ - sterile control with biphenyl amendment. Error bars represent the standard deviation between duplicate samples.

The biphenyl amendment appeared to slightly increase the rate at which the trichlorobiphenyl was degraded. Because the starting concentrations were somewhat different for the biphenyl-amended and unamended microcosms, kinetic parameters for the degradation of 2,3',4'-PCB in each treatment were calculated for comparison. These rates were calculated using the Michaelis-Menten equation, the simplest and most commonly used model for enzyme-catalyzed reactions (Equation 6.1).

$$\frac{dC}{dt} = -\frac{v_{\max} C}{K_m + C} \quad \text{Equation 6.1}$$

In Eqn 6.1, $v_{\max}$ =maximum reaction rate (1/wk), K_m =Michaelis constant (mg/kg), C =PCB concentration in soil (mg/kg), and t =time (wk). Values for $v_{\max}$ and K_m were determined by performing nonlinear curve fitting to the experimental data using SimuSolv™, a modeling and simulation package (Dow Chemical Company). Simusolv employed a Gear method to solve the differential equations and maximized the log of the likelihood function (LLF) to optimize the unknown parameters and discriminate between models. Model curves were obtained via numerical integration of Equation 6.1 using *Engineering Equation Solver*. The model curves are shown in Figure 6.3 and the constants obtained for $v_{\max}$ and K_m are presented in Table 6.1. The model fits for each substrate depletion curve are quite good, with roughly 97% of the variation in the experimental data explained by the model. It is interesting to note that the maximum rate ($v_{\max}$) of degradation for unamended microcosms was greater than that for biphenyl-amended microcosms. However, due to the low K_m value for biphenyl-amended microcosms, the overall degradation of 2,3',4'-PCB is more rapid in biphenyl-amended than in unamended microcosms. At lower PCB concentrations that might reasonably be found in environmental matrices, the advantage that a low K_m provides would be even more pronounced.

Table 6.1 Kinetic parameters for the degradation 2,3',4'-PCB

Treatment	$v_{\max}$ (1/wk)	K_m (mg/kg)	$v_{\max}/K_m$ (mg/kg wk)	Percent Variation Explained (%)
Unamended	67.4	89.9	0.75	97.2
Biphenyl-amended	54.4	35.4	1.54	97.6

No researchers have reported rate constants determined based on a PCB depletion curve. However, Fairbanks et al. determined first order rate constants for $^{14}\text{CO}_2$ evolution from soils amended with ^{14}C -PCBs and sewage sludge (6). For a concentration of 50 mg/kg PCBs in soil, rate constants of $2.8 - 25.9 \text{ wk}^{-1}$ were found. Focht and Brunner also calculated rate constants based on $^{14}\text{CO}_2$ evolution and obtained a rate constant of 2.7 wk^{-1} in low organic matter soil (7). For substrate concentrations much less than K_m , the Michaelis-Menten equation reduces to the first order rate expression given in Equation 6.2

$$k_{\text{firstorder}} = \frac{v_{\text{max}}}{K_m} \quad \text{Equation 6.2}$$

The parameters determined in this work can be compared to those determined by other researchers using the ratio of v_{max}/K_m . Values for this ratio were calculated using the determined kinetic parameters and are given in Table 6.1. The rate of degradation of the trichlorobiphenyl, while indicating a lower rate of degradation are on the same order of magnitude of the results of other researchers described above.

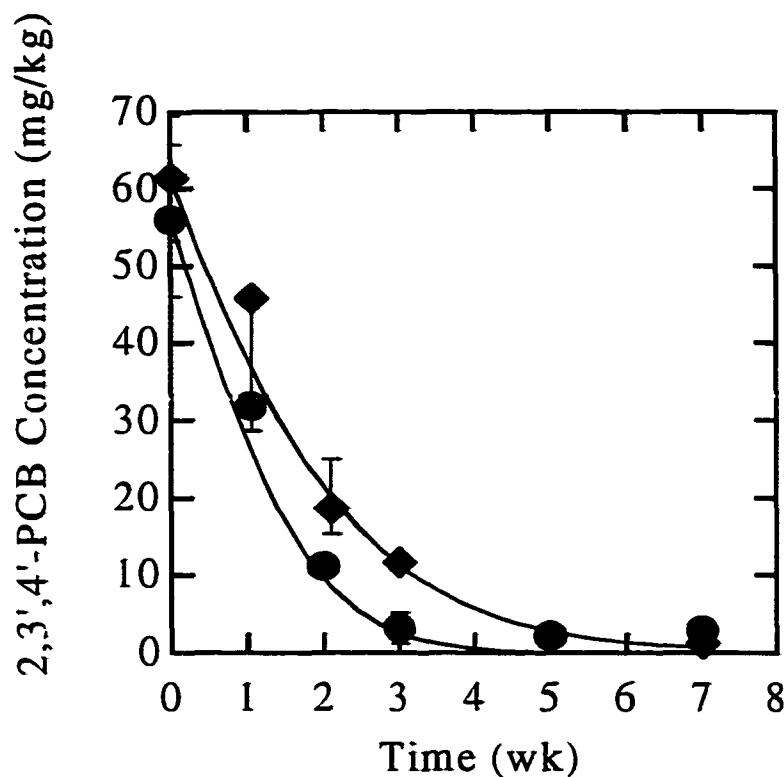


Figure 6.3 Model of 2,3',4'-PCB degradation. ● - biphenyl-amended, ◆ - unamended. Error bars represent the standard deviation between duplicate samples.

The increase in overall degradation rate observed with biphenyl amendment suggests that the biphenyl is inducing the biphenyl dioxygenase enzyme that carries out the cleavage of one of the biphenyl rings and supporting the growth of the *bph*-expressing population. However, the trichlorobiphenyl was also completely degraded in the unamended microcosms, and thus the necessary enzymes are also induced without the presence of biphenyl. Cometabolism is thus not driving the degradation of trichlorobiphenyl in our system. Preliminary experiments showed the probable presence of both a 2,3- and a 3,4-biphenyl dioxygenase enzyme system in the SRS soil

microorganisms (Section 4.A.3.b.). Both of these enzyme systems would be capable of catalyzing the degradation of the 2,3',4'-PCB. One possible scenario that could explain the increased degradation rate observed with biphenyl amendment is that only one of these two dioxygenase enzymes is induced by the 2,3',4'-PCB, but both are induced by the presence of the biphenyl and 2,3',4'-PCB combination. Another possibility is that both enzymes are induced in each case, but are induced more rapidly or to a greater extent in the presence of biphenyl.

Figures 6.4 and 6.5 show the concentrations of 3,3',4,4'- and 2,2',4,4',5,5'-PCB in the aerobic soil microcosm experiments. No degradation of either of these congeners occurred in the 7-wk experiment. This lack of degradation of more highly chlorinated congeners is in agreement with results obtained by most researchers. In a small number of cases, aerobic degradation of these compounds has been reported. Williams and co-workers observed 22 and 61% degradation of 2,2',4,4',5,5'-PCB by pure cultures of *Alcaligenes eutrophus* H850 and *Burkholderia* sp. LB400 (18). These two organisms have been shown to have nearly identical genes coding for the PCB-degradative enzymes (3, 4). However, this type of degradative ability is extremely rare and has not been described under conditions that begin to approximate real environments (e.g., soil microcosms or field studies with indigenous organisms).

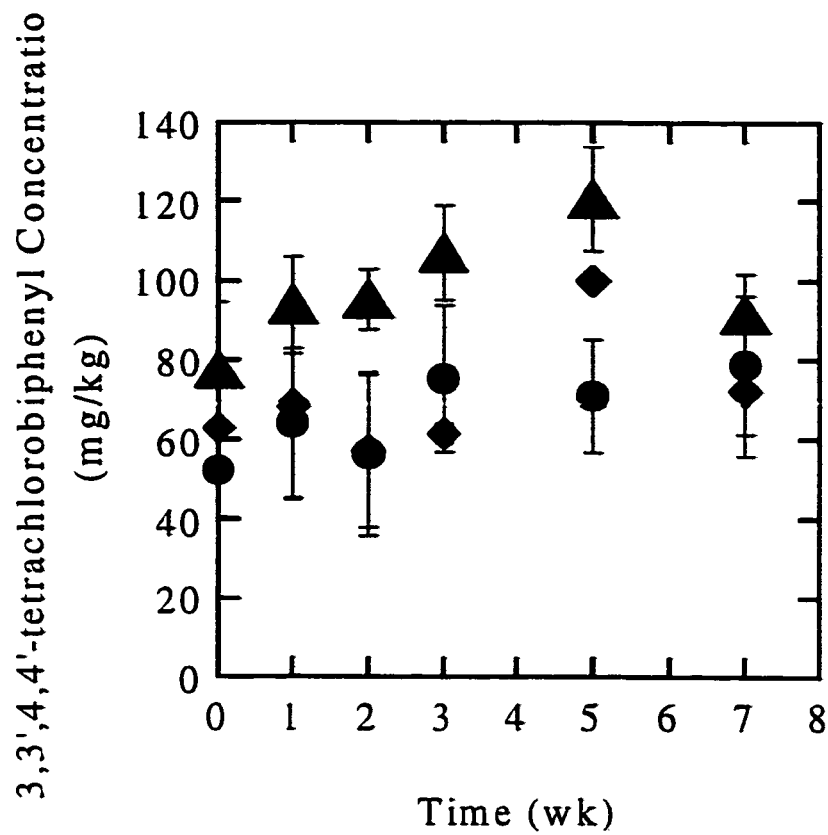


Figure 6.4 Degradation of 3,3',4,4'-tetrachlorobiphenyl. ◆ - unamended soil microcosms, ● - biphenyl-amended microcosms, ■ - sterile control microcosms. Error bars represent the standard deviation between duplicate samples.

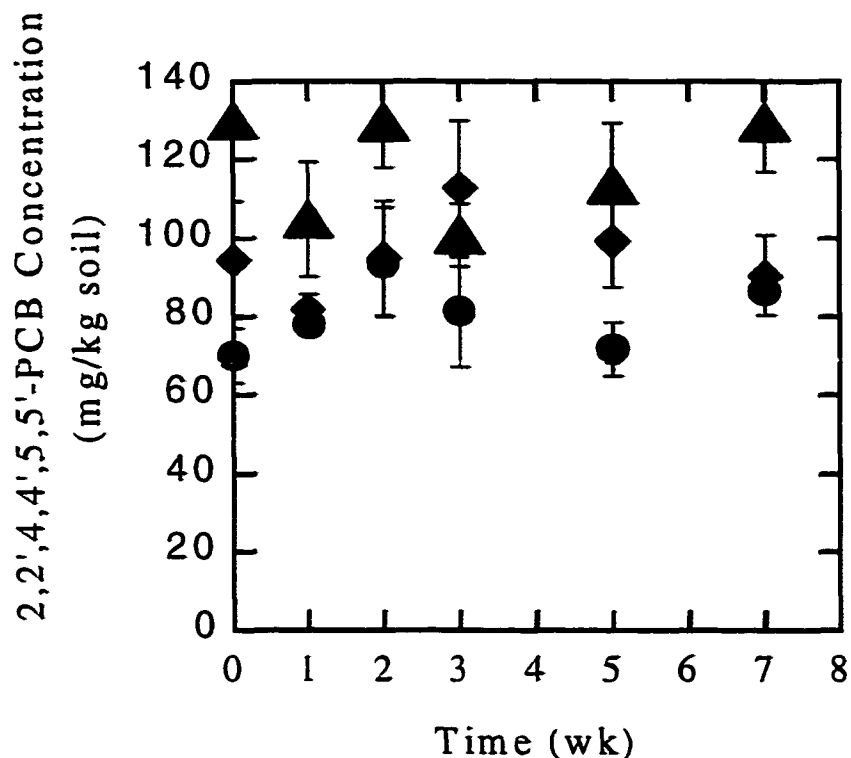


Figure 6.5 Degradation of 2,2',4,4',5,5'-hexachlorobiphenyl. ♦ - unamended soil microcosms, ● - biphenyl-amended microcosms, and ■ - Sterile control microcosms. Error bars represent the standard deviation between duplicate samples.

6.C. Microorganisms

Once the aerobic degradative capabilities of the soil microbial community had been determined, experiments were conducted to characterize the microbial populations carrying out the observed degradation.

6.C.1. Plate Counts

As with the preliminary experiments, traditional plating methods using both ½ tryptic soy agar and mineral media plates with biphenyl as the sole carbon source were attempted for

the enumeration of the microbial population. Figures 6.6 and 6.7 show the plate count results for unamended and biphenyl-amended microcosms on ½ TSA and biphenyl plates, respectively. A large increase in the number of culturable cells on ½ TSA occurred in both treatments at wk 2. No such spike was observed in biphenyl plates. These results seem opposite to what could be expected intuitively. If both 2,3',4'-PCB and biphenyl are being degraded, any spike in microbial concentrations would be expected to be due to increases in the biphenyl-degrading microorganisms. Sterile controls were also plated on both media to ensure sterility; no growth was observed on either media.

In both treatments, the microorganisms from samples taken after 3 wk were unable to grow on either of these culture media. Because degradation of both biphenyl and 2,3',4'-PCB was essentially complete by week 3, the possibility that the levels of the microorganisms had indeed decreased over this time is feasible. However, this phenomenon has also been observed by Kieft and co-workers in a study investigating the addition of microorganisms to soil microcosms (13). In their work, a decline in the number of culturable cells below detection levels was observed within 16 d of inoculation. However, they observed that total direct counts and total PLFAs remained constant. While these experimental conditions are somewhat different from this work (no microorganisms were added in our study), their results substantiated the inability of our plate-counting methods to accurately enumerate the microbial population during both the aerobic treatability phase and during the preliminary experiments. With these two pieces of evidence, the need for alternative methods for enumeration of the mixed cultures and environmental microorganisms in the soil microcosms was highlighted and other methods were employed.

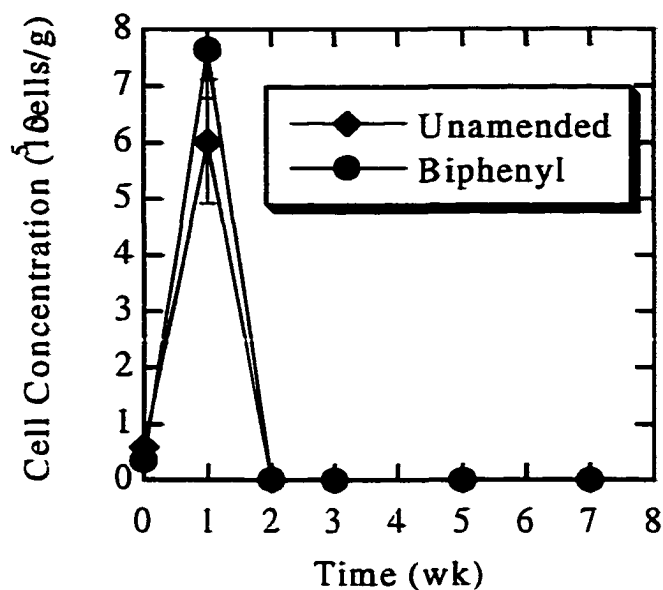


Figure 6.6 Enumeration of microbial population on 1/2 TSA plates. The limit of detection for the plate counting method was 1×10^1 cells/g soil. Error bars represent the standard deviation between duplicate samples.

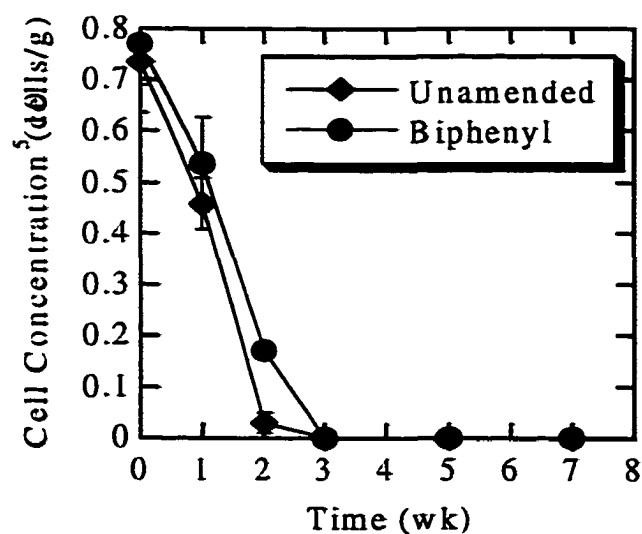


Figure 6.7 Enumeration of microbial population on mineral medium plates with biphenyl as a sole carbon source. The limit of detection for the plate counting method was 1×10^1 cells/g soil. Error bars represent the standard deviation between duplicate samples.

6.C.2. PLFA/FAME analysis

Since traditional microbial enumeration methods were unable to be used for this work, several alternative methods were used to characterize and enumerate the microbial community. As in the anaerobic phase of this work, the PLFA/FAME method was used to describe changes in the microbial population. The same specific fames were used for analysis (Table 5.1). By summing the amounts of each FAME, it was determined that total biomass remained constant in both unamended and biphenyl-amended microcosms over the of the 7-wk experiment (Table 6.2). Concentrations of PLFAs were low (116 – 443 pmol/g), but of a magnitude and diversity similar to others described from sandy clay and sand aquifer material (8, 9, 16). Phelps et al. observed PLFA concentrations of 32 to 298 pmol/g soil from aquifer materials associated with a TCE plume near the Savannah River (16). In contrast, Langworthy and co-workers determined total biomass concentrations of 1.1×10^5 – 4.6×10^5 pmol/g in a riverine sediment contaminated with PAHs (14).

Table 6.2 Concentration of total biomass in aerobic microcosms. Values are pmol/g $\pm$ S.D.
*Only a single sample was analyzed so no S.D. could be determined.

Time (wk)	Unamended Microcosms	Biphenyl-Amended Microcosms
0	264.6 $\pm$ 94.9	
1	282.9 $\pm$ 18.7	319.7 $\pm$ 110.1
2	154.2 $\pm$ 69.7	183.3 *
3	295.2 $\pm$ 64.8	195.3 $\pm$ 25.8
5	204.8 $\pm$ 55.2	343.8 *
7	322.1 $\pm$ 97.5	207.4 $\pm$ 24.1

Even though total microbial biomass was unchanged throughout the experiment for both treatments, specific changes in fatty acid methyl ester profiles occurred in biphenyl-amended and unamended microcosms. The most significant changes were observed in

biphenyl-amended microcosms. Variations in proportions of specific fatty acids between samples and treatments can indicate two things: 1) changes are occurring in the composition of the lipid-contributing microbial community, and/or 2) no changes in overall population composition occurred, but changes in the lipid content of the microorganisms changed. While neither option can be completely ruled out, many researchers have analyzed FAME profile data based on the first assumption. Multiple variable ANOVA using Tukeys HSD for comparison between means was used to determine significant changes in FAMES between treatments and time points.

PLFA/FAME analysis on soil samples from the Savannah River Site soil indicated a lack of mid-chain branched PLFAs characteristic of gram-positive bacteria including *Rhodococcus* and other filamentous actinomycetes. One of the most well studied PCB-degrading bacteria is *Rhodococcus gloverulus* (formerly *Corneybacterium* MB1 and *Acinetobacter* P6) (2, 5). Mid-chain branched PLFA are also characteristic of sulfate-reducing bacteria, which would not be expected to proliferate under the aerobic conditions observed in the microcosms. In biphenyl-amended microcosms, an increase of nearly 65% in straight-chain fatty acids of even length (16:1 and 18:1) in biphenyl-amended microcosms was observed. This indicates an increase in *Pseudomonas* and other gram-negative, related aerobes that have been shown to have high levels of these compounds in their cell membrane (Figure 6.8) (11). No significant increase or decrease was observed in unamended microcosms over the course of the 7-wk experiment.

In addition to the monounsaturates (16:1 ω 7 and 18:1 ω 7), cy17:0 increased slightly in both biphenyl-amended and unamended microcosms. Cyclopropyl 17:0 and cyclopropyl 19:0 have been associated with anaerobic bacteria from members of the genus *Clostridium*, *Vibrio*,

and *Enterobacteriaceae*, which are not typically observed in soil environments (11). However, cyclopropyl 17:0 is also distributed among aerobic bacterial genera including *Pseudomonas* and other similar Gram-negative bacteria. In these genera, *cy17:0* can act as a marker for microorganisms undergoing nutritional or environmental stress (13). Since complete degradation of the trichlorobiphenyl was observed, nutritional deprivation was not likely occurring, but other sources of stress may have existed in the microcosms. The presence of *cy17:0* was not detected at time zero in the soil microcosms, but increased slightly after one week and remained essentially constant after this time. This may indicate an inhibitory effect of either the higher chlorinated PCBs or the hexane used to dose the PCBs with. To investigate this question, the FAME analysis for treatment 17 where no PCBs were dosed, but hexane was used was investigated. In the hexane-only treatment, a similar increase in *cy17:0* was observed, with gradually declining concentrations over the course of the 60 wk anaerobic phase. These similarities provide evidence that the observed increase in *cy17:0* was due to the inhibitory effect of hexane addition, rather than as a contribution to the increase in aerobic microorganisms.

While the aerobic community appeared to increase, decreases in the PLFAs *i14:0* and *i15:0* were observed (Figure 6.9). Fatty acids *i14:0*, *i15:0*, and *i16:0* are indicative of both *Bacillus*-type, gram-positive organisms or anaerobic gram-negative bacteria (14). These losses could indicate a loss in either group of organisms. However, no loss was observed in other fatty acids typically found in *Bacillus*-type organisms but less prevalent in anaerobic organisms (*a15:0* and *16:0*). Thus, the decrease of *i14:0* and *i15:0* is likely due to a decrease in the anaerobic microbial community. For the biphenyl-amended microcosms, the levels of *i14:0* and *i15:0* fell below the detection limit at wk 5. There was a similar decrease of these PLFAs

in unamended microcosms; however, these populations seemed to increase again by the end of the 7 wk. In addition to those decreases, the PLFA 18:2 ω 6,9, found in algae, fungi, and protozoa, was undetectable by week 7 in the biphenyl-amended microcosms (Figure 6.10). Hanson et al. observed a similar decrease in the PLFA 18:2 ω 6,9 after exposure of soil samples to 100 μ g toluene/mL (12). With this loss of FAMES that are indicative of anaerobes and eukaryotes it is apparent that, a decrease in overall microbial diversity occurred in biphenyl-amended microcosms.

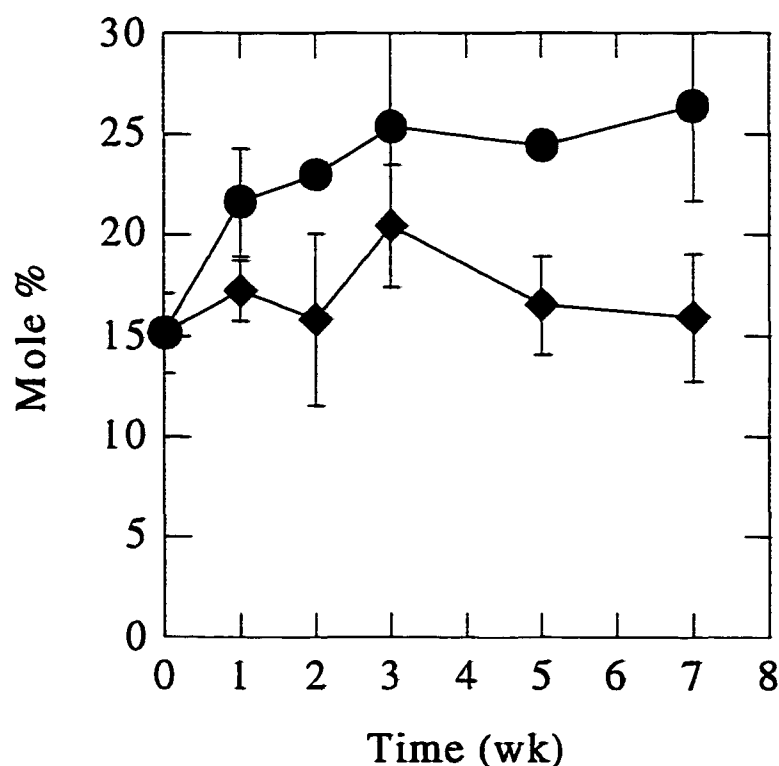


Figure 6.8 Changes in total monounsaturated (16:1 ω 7 + 18:1 ω 7) FAMES in unamended and biphenyl-amended microcosms. ● - unamended microcosms, ◆ - biphenyl-amended microcosms. Detection limit is 0.01 pmol/g for each fatty acid (approximately 0.1%). Error bars represent the standard deviation between duplicate samples.

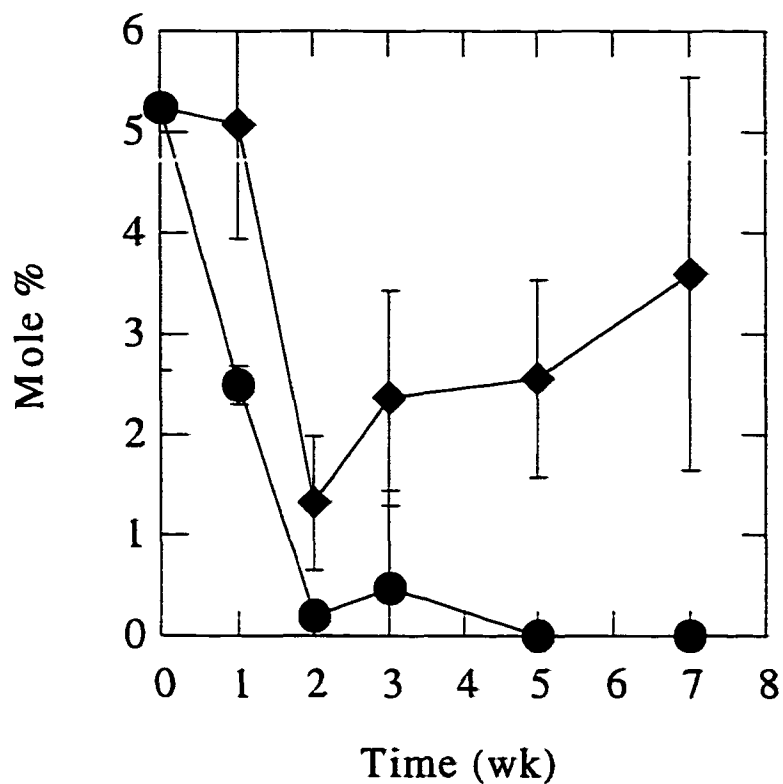


Figure 6.9 Changes in mole % of *i14:0+i15:0* in unamended and biphenyl-amended microcosms. ● - unamended microcosms, ◆ - biphenyl-amended microcosms. Detection limit is 0.01 pmol/g for each fatty acid (approximately 0.1%). Error bars represent the standard deviation between duplicate samples.

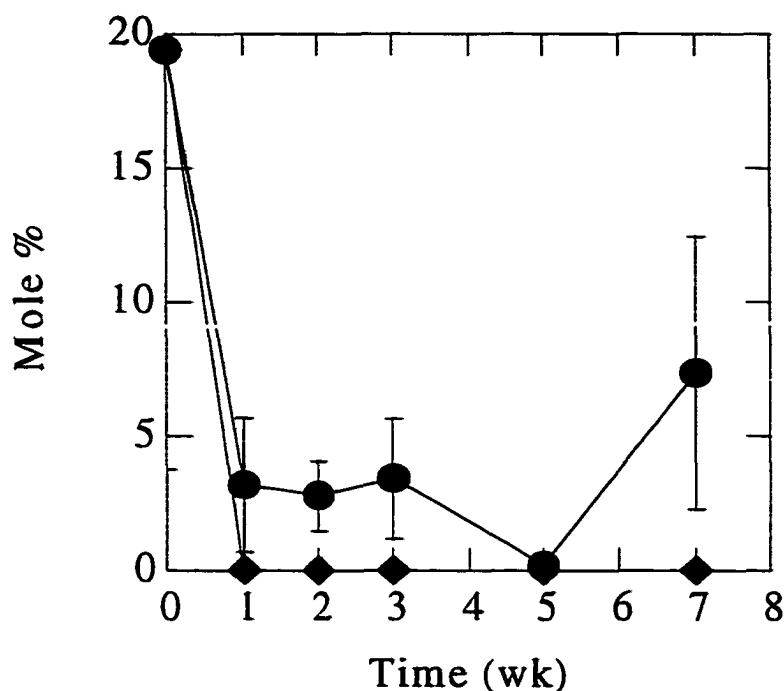


Figure 6.10 Change in mole % of 18:2 ω 6,9 in biphenyl-amended and unamended microcosms. ● - unamended microcosms, ◆ - biphenyl-amended microcosms. Detection limit is 0.01 pmol/g for each fatty acid (approximately 0.1%). Error bars represent the standard deviation between duplicate samples.

In addition to looking at changes in specific FAMES, we also wished to know if samples from the two treatments could be distinguished from each other and from the time zero samples. PCA analysis of FAME profiles from each sample revealed two significant components of variation; 85.4% of the variation between samples was described by the first PC and 8.6% by the second PC. Inclusion of additional principal components increased the variation explained by less than 1%. Principal Components 1 and 2 mapped the majority of samples into one group, the exceptions being samples from time zero and the unamended treatment at week 1 and week 7 (Figure 6.11). Samples with eigenvalues greater than 0.9 for

Principal Component 1 were enriched in 16:0 and 18:1 ω 7, while those with eigenvalues less than 0.85 were enriched in *i*16:0 and 18:2 ω 6. Those samples with eigenvalues less than -0.2 for Principal Component 2 were also enriched in *i*15:0 and *i*14:0. These results agree with the results described above with time zero and unamended samples being more enriched in anaerobic markers (*i*14:0 and *i*15:0) and eucaryotic markers (18:2 ω 6,9) than biphenyl-amended microcosms. Even though a reduction in diversity occurred in biphenyl-amended microcosms, it is interesting to note that unamended samples at week 2, 3, and 5 are more the biphenyl-amended treatments than to the initial or final unamended samples. This indicates that the community composition in the unamended microcosms changed over the course of the experiment but returned somewhat to their original state. This ability has been proposed as an indication that a proposed treatment endpoint has been reached in in-situ bioremediation (17). Because both the anaerobic biomarkers (*i*14:0+*i*15:0) and the eukaryotic biomarker (18:2 ω 6) represented only a small fraction of the overall concentration at time zero, changes in these specific fatty acids had less effect on the PCA analysis clustering.

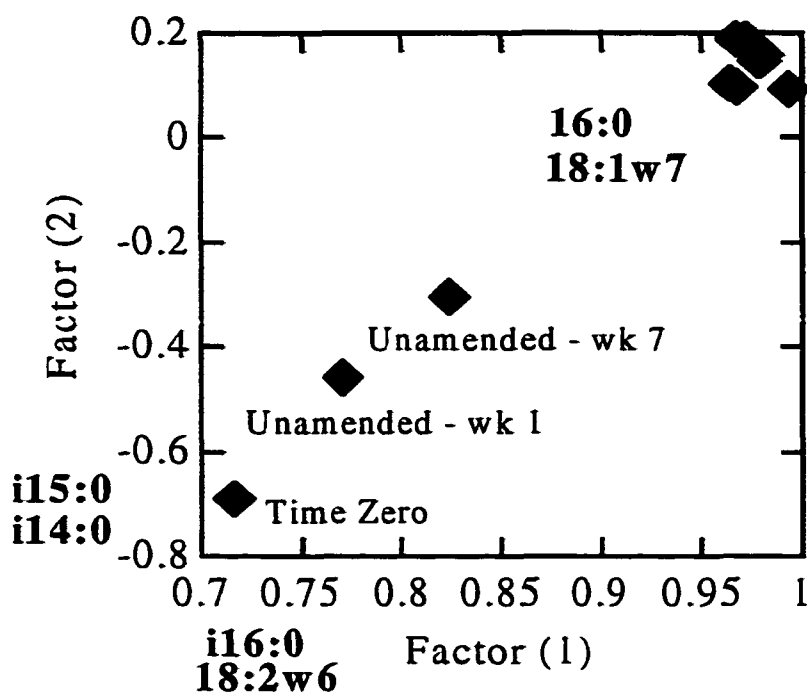


Figure 6.11 Principal component analysis of aerobic fatty acid methyl ester profiles

6.C.3. PCR with Specific Primers

The PLFA/FAME analysis method was able to produce a valuable overall phenotypic description of the microbial community. However, more specific information was desired to describe the changes in specific populations carrying out the degradation of the biphenyl and trichlorobiphenyl. To begin this process, primers specific for the third gene in the biphenyl dioxygenase pathway, 4',chloro-2,3-dihydroxybiphenyl dioxygenase (*bphC*) were used in a polymerase chain reaction procedure to determine the presence or absence of this gene in the microbial population at specific sampling points. The primers chosen were developed by Erb et al. to be specific for microorganisms with *bphC* genes similar to *Pseudomonas* species Q1

(a well-studied biphenyl degrader) but to also amplify the *bphC* gene from nearly all of the known cultured biphenyl degraders. Using this primer set, DNA extracted from biphenyl-amended and unamended samples at time zero and the final time point (wk 7) was used to perform PCR. Figure 6.12 shows an agarose electrophoresis gel with the amplified PCR products from these reactions. In addition to the biphenyl dioxygenase specific primers, universal PCR primers were used to test the quality of the DNA. All samples were amplified using this universal primer set. At time zero, amplification of *bphC* occurred, indicating the presence of this gene in the microbial population. At wk 7 in both the unamended and biphenyl-amended microcosms, no detectable amplification occurred. This is an unexpected result as biphenyl and trichlorobiphenyl degradation occurred in both treatments over the course of the seven weeks; this would be expected to correspond with an increase (or a steady concentration) in the responsible enzyme. One possible explanation is that the microbial community composition shifted with an increase in microorganisms whose *bphC* gene is less homologous to known degraders and thus not detectable with this primer sequence. As seen in the preliminary experiments, the indigenous microorganisms have unique PCB-degrading abilities (ability to cleave at both a 2,3 and a 3,4 site on the biphenyl ring), which may not correspond to those observed to date by other researchers.

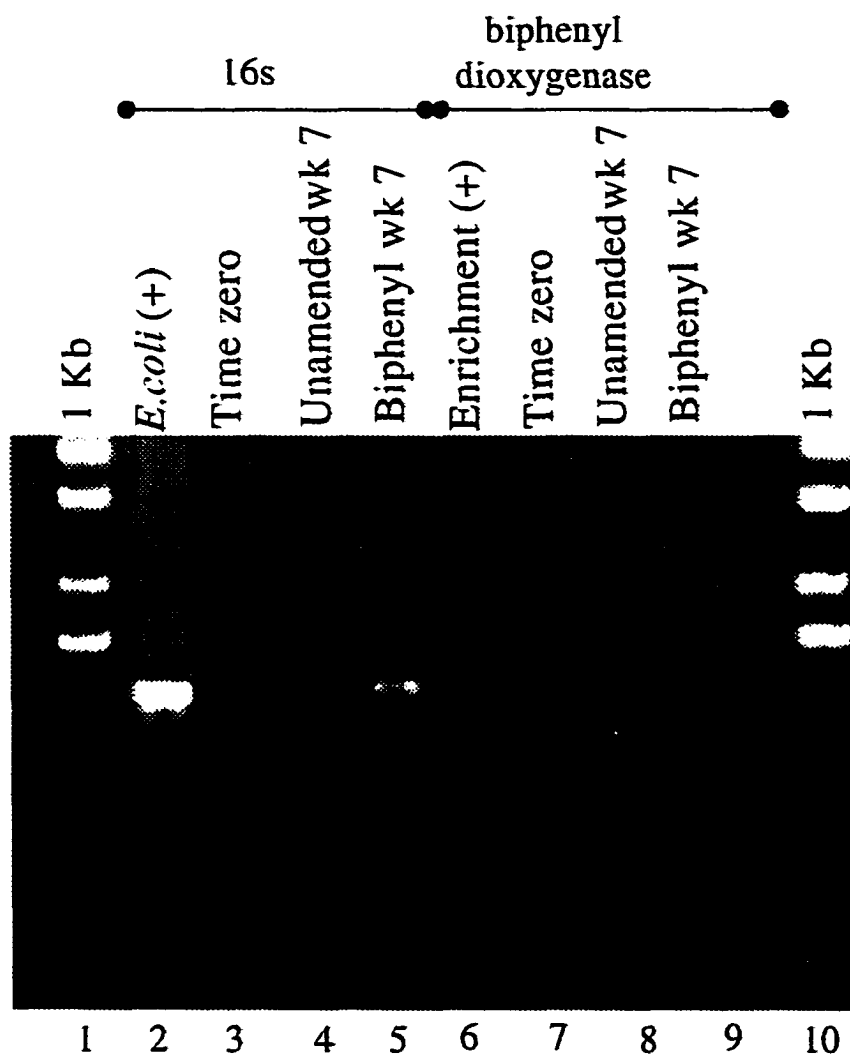


Figure 6.12 Agarose gel electrophoresis of PCR products using general (16S) and specific (*bph*) primers. Lanes one and ten are the 1 Kb marker (Gibco-BRL), Lanes 2-5 were run using universal bacterial primers, Lanes 6-8 were run using a primer specific for 4',chloro-2,3-dihydroxybiphenyl dioxygenase.

6.C.4. Denaturing Gradient Gel Electrophoresis

Since the primers for the commonly followed *bphC* gene were not suitable to chart the course of microbial community change during the aerobic phase, denaturing gradient gel electrophoresis was utilized. This method produced a snapshot of the community diversity in a particular sample using gel electrophoresis of PCR products

amplified from total community DNA. To do this, universal primers specific to all bacteria were utilized. Once PCR had been used to amplify total community DNA, the products from time zero and the final time point (wk 7) samples were compared based on their banding patterns. Figure 6.13 shows this DGGE gel picture. Each band represents a specific microorganism or group of microorganisms with similar DNA sequences in the amplified region. Differences in banding patterns indicate differences in microbial community composition between samples. Samples were run in duplicate in adjacent lanes. Differences can be seen between the time zero samples and the biphenyl and unamended samples at wk 7. Bands 1, 2, 3, 6 and 7 are prominent in time zero and unamended samples while Bands 4 and 5 are prominent in biphenyl-amended samples. Based on similar banding patterns observed in time zero samples used on a DGGE gel with anaerobic samples (Figure 5.16) representative microorganisms of many bands of the aerobic gel could be inferred. Table 6.3 shows the relation between bands on the two gels and the microorganisms with the closest sequence match to the band sequenced.

In complex environmental matrices, each band is expected to contain more than 1 species. To test this, more than one clone was sequenced from several bands; each clone having a different sequence identity. Of the bands sequenced from the aerobic samples, nearly all were representatives of the α - and γ -proteobacteria classes. While only one of the strains matched (Band 2) with a strain observed in PCB-contaminated soil, all of these genera contain at least one known PCB-degrading strain (2, 4). In particular, one of the most well studied strains, *Acinetobacter* P6, is from the same genera as several representatives of the microbial community. In addition, the sequencing of these bands, which can be thought of as the major constituents of the soil microbial community (in

numbers), reinforces the conclusions drawn from the PLFA/FAME analysis. High concentrations of *Pseudomonas*-type species are present and the unamended microcosm samples are more similar to the time zero samples than are the biphenyl-amended samples. Sequencing of the bands not corresponding to bands already sequenced in the anaerobic samples will provide further information about community changes during the aerobic phase.

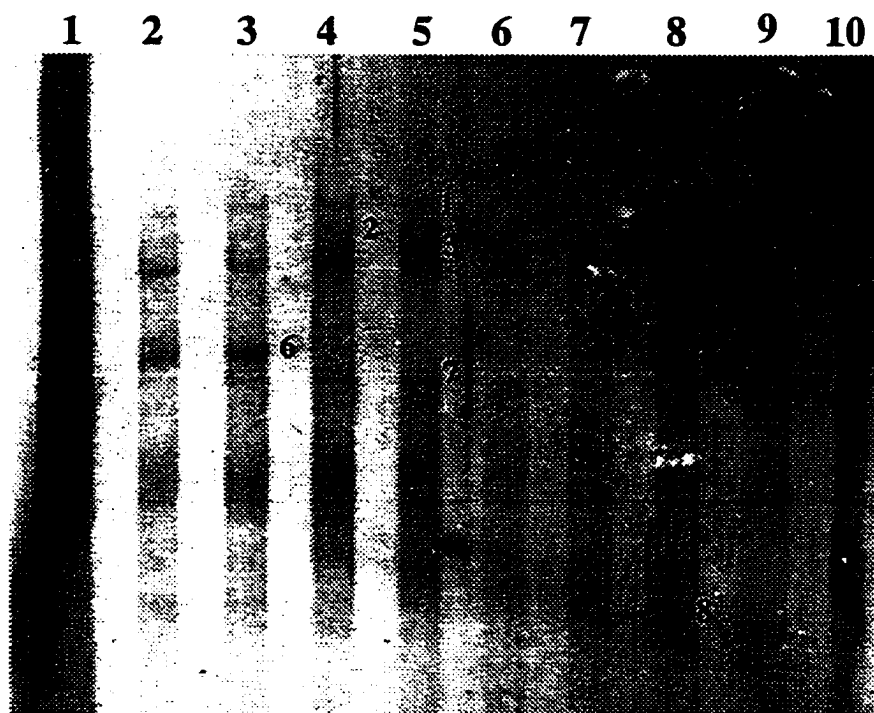


Figure 6.13 Denaturing Gradient Gel Electrophoresis of aerobic samples. A denaturing gradient of 50-80% was utilized. Lane 1, *E. coli*. Lanes 2 and 3, time zero, duplicate 1. Lanes 4 and 5, time zero, duplicate 2. Lanes 6 and 7, unamended treatment at wk 7. Lanes 8 and 9, biphenyl-amended treatment at wk 7. Lane 10, 1 Kb size marker.

Table 6.3 Identity of bands sequenced from aerobic gel

Aerobic band	Anaerobic band (Table 5.3)	Microorganism	% Similarity
1	2	<i>Pseudomonas saccharophila</i>	100%
2	5	Unidentified γ -proteobacterium	96%
3	3	<i>Comamonas acidovorans</i> strain MC1	92%
4	-	-	-
5	-	-	-
6	7	<i>Comamonas</i> sp. D22	100%
		<i>Acinetobacter</i> sp.	100%
7	8	<i>E. coli</i>	100%
8	9	<i>Acinetobacter</i> sp. PIL001	98%

6.D. Conclusions

The aerobic degradation portion of the treatability study investigated the effect of biphenyl amendment on the degradation of the three congener mixture. In contrast to the results of some researchers, biphenyl was not required for the induction of PCB-degrading enzymes. The trichlorobiphenyl, 2,3',4'-PCB, was degraded in both unamended and biphenyl-amended microcosms. However, 2,3',4'-PCB was degraded slightly faster in biphenyl-amended microcosms, indicating that biphenyl amendment either enriched for microorganisms with superior degradative capabilities or was a better inducer of the PCB-degrading enzyme system than was the trichlorobiphenyl. No degradation of either the 3,3',4,4'- or 2,2',4,4',5,5'-PCB was observed in any treatment.

Fatty acid methyl ester profiles, used to describe changes in overall microbial community structure, indicated a significant change in the microbial population in biphenyl-amended microcosms. A decrease in markers for anaerobic microorganisms and eucaryotes and an increase in markers representative of aerobic microorganisms was observed in biphenyl-amended microcosms. No overall change in total biomass levels in either treatment was observed, contradicting the apparent decrease in microbial concentration observed using plate counts. DGGE analysis confirmed the presence of aerobic microorganisms, with most organisms clustering within the α - and γ -proteobacteria classes. These groups of organisms contain many well studied biphenyl degraders. Differences in banding patterns were observed between biphenyl-amended and the unamended and time zero samples, confirming the PLFA/FAME analysis that biphenyl amendment induced changes in microbial community composition.

6.E. References

1. **Abramowicz, D. A.** 1990. Aerobic PCB biodegradation and anaerobic PCB dechlorination in the environment.
2. **Asturias, J. A., E. Moore, M. Yakmov, S. Klatte, and K. N. Timmis.** 1994. Reclassification of the polychlorinated biphenyl-degraders *Acinetobacter* sp. strain P6 and *Corynebacterium* sp. strain MB1 as *Rhodococcus gloverulus*. Systemic Applied Microbiology. 17:226-231.
3. **Bedard, D. L., R. E. Wagner, M. J. Brennan, M. L. Haberl, and J. F. Brown.** 1987. Extensive degradation of Aroclors and environmentally transformed

- polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Applied and Environmental Microbiology. 53:1094-1102.
4. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. Journal of Industrial Microbiology. 1:23-29.
 5. **Brunner, W., F. H. Sutherland, and D. D. Focht.** 1985. Enhanced biodegradation of polychlorinated biphenyls in soil by analog enrichment and bacterial inoculation. Journal of Environmental Quality. 14:324-328.
 6. **Fairbanks, B. C., G. A. O'Connor, and S. E. Smith.** 1987. Mineralization and volatilization of polychlorinated biphenyls in sludge-amended soils. Journal of Environmental Quality. 16:18-25.
 7. **Focht, D. D., and W. Brunner.** 1985. Kinetics of biphenyl and polychlorinated biphenyl metabolism in soil. Applied and Environmental Microbiology. 50:1058-1063.
 8. **Franzmann, P. D., B. M. Patterson, T. R. Power, P. D. Nichols, and G. B. Davis.** 1996. Microbial biomass in a shallow, urban aquifer contaminated with aromatic hydrocarbons: analysis by phospholipid fatty acid content and composition. Journal of Applied Bacteriology. 80:617-625.
 9. **Ghiorse, W. C., and J. T. Wilson.** 1988. Microbial ecology of the terrestrial subsurface. Advances in Applied Microbiology. 33:107-172.
 10. **Gibson, D. T., D. L. Cruden, J. D. Haddock, G. J. Zylstra, and J. M. Brand.** 1993. Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. LB400 and *Pseudomonas pseudoalcaligenes* KF707. Journal of Bacteriology. 175:4561-4564.

11. **Haack, S. K., H. Garchow, D. A. Odelson, L. J. Forney, and M. J. Klug.** 1994. Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. *Applied and Environmental Microbiology*. **60**:2483-2493.
12. **Hanson, J. R., J. L. Macalady, D. Harris, and K. M. Scow.** 1999. Linking toluene degradation with specific microbial populations in soil. *Applied and Environmental Microbiology*. **65**:5403-5408.
13. **Kieft, T. L., D. B. Ringelberg, and D. C. White.** 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium. *Applied and Environmental Microbiology*. **60**:3292-3299.
14. **Langworthy, D. E., R. D. Stapleton, G. S. Sayler, and R. H. Findlay.** 1998. Genotypic and phenotypic responses of a riverine microbial community to polycyclic aromatic hydrocarbon contamination. *Applied and Environmental Microbiology*. **64**:3422-3428.
15. **Maeda, M., S.-Y. Chung, E. Song, and T. Kudo.** 1995. Multiple genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase in the gram-positive polychlorinated biphenyl-degrading bacterium *Rhodococcus erythropolis* TA421, isolated from a termite ecosystem. *Applied and Environmental Microbiology*. **61**:549-555.
16. **Phelps, T. J., D. Ringelberg, D. Hedrick, J. Davis, C. B. Fliermans, and D. C. White.** 1988. Microbial biomass and activities associated with subsurface environments contaminated with chlorinated hydrocarbons. *Geomicrobiology Journal*. **6**:157-170.

17. **White, D. C., and D. B. Ringelberg.** 1998. Signature lipid biomarker analysis, p. 255-272. *In* R. S. Burlage, R. Atlas, d. Stahl, G. Geesey, and G. Sayler (ed.), *Techniques in microbial ecology*, vol. 1. Oxford University Press, New York.
18. **Williams, W. A., J. H. Lobos, and W. E. Cheetham.** 1997. A phylogenetic analysis of aerobic polychlorinated biphenyl-degrading bacteria. *International Journal of Systematic Bacteriology*. **47**:207-210.

CHAPTER 7

TRANSITION TO AEROBIC CONDITIONS

7.A. Introduction

To develop a successful sequential treatment strategy, a method for switching from anaerobic to aerobic conditions must be used that provides enough oxygen for aerobic degradation of the remaining PCBs without inhibiting the growth of the microorganisms carrying out the degradation. Providing sufficient oxygen for bioremediation is often a challenge because O_2 is sparingly soluble in water (approximately 10 mg/L under 1 atm air and approximately 40 mg/L under 1 atm O_2) (1). On the other hand, hydrogen peroxide is very soluble in water and is relatively easy to deliver to a variety of matrices. It is, however, toxic to microbial cells above certain levels. Hydrogen peroxide is a powerful oxidizing agent that can destroy cell constituents very rapidly. Pardieck et al. found that H_2O_2 was toxic to subsurface microorganisms at levels as low as 0.003% (5). The experiments described in this chapter investigated methods for initiation of aerobic conditions in anaerobic microcosms using air or hydrogen peroxide as an oxygenation agent. Both the viability and ability of the indigenous organisms to degrade PCBs after oxygenation was evaluated.

7.B. PCB Degradation

Before the switch from anaerobic to aerobic conditions was made in the full treatability study, a set of experiments investigating the use of either air or hydrogen peroxide for oxygenation was performed. A set of microcosms was set-up under anaerobic conditions and allowed to remain under those conditions for one week. The microcosms were then dosed with either air or one of 3 levels of hydrogen peroxide, 0.1, 0.25, or 0.5%. To eliminate the large time period required to achieve anaerobic conditions (as described in Chapter 5), microcosms were sparged with an $\text{H}_2:\text{CO}_2:\text{N}_2$ gas mixture. The presence of reducing conditions was confirmed by using the redox indicator resazurin. After sparging, the medium changed from purple to a pink color, indicating slightly reducing conditions. These conditions were expected to simulate those encountered in the anaerobic phase. The degradation of trichlorobiphenyl and any changes in the microbial community concentration were followed over the course of 7 wk. This time was chosen to match that of the aerobic microcosm experiments (Chapter 6).

Figure 7.1 shows the degradation of 2,3',4'-PCB in soil microcosms in the 7 wk of aerobic conditions following a week under anaerobic conditions. No degradation of the trichlorobiphenyl was observed during the week under anaerobic conditions. Degradation occurred in all treatments after the switch from anaerobic to aerobic conditions, and occurred at nearly the same rate and extent in each. While degradation occurred in each microcosm, the extent of degradation in the 7 wk time period was less than that observed in the aerobic microcosms alone (Figure 6.1). In Figure 6.1, total degradation of 2,3',4-trichlorobiphenyl was observed by week 3. This decrease in the

rate of degradation could be due either to the week under anaerobic conditions, to the effect of each of the oxygenation treatments, or to a combination of the two. However, since nearly the same rate was observed for all treatments, it is reasonable to think that the decrease in degradation rate compared to those previously observed would be due to a factor encountered by all microcosms. Thus, the week under anaerobic conditions may have been the cause of the decrease in degradation rates. Under the conditions encountered by the microorganisms in the anaerobic phase experiments described in Chapter 5, no overall loss of microorganisms or aerobic biomarkers was observed over the course of the experiment. However, a loss of biphenyl-degrading organisms may have occurred both during the anaerobic phase in Chapter 5 experiments and in the week under anaerobic conditions used in this study.

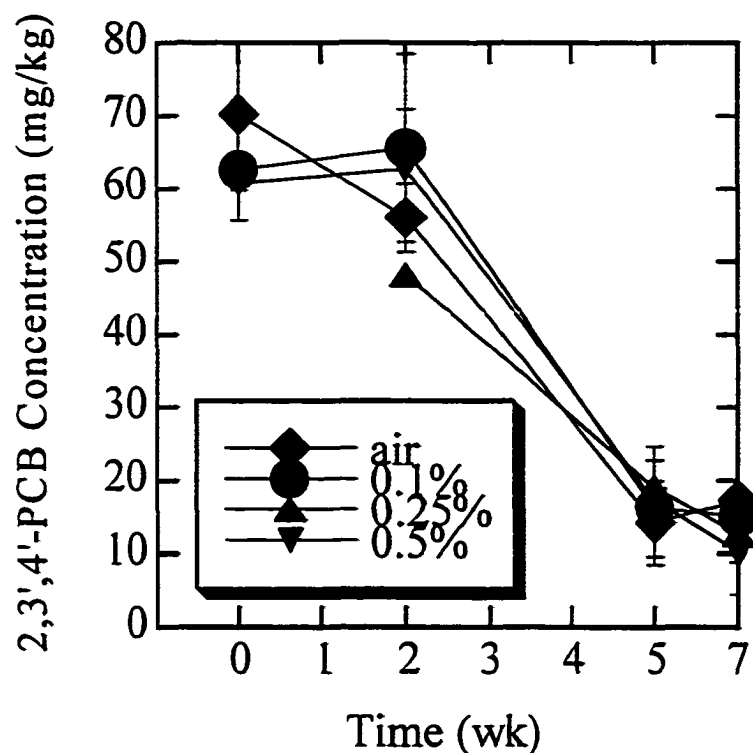


Figure 7.1 Degradation of 2,3',4'-PCB in oxygenation experiment microcosms. Error bars represent the standard deviation between duplicate samples.

Dissolved oxygen was monitored in the microcosms over the course of the seven weeks (Figure 7.2). No DO concentration values were obtained at wk 5 because the DO probe was being repaired at this time point. Addition of hydrogen peroxide did not provide the theoretical amount of dissolved oxygen for each level (47 mg/L, 117 mg/L, 235 mg/L). However, initial DO concentration trends were consistent with the levels added. Degradation of the hydrogen peroxide may have occurred in the soil, reducing the amount of oxygen available for use by the microorganisms. Anid et al. observed initial

oxygen levels proportional to added concentration of H_2O_2 but, in agreement with this work, lower than theoretical amounts (1).

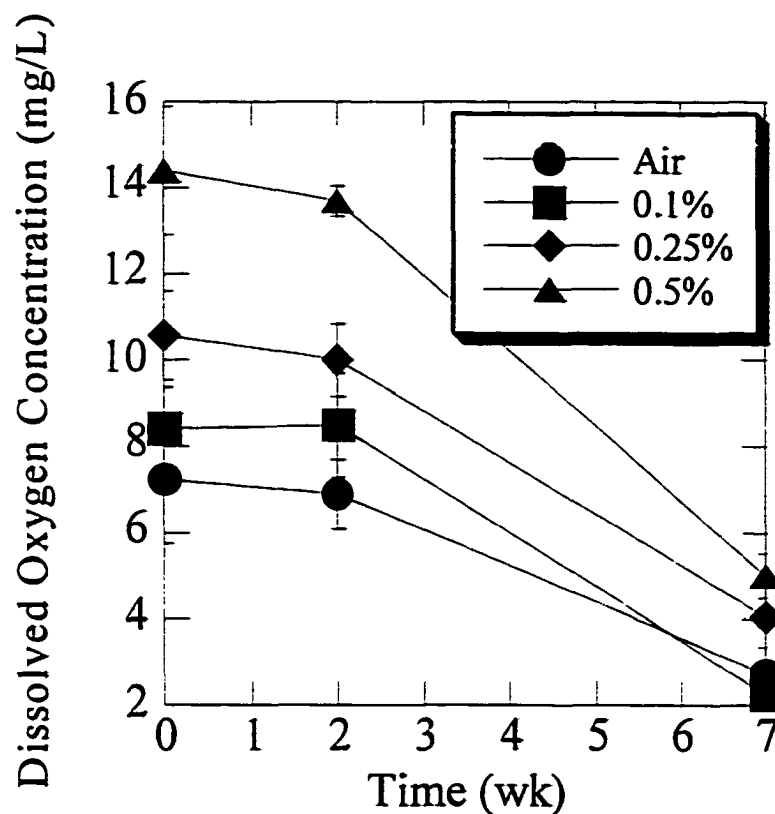


Figure 7.2 Dissolved oxygen concentration levels in microcosms over the 7 wk degradation period. Error bars represent the standard deviation between duplicate samples.

A decrease in dissolved oxygen levels was observed in all treatments and this decrease mirrored the timing and rates observed in the degradation of 2,3',4'-PCB (Figure 7.1). A 2-wk lag period was observed in both PCB degradation and DO utilization. In agreement with the PCB concentration results, little dependence of the final extent of DO utilization on hydrogen

peroxide concentration was observed. It is possible that within the range of H_2O_2 concentrations used for this work, the microorganisms experienced little difference in toxicity or enhancement between the different treatments. Some differences in rates of DO utilization were apparent. The air-amended treatment appeared to have a slightly slower rate of DO utilization than the other treatments and the 0.5% H_2O_2 addition appeared to have a higher rate of utilization. Anid et al. also found that rates of oxygen utilization were proportional to the added amount of hydrogen peroxide, although the range of hydrogen peroxide concentrations (0.0171% - 0.171%) utilized in their work was lower than that utilized in the present work. Few studies have reported such high tolerance for H_2O_2 concentrations over 0.1% (5). Two factors may have contributed to the observed tolerance of the indigenous microorganisms. First, the degradation of the H_2O_2 by soil components decreased the concentration of the H_2O_2 “seen” by the microbial population. Specifically, high concentrations of iron, nickel, and copper can catalyze this decomposition. Second, the indigenous organisms may already have efficient catalase enzymes to deal with hydrogen peroxide toxicity.

7.C. Intermediate Investigation

The production of intermediates expected from the aerobic degradation of PCBs, i.e., chlorobenzoic acids, was investigated using HPLC. The aqueous phase of the soil slurry microcosms were extracted with ethyl acetate and the dried samples run on the HPLC. Several intermediates were detected in all of the samples, some of which could be identified on the basis of retention time to be chlorobenzoic acids. Most microorganisms possess a biphenyl dioxygenase that cleaves the biphenyl molecule at a vacant 2,3-site (2, 3). As discussed in the preliminary results, a few microorganisms have been shown to possess a biphenyl dioxygenase

enzyme able to cleave at vacant 3,4-sites. Figure 7.3 shows the expected products from both types of cleavage of the biphenyl ring. Preliminary results indicated the presence of both of these types of biphenyl dioxygenase enzymes, so both 2- and 3,4-chlorobenzoic acids were specifically investigated. Both types of cleavage produce 3,4-dichlorobenzoic acid from the aerobic degradation of 2,3',4'-PCB while only 2,3-cleavage produces 2-chlorobenzoic acid.

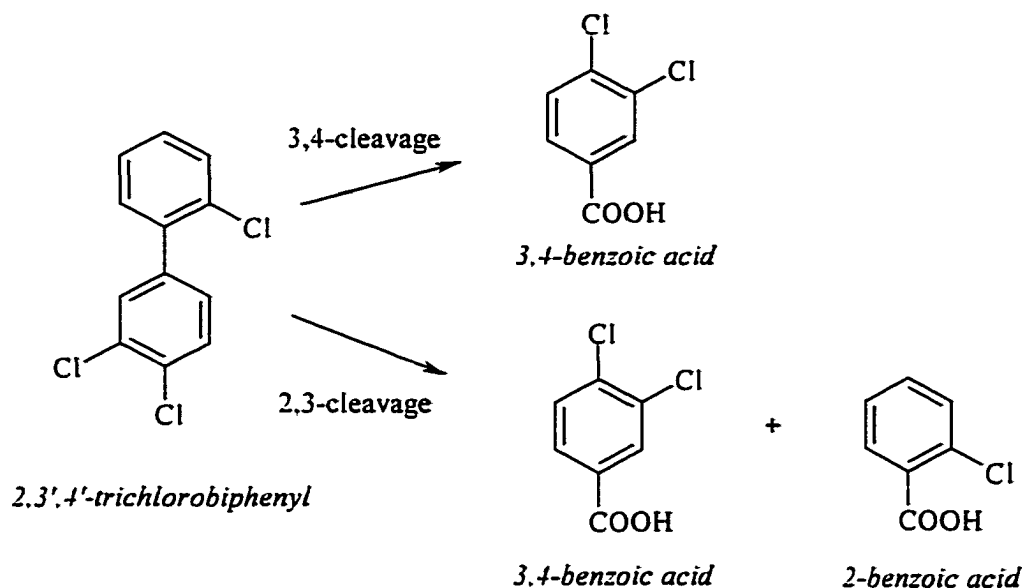


Figure 7.3 Degradation pathway of PCBs to chlorobenzoic acids

Both 2- and 3,4-chlorobenzoic acids were detected in all treatments. The production of these two compounds occurred at wk 2 in the 0.5% H₂O₂ amendment, and at wk 5 in all other treatments. These compounds increased and then decreased at the end of the 7-wk in all treatments except the 2-chlorobenzoic acid in 0.1% H₂O₂ treatments that did not decrease (Figure 7.4 and 7.5). Hydrogen peroxide treatments with 0.25% and 0.5% amendment produced the highest concentrations of both chlorobenzoic acids. Different oxygenation treatments induced the production of different ratios of the two compounds. Unamended and

0.1% H₂O₂-amended treatments produced higher concentrations of 3,4-dichlorobenzoic acid than 2-CBA; the opposite was true of 0.25% and 0.5% H₂O₂-amended treatments. Since both compounds were detected, we cannot determine if both cleavage mechanisms are acting or if cleavage is occurring solely at the 2,3 site. In addition, the decrease in these concentrations near the end of the experiment indicates that chlorobenzoic acid utilizing microorganisms are present in the microbial community. Several of the genotypes isolated using DGGE and sequence analysis (Chapter 5) contain strains able to utilize chlorobenzoic acids including *Comamonas* and *Pseudomonas* species (6). In agreement with this work, Flanagan and May observed chlorobenzoic acid metabolites at low concentrations and only transiently. Of these two compounds, 2-chlorobenzoic acid is expected to be a less effective inhibitor of PCB degradation and 3,4-dichlorobenzoic acid is expected to be a very strong inhibitor of PCB degradation (4). This is hypothesized to be due to inhibition by metabolites of 3,4-chlorobenzoic acid degradation (chlorocatechol, carboxy-1,2-benzoquinone).

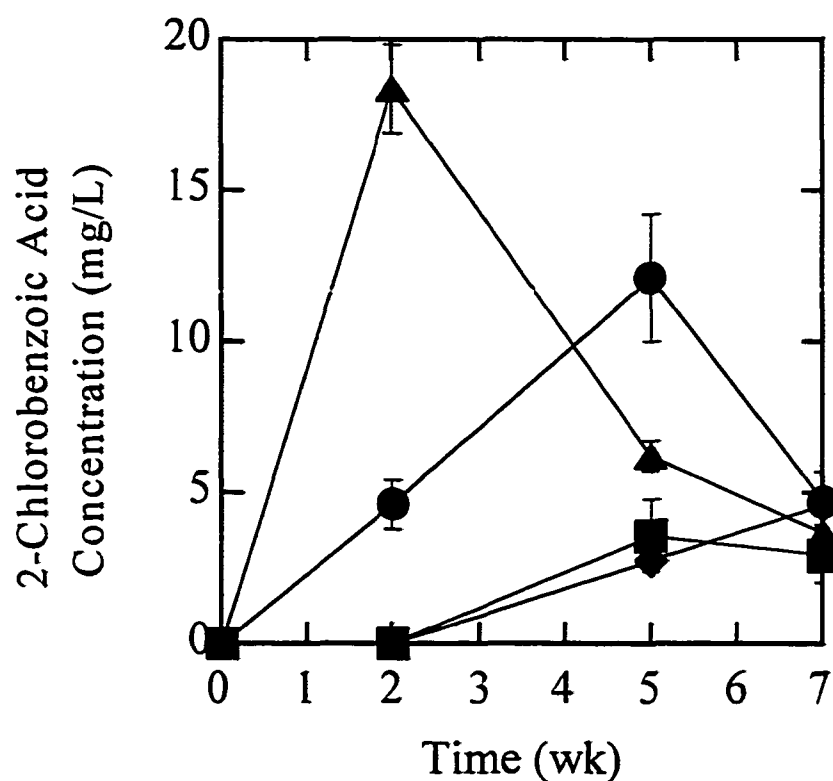


Figure 7.4 Detection of 2-dichlorobenzoic acid in oxygenation experiment microcosms. ■ - air amendment, ◆ - 0.1% H₂O₂ amendment, ● - 0.25% H₂O₂ amendment, and ▲ - 0.5% H₂O₂ amendment. Error bars represent the standard deviation between duplicate samples.

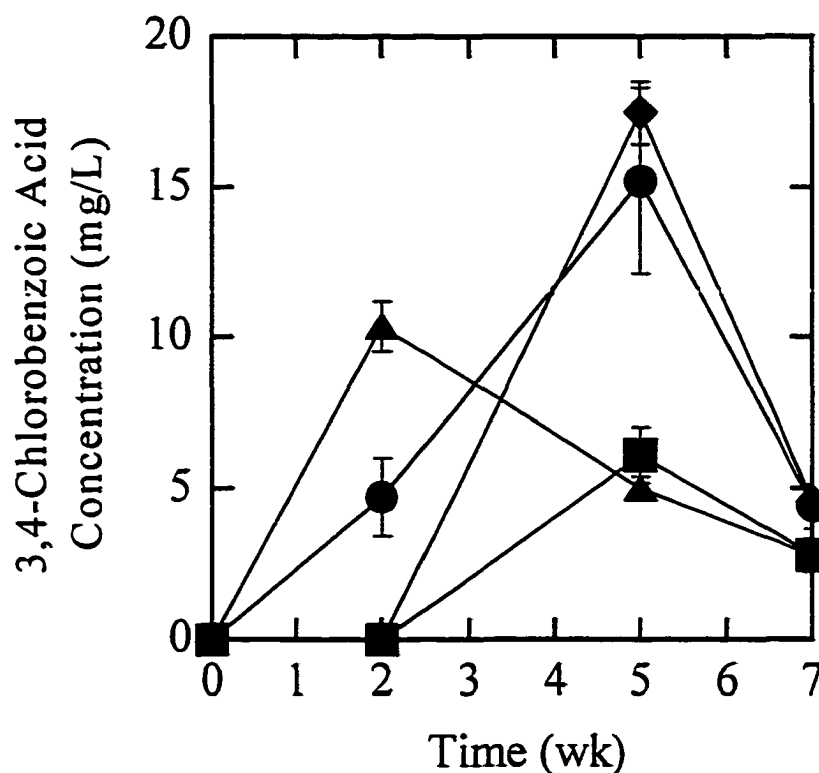


Figure 7.5 Detection of 3,4-dichlorobenzoic acid in oxygenation experiment microcosms. ■ - air amendment, ◆ - 0.1% H₂O₂ amendment, ▲ - 0.25% H₂O₂ amendment, and ● - 0.5% H₂O₂ amendment. Error bars represent the standard deviation between duplicate samples.

Although chlorobenzoic acids were produced by this degradation process, their concentrations never reached above 25 mg/L for both intermediates. The theoretical amount of 2-chlorobenzoic acid production for the amount of PCB degradation observed is approximately 150 mg/L. Thus, the concentration of these compounds could not fully account for the amount of 2,3',4'-PCB degradation observed. Several other metabolites were also observed in the HPLC analysis. These metabolites differed between air treated microcosms and hydrogen peroxide treated microcosms. In each case, a relatively large peak was produced and then

partially degraded within the 7 wk time period. The peak detected in air-treated microcosms was roughly twice the area of both the chlorobenzoic acids detected and the peak detected in hydrogen peroxide-amended treatments. The maximum peak area occurred at wk 5, this was the same time that the CBA concentrations reached a maximum in air-treated microcosms. To attempt to identify the peaks observed, fractions containing the two peaks (one from air treated microcosms and one from hydrogen peroxide treated microcosms) were collected and concentrated. These fractions were then run using an electrospray method. This method produces values for possible molecular ions present in the sample. If a mixture of compounds is present, then the molecular ion for each will be detected in the analysis. Several possible molecular ions (110, 176, 216, and 370) were present in samples from the peak fraction from air-treated samples. For three of the four possible molecular ions, no positive identification could be made, but the presence of these compounds may have been the cause of the inhibition of PCB degradation as compared to the aerobic treatment alone. Catechol has a molecular ion of 110 and is a known degradation product of an oxygenolytic dehalogenation which occurs during attack of 2-chlorobenzoate by a 2-CBA-specific 1,2-benzoate dioxygenase (4). The peak with a mass of 110 was the dominant peak in the electrospray analysis. To confirm that the peak was indeed catechol, a standard was prepared and run on the HPLC. The retention time matched with the unidentified peak, thus the major peak in air treated microcosms is likely catechol. Electrospray analysis of the peak fraction from the hydrogen peroxide-treated sample provided no information on molecular ions as the concentration was too low for detection.

7.D. PLFA/FAME analysis

The PLFA/FAME analysis method was again used to describe changes in microbial community concentration throughout the course of the oxygenation experiments. The same FAMES were utilized as with the anaerobic and aerobic phases (Table 5.1). In contrast to the results of the aerobic phase experiments, few changes were observed in the mole percent of specific fatty acids of the microbial community. A reduction in anaerobic biomarkers (i14:0+i15:0+i16:0) of approximately 50% was observed in all treatments after the switch from anaerobic to aerobic conditions at time zero. However, during the length of the aerobic period, no further decrease in anaerobic markers was observed (Figure 7.6). Also, no increase in the mole percent of aerobic biomarkers was observed with any of the treatments.

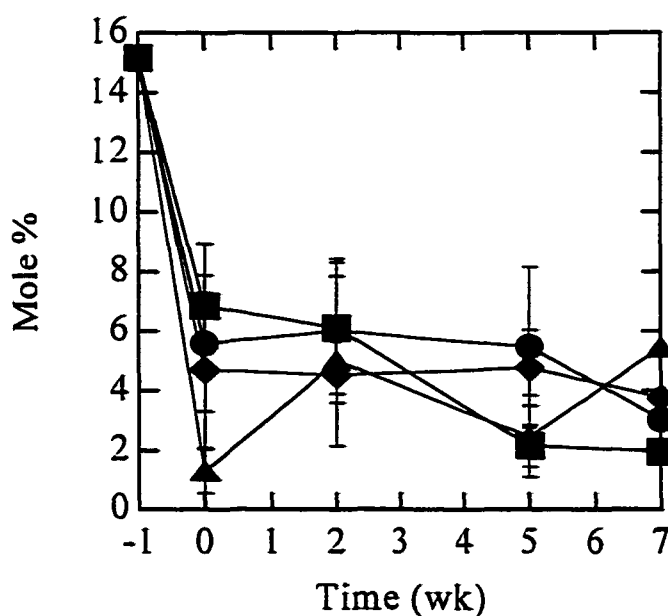


Figure 7.6 Change in mole percent of anaerobic biomarkers (i14:0+i15:0+i16:0). Conditions were switched from anaerobic to aerobic at time zero. ■ - air amendment, ◆ - 0.1% H₂O₂ amendment, ▲ - 0.25% H₂O₂ amendment, and ● - 0.5% H₂O₂ amendment. Error bars represent the standard deviation between duplicate samples.

In addition to the changes in anaerobic biomarkers, significant changes in total lipid content were observed. Figure 7.7 shows the change in total biomass for each of the four oxygenation treatments. As with the degradation of 2,3',4'-PCB in these samples, the change in PLFA content followed the same trend for each sample with one notable exception in the air-treated microcosms. There was an initial significant decrease in the total biomass concentration of each of the samples after dosing with either air or hydrogen peroxide. The concentration of total organisms leveled off by wk 5 and then decreased again by wk 7. The first decrease is likely due to an inhibitory effect of the hydrogen peroxide or to the loss of anaerobic microorganisms without the rebound of aerobic microorganisms that had been harmed by the initiation of anaerobic conditions. The increase observed in the total biomass content in air-treated microcosms is a well-described phenomenon due solely to the disturbance of the microcosms by the air sparging and is probably not due to any significant increase in the microbial population. However, after this initial disturbance, the levels of total biomass in the soil in air-treated microcosms also decreased in a manner similar to the other treatments. This indicates that the decrease in microorganisms may be due to a loss of the anaerobic population. The second decrease could also be caused by the build-up of inhibitory degradation products near the end of the experiment. Interestingly, no such phenomenon was observed in the aerobic phase only treatment.

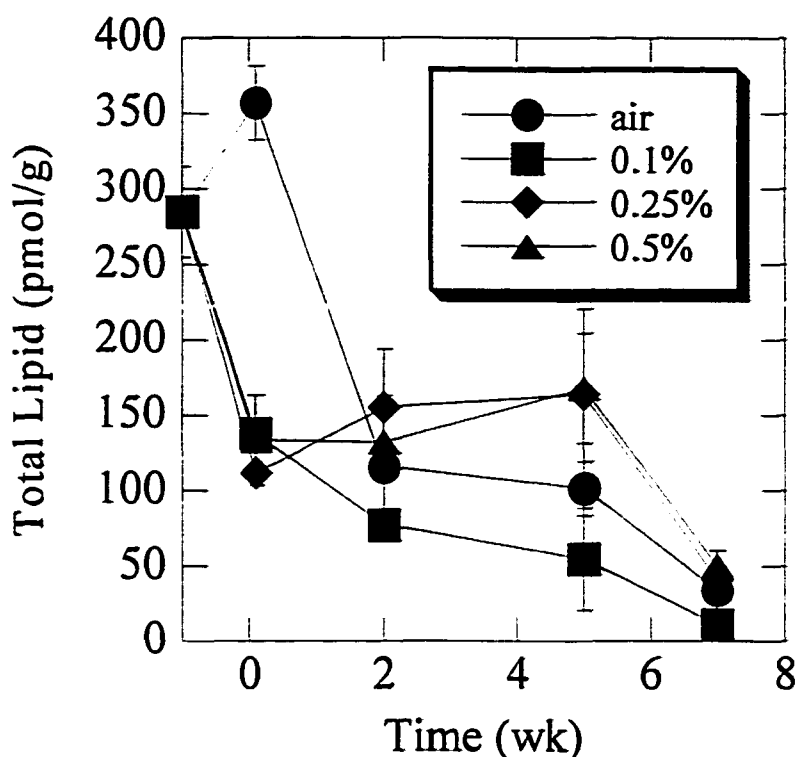


Figure 7.7 Change in total biomass content of oxygenation experiment microcosms. Error bars represent the standard deviation between duplicate samples.

7.E. Sterile Controls

To ensure that the observed degradation was not caused by an abiotic reaction with the trichlorobiphenyl, sterile control experiments were performed. The same four treatments were investigated as described previously. Microcosms were autoclaved on three consecutive days and then amended with 50 mg/kg 2,3',4'-PCB. Figure 7.8 shows the results of this sterile control experiment. No significant losses of 2,3',4'-PCB were observed in the sterile control. The DO concentration was also monitored in the sterile control microcosms. Decreases in DO concentrations of less than 5% occurred over the 7 wk experimental

period, indicating that oxygen utilization in the oxygenation experiment microcosms was due primarily to biotic processes.

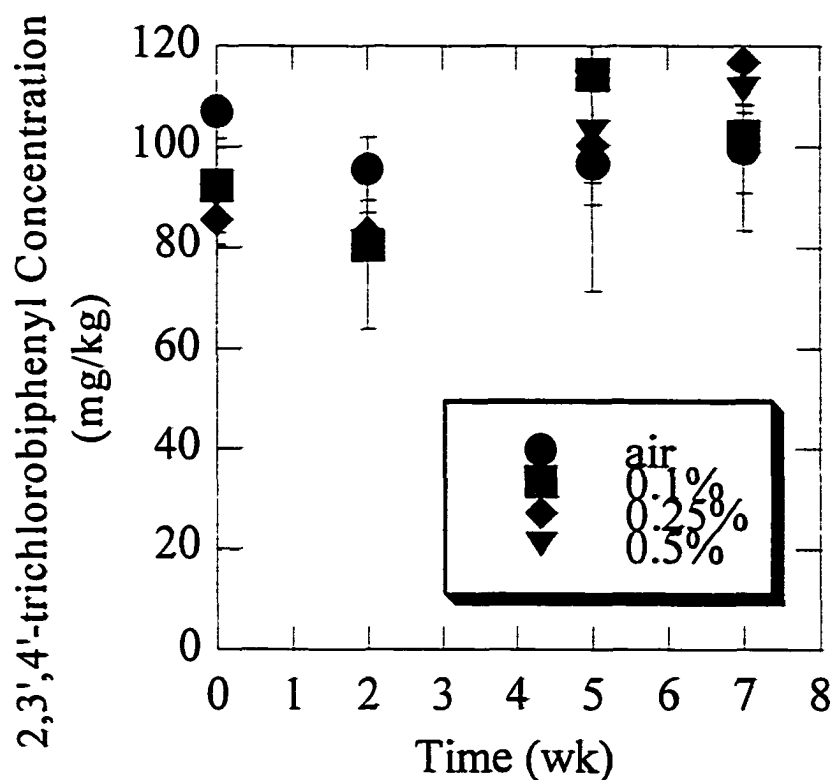


Figure 7.8 Degradation of 2,3',4'-trichlorobiphenyl in sterile control experiments. Error bars represent the standard deviation between duplicate samples.

7.F. Conclusions

While extensive 2,3',4'-trichlorobiphenyl degradation occurred in air-amended and hydrogen peroxide-amended treatments, the rate and extent of this degradation was less than that observed in the aerobic treatments alone. No significant differences were observed in

degradation rate and extent or in total biomass levels between the four treatments. While the only difference in community composition that could be determined using the lipid analysis was a loss of anaerobic microorganisms, changes in community composition towards those organisms with more effective means of dealing with hydrogen peroxide toxicity but less efficient at degrading PCBs could have occurred. Small differences in DO utilization were observed with higher rates of utilization observed with higher levels of hydrogen peroxide amendment. The fact that these differences in rates did not correspond to differences in PCB degradation may indicate that oxygen is not limiting in this system.

Chlorobenzoic acids, the metabolic by-products of PCB degradation, were observed with both the 2- and 3,4-chlorobenzoic acids detected in all treatments but not at stoichiometric concentrations. A decrease in these compounds by the end of the experimental period indicated the presence of microorganisms able to utilize chlorobenzoic acids. However, other unidentified metabolites were detected that differed between air- and hydrogen peroxide-amended treatments. A metabolite of the degradation of 2-chlorobenzoic acid, catechol, was observed in air-treated microcosms only. Even though differences in metabolic by-products were observed, the overall rate of degradation of 2,3',4'-trichlorobiphenyl was the same between treatments. This and the fact that non-stoichiometric amounts of chlorobenzoic acids were produced indicates that another pathway for aerobic PCB degradation may be utilized by the microorganisms in this system. It seems likely that the decrease in rate and extent of degradation were due to both the initiation of anaerobic conditions prior to oxygenation and the build-up of inhibitory metabolic intermediates.

7.G. References

1. **Anid, P. J., B. P. Ravest-Webster, and T. M. Vogel.** 1993. Effect of hydrogen peroxide on the biodegradation of PCBs in anaerobically dechlorinated river sediments. *Biodegradation*. 4:241-248.
2. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. *Journal of Industrial Microbiology*. 1:23-29.
3. **Boyle, A. W., C. J. Silvin, J. P. Hassett, J. P. Nakas, and S. W. Tanenbaum.** 1992. Bacterial PCB biodegradation. *Biodegradation*. 3:285-298.
4. **Guilbeault, B., M. Sondossi, D. Ahmad, and M. Sylvestre.** 1994. Factors affecting the enhancement of PCB degradative ability of soil microbial populations. *International Biodeterioration and Biodegradation*. 33:73-91.
5. **Pardieck, D. L., E. J. Bower, and A. T. Stone.** 1992. Hydrogen peroxide use to increase oxidant capacity for in situ bioremediation of contaminated soils and aquifers. *Journal of Contaminant Hydrology*. 9:243-269.
6. **Peel, M. C., and R. C. Wyndham.** 1999. Selection of *clc*, *cba*, and *fc**b* chlorobenzoate-catabolic genotypes from groundwater and surface waters adjacent to the Hyde Park, Niagra Falls, chemical landfill. *Applied and Environmental Microbiology*. 65:1627-1635.

CHAPTER 8

SEQUENTIAL ANAEROBIC/AEROBIC TREATMENT

8.A. Introduction

PCB contamination nearly always contains a mixture of both highly and less chlorinated congeners. As shown in this work and the work of others, anaerobic dechlorination removes chlorine substituents, but primarily from highly chlorinated PCBs (e.g., 2,2',4,4',5,5'-hexachlorobiphenyl). Conversely, aerobic degradation of PCBs transforms primarily those congeners with low chlorine number (e.g., 2,3',4'-trichlorobiphenyl). The indigenous organisms in this work were unable to aerobically degrade congeners with more than three chlorines. Thus, a combination of anaerobic and aerobic treatment appeared to be a promising solution to the problems encountered with each treatment phase alone. Previous chapters (Chapters 5 and 6) report on PCB degradation in each treatment phase alone. This chapter presents experimental results from a sequential treatment process that was designed and evaluated using results from previous chapters.

The sequential experimental procedure consisted of a 60-wk anaerobic phase, addition of 0.25% H₂O₂ to aerate the microcosms, and a 7-wk aerobic phase. For the anaerobic portion of the sequential treatment, the same electron donor treatments were utilized as described in Chapter 5: no exogenous donor, methanol amendment and butyrate amendment. In addition to these treatments, three additional treatment options

were utilized. These involved addition of biphenyl, butyrate +biphenyl, or methanol + biphenyl at the beginning of the anaerobic phase. These treatments were intended to complete the statistical design described in Chapter 3 (Table 3.1). The biphenyl was expected to be degraded only in the aerobic phase; this was not the case and the influence of the biphenyl degradation on both the anaerobic and aerobic phase will be discussed further in Sections 8.B and 8.C. Corresponding sterile controls for each treatment were also investigated. Of particular interest in the sequential treatment process was the effect that the anaerobic phase and oxygenation treatments had on the aerobic microorganisms required for the aerobic portion of the treatment.

8.B. Anaerobic Phase

8.B.1. PCB Concentration

Duplicate microcosms were sacrificed for sampling at 0, 4, 12, 22, 30, and 60 wk. The concentration of the dosed hexachlorobiphenyl (2,2',4,4',5,5') over the 60-wk anaerobic period is shown in Figures 8.1 and 8.2. Significant dechlorination occurred (20%-33%) in all of the microcosms. Nearly the same rate and extent of dechlorination were observed for the unamended, butyrate- and methanol-amended treatments as were observed in the anaerobic-only study (Figure 8.1). The rate of dechlorination of 2,2',4,4',5,5'-PCB in the biphenyl-amended, butyrate+biphenyl amended and methanol+biphenyl amended microcosms is shown in Figure 8.2. Biphenyl-amended microcosms displayed the largest amount of dechlorination of all treatments, with roughly 33% dechlorination observed. This amount of dechlorination was significantly higher than all other treatments at a significance level of $p=0.05$. Those microcosms

amended with biphenyl plus either methanol or butyrate did not display the same extent of dechlorination as biphenyl-only microcosms.

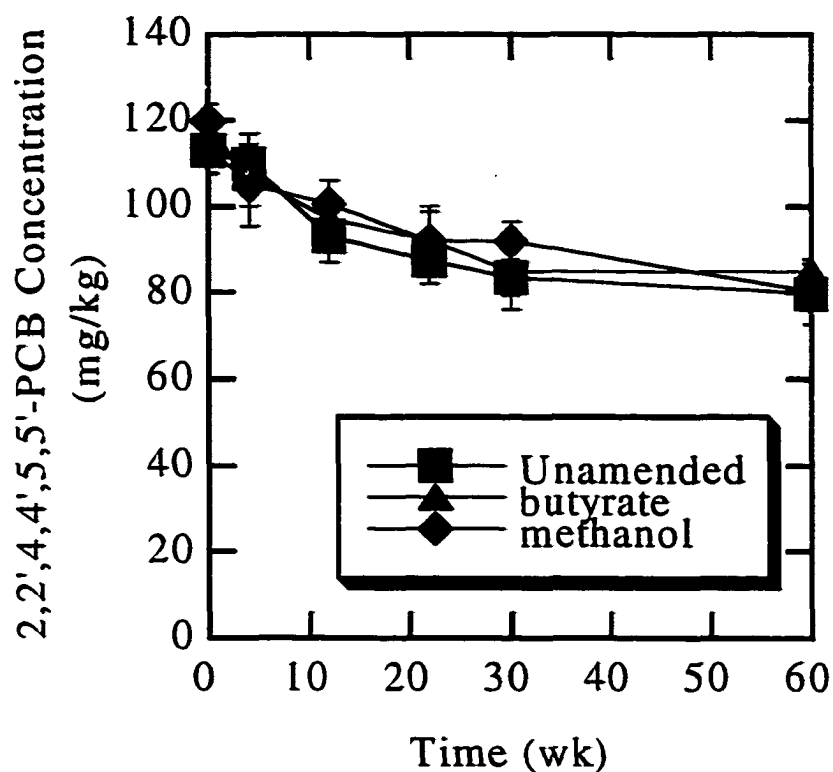


Figure 8.1 Degradation of 2,2',4,4',5,5'-PCB in anaerobic phase microcosms. Error bars represent the standard deviation between duplicate samples.

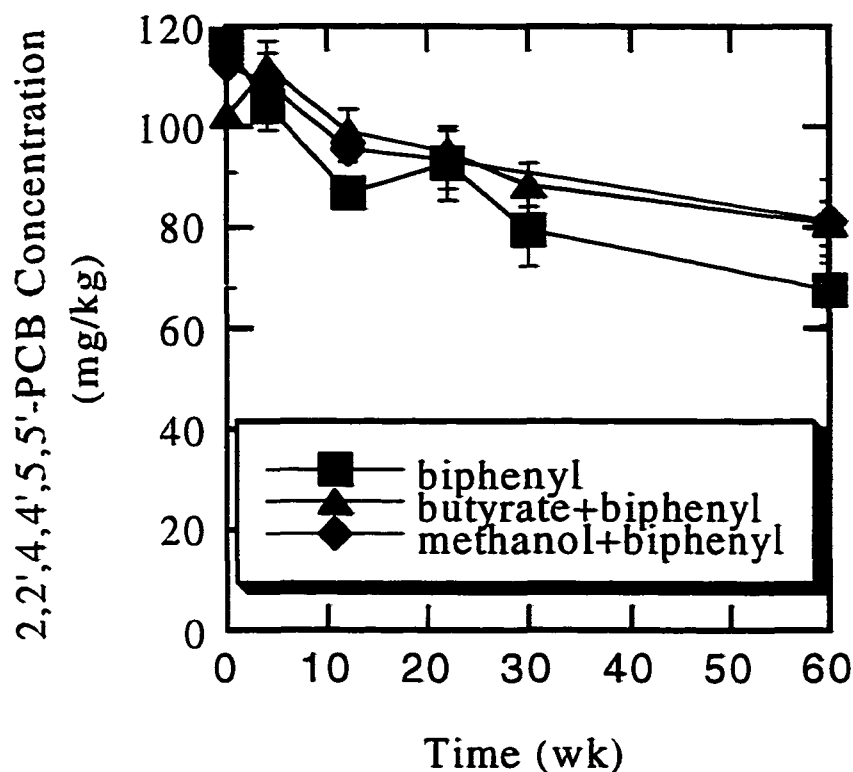


Figure 8.2 Degradation of 2,2',4,4',5,5'-PCB in anaerobic phase microcosms. Error bars represent the standard deviation between duplicate samples.

The same dechlorination pattern observed in the anaerobic-only experiments (Chapter 5) was also observed in each of the treatments used for the sequential phase of treatment. Both *meta* and *para* dechlorination to 2,2',4,4',5,5'-pentachlorobiphenyl followed by a second *para* dechlorination of 2,2',4,5,5'-PCB to 2,2',5,5'-tetrachlorobiphenyl was observed in all microcosms. For those treatments that were essentially duplicates of those described in Chapter 5 (no amendment, butyrate, and methanol amended), these results provided additional confirmation of the previously observed trends. For treatments that were different (biphenyl, biphenyl+butyrate,

biphenyl+methanol), the same dechlorination pattern was observed. This indicated that each treatment option enriched similar dechlorinating microbial populations.

The primary dechlorination product was 2,2',4,5,5'-PCB obtained by *para* dechlorination. The *meta* dechlorination product, 2,2',4,4',5-PCB was produced at a concentration of roughly a third of the 2,2',4,5,5'-PCB concentration (Figures 8.3 and 8.4).

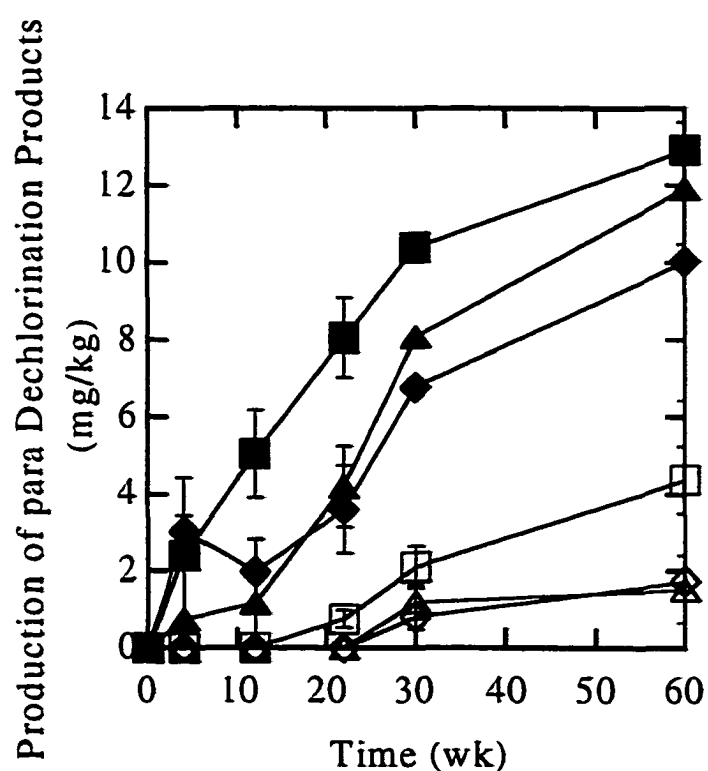


Figure 8.3 Production of 2,2',4,4',5-PCB in anaerobic phase microcosms(■ - unamended microcosms, ◆ - butyrate-amended, ▲ - methanol-amended) and 2,2',5,5'-PCB (□ - unamended, ◇ - butyrate-amended, and △ - methanol-amended). Error bars represent the standard deviation between duplicate samples.

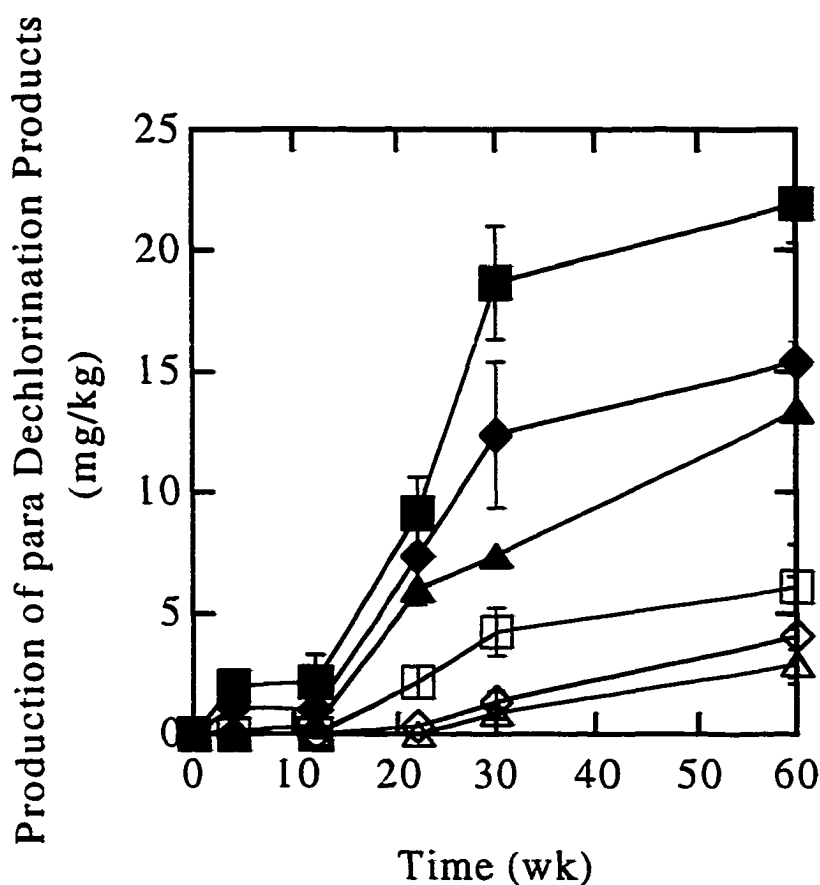


Figure 8.4 Production of 2,2',4,4',5'-PCB in anaerobic microcosms, biphenyl-amended (■ - biphenyl-amended microcosms, ◆ - butyrate+biphenyl amended, ▲ - methanol+biphenyl amended) and 2,2',5,5'-PCB (□ - unamended, ◇ - butyrate-amended, and △ - methanol-amended). Error bars represent the standard deviation between duplicate samples.

The amount of production of 2,2',4,4',5,5'-PCB observed was consistent with the hexachlorobiphenyl dechlorination results in that unamended treatments and biphenyl-amended treatments had the greatest production of the *para* dechlorination product. An additional *para* dechlorination was observed in some treatments to produce 2,2',5,5'-PCB (Figures 8.3-8.4). In the anaerobic-only results (Chapter 5), the rate of dechlorination in the unamended microcosms appeared to be slowing by wk 60 while the

butyrate- and methanol-amended treatments appeared to be continuing at roughly the same rate. This slowing in dechlorination rate in unamended treatments was less pronounced in this phase of treatment but still appeared to be occurring. Again, biphenyl-amended treatments were the most successful at dechlorinating the hexachlorobiphenyl with 21.9 ± 5.7 mg/kg 2,2',4,5,5'-PCB produced. The final concentrations of each dechlorination product produced from dechlorination of the original hexachlorobiphenyl dosed are shown in Table 8.1. The final carbon balances were not significantly different from the amount of hexachlorobiphenyl dosed on a mole basis. The carbon balances could not be confirmed using the production of chloride because of the naturally high chloride concentrations in the soil as discussed in Chapter 5.

Table 8.1 Dechlorination of 2,2',4,4',5,5'-hexachlorobiphenyl. Values are mmol/kg $\pm$ std dev.

Congener	Unamended	Butyrate	Methanol	Biphenyl	Butyrate+ Biphenyl	Methanol+ Biphenyl
2,2',4,4',5,5	0.221 ± 0.014	0.236 ± 0.008	0.222 ± 0.006	0.188 ± 0.019	0.225 ± 0.012	0.226 ± 0.023
2,2',4,5,5'	0.046 ± 0.007	0.026 ± 0.006	0.025 ± 0.003	0.062 ± 0.016	0.028 ± 0.01	0.038 ± 0.019
2,2',4,4',5	0.014 ± 0.003	0.009 ± 0.003	0.007 ± 0.002	0.019 ± 0.004	0.009 ± 0.002	0.012 ± 0.004
2,2',5,5'	0.015 ± 0.003	0.006 ± 0.001	0.0053 ± 0.003	0.021 ± 0.006	0.014 ± 0.007	0.010 ± 0.002
Total	0.296 ± 0.027	0.277 ± 0.018	0.259 ± 0.014	0.289 ± 0.045	0.276 ± 0.031	0.286 ± 0.048
Dosed	0.342 ± 0.051	0.324 ± 0.002	0.318 ± 0.064	0.389 ± 0.113	0.284 ± 0.016	0.313 ± 0.054

As observed with the anaerobic dechlorination studies, little degradation or dechlorination of 3,3',4,4'-PCB occurred (insignificant at the $p=0.1$ level). Only in

biphenyl-amended treatments were 3,3',4'- and 3,4,4'-trichlorobiphenyl, the dechlorination products of 3,3',4,4'-PCB, routinely observed. Less than 5% of the original tetrachlorobiphenyl was dechlorinated to these products by the end of the 60 wk.

In contrast, the trichlorobiphenyl (2,3',4'-PCB) dosed at time zero was partially degraded in the first 22 wk of the anaerobic phase (Figures 8.5-8.6). This phenomenon was also observed in the anaerobic-only study and seems to contradict the often-reported phenomenon that less chlorinated congeners are not degraded anaerobically (2). However, during this initial time period, conditions were not sufficiently reduced to prevent aerobic degradation of the trichlorobiphenyl. By wk 22, redox conditions in the microcosms had reached -10 to 0 mV and remained near these levels for the remainder of the sampling period in all microcosms (Figure 8.5 inset). Those microcosms amended with biphenyl displayed a greater extent of degradation of the 2,3',4'-PCB than those not amended with biphenyl (an average of 35% without biphenyl and 50% with biphenyl) in the first 22 wk of the anaerobic phase. This agrees with observations made during the aerobic-only experiments that biphenyl amendment increased the rate of degradation of the trichlorobiphenyl.

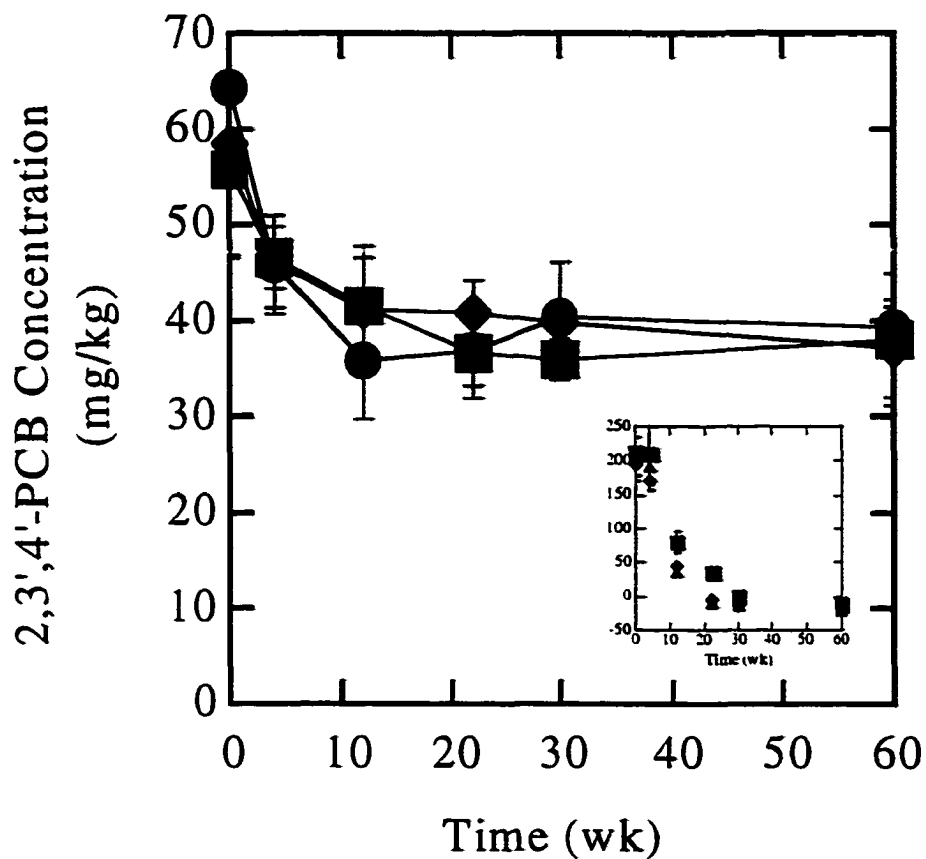


Figure 8.5 Degradation of 2,3',4'-trichlorobiphenyl during the anaerobic phase microcosms. ■ - unamended microcosms, ◆ - butyrate-amended microcosms, ▲ - methanol-amended microcosms. Error bars represent the standard deviation between duplicate samples.

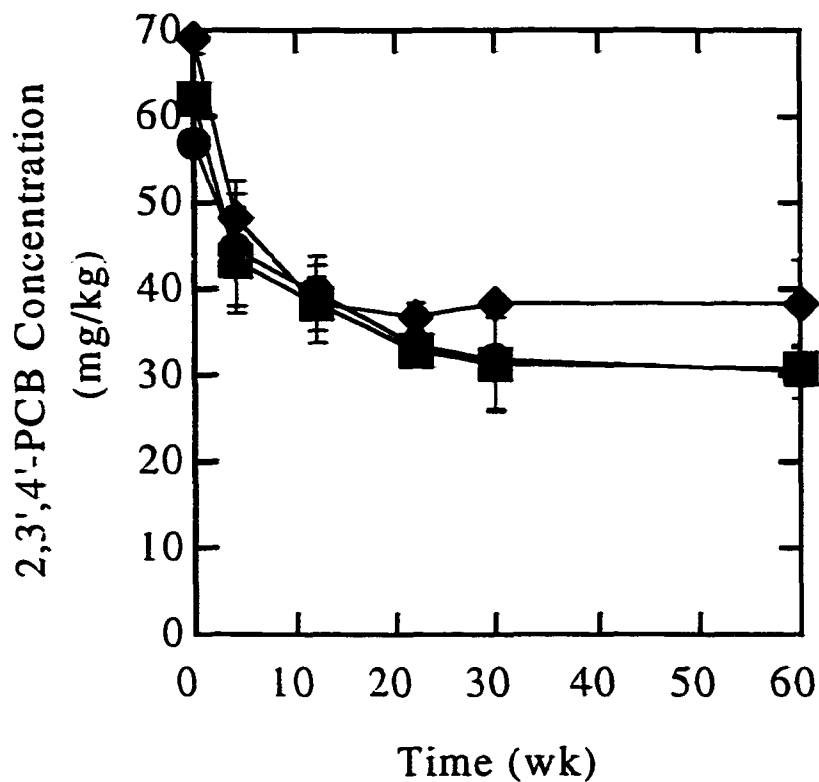


Figure 8.6 Degradation of 2,3',4'-PCB in anaerobic phase microcosms. ■ - biphenyl-amended microcosms, ◆ - butyrate+biphenyl amendment, ▲ - methanol+biphenyl amendment. Error bars represent the standard deviation between duplicate samples.

While biphenyl was originally added to serve as an inducer of the aerobic portion of degradation, because of the trouble encountered with initiating anaerobic conditions in the microcosms, it was nearly completely degraded during the first 4 wk of the sampling period (Figure 8.7). No further degradation occurred in the remaining weeks of the anaerobic phase. A slight decrease in the biphenyl concentration was observed in sterile controls. This decrease represented less than a 10% decrease of the total dosed biphenyl concentration. There was no difference in the rate or extent of degradation of the

biphenyl between treatments. In addition, the biphenyl appeared to be serving in some capacity during the anaerobic phase to enhance the dechlorination observed in the biphenyl only amended microcosms, but not in those amended with both biphenyl and butyrate or biphenyl plus methanol. Biphenyl was nearly completely degraded in these microcosms by wk 4, yet little dechlorination was observed at this point. Thus, biphenyl is likely not serving as an electron donor but could be stimulating the growth of certain organisms able to dechlorinate PCBs. Once the biphenyl was depleted at wk 4, a different electron donor would be required for dechlorination. This electron donor could either be biphenyl degradation products or an inorganic electron donor. Since it seemed possible from the unamended treatments that an inorganic electron donor was being utilized in the unamended treatments the same donor might be involved in dechlorination in the biphenyl-amended treatments.

Several researchers have investigated the enhancement of anaerobic dechlorination with biphenyl but have obtained mixed results. In a work by Rhee and co-workers, the rate and extent of dechlorination of Aroclor 1254 was greater in nonenriched sediments than in biphenyl-enriched sediments (10). Different patterns of dechlorination were also observed between the two treatments.

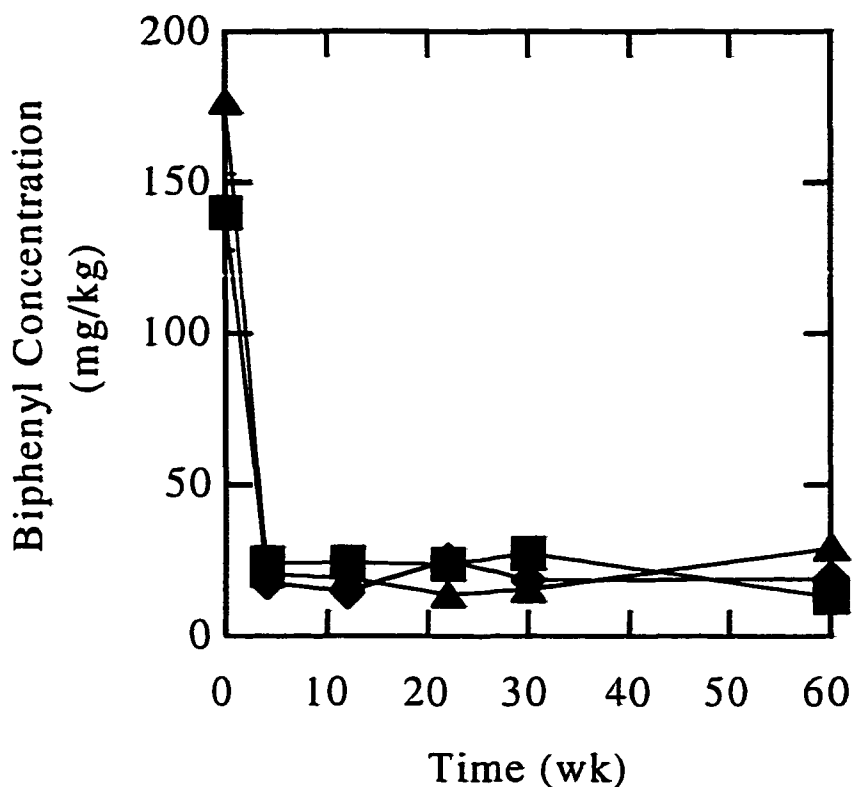


Figure 8.7 Degradation of biphenyl in anaerobic phase microcosms. ■ - biphenyl-amended microcosms, ◆ - butyrate+biphenyl amendment, ▲ - methanol+biphenyl amendment. Standard deviations were less than the size of the markers. Error bars represent the standard deviation between duplicate samples.

The number of chlorines per biphenyl was plotted for each of the different treatments to investigate the overall reduction in higher chlorinated congeners. In Figures 8.8 and 8.9 the number of chlorines per biphenyl is shown without inclusion of either biphenyl or the trichlorobiphenyl. The number of chlorines per biphenyl again decreased in all treatments, but decreased significantly more in the biphenyl only treatments.

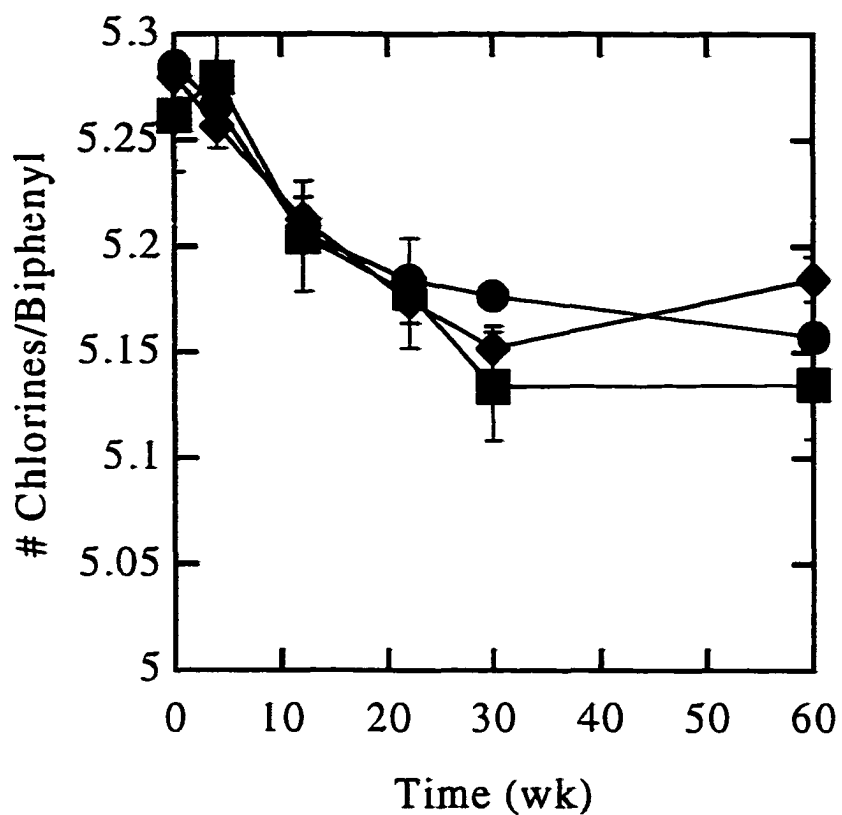


Figure 8.8 Change in the number of chlorines per biphenyl. ■ - unamended microcosms, ◆ - butyrate-amended, ▲ - methanol-amended. Error bars represent the standard deviation between duplicate samples.

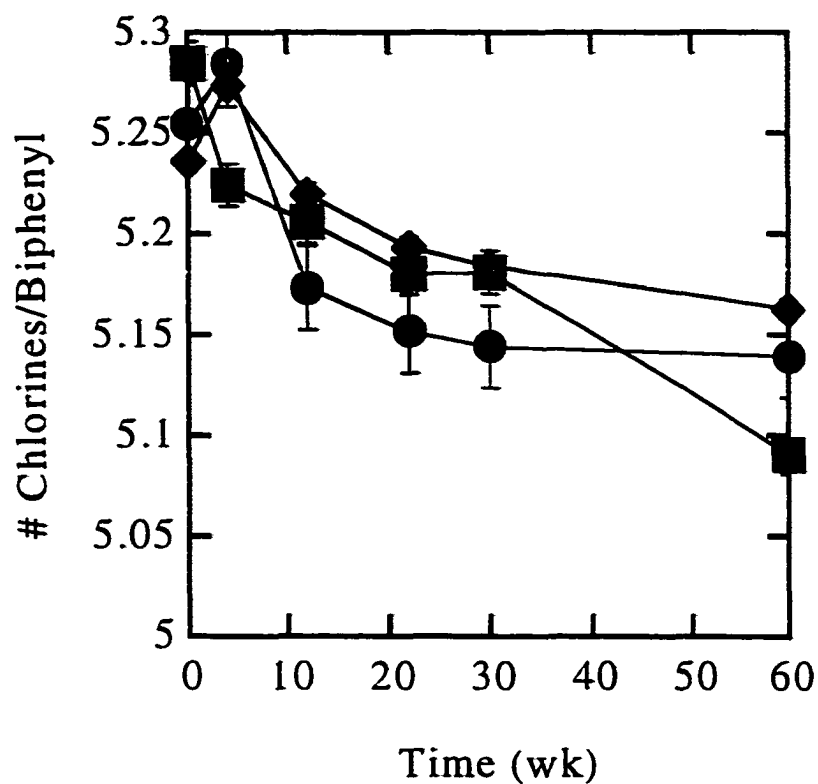


Figure 8.9 Change in the number of chlorines per biphenyl. ■ - biphenyl-amended microcosms, ◆ - butyrate+biphenyl amended microcosms, ▲ - methanol+biphenyl amended microcosms. Error bars represent the standard deviation between duplicate samples.

8.B.2. Electron Donor Concentration

As with the anaerobic-only treatments in Chapter 5, the concentration of butyrate and methanol were determined at each sampling point. The same dosing schedule was utilized for these microcosms and is shown in Figures 8.10 and 8.11 with the electron donor concentrations. No significant degradation of the methanol and butyrate was

observed in sterile controls. Differences in the initial rate of electron donor utilization were observed between those microcosms amended with biphenyl and those not amended with biphenyl. In microcosms amended with biphenyl, the initial utilization rate of electron donor was decreased compared to those microcosms not amended with biphenyl. By wk 4, the utilization of electron donor was essentially the same between all treatments, and matched that described in Chapter 5. This timing corresponds to the utilization of biphenyl which was complete by wk 4, indicating that the microbial population was better able to use biphenyl than butyrate or methanol. While both butyrate and methanol are more simple chemically and would thus seem like better substrates, the microbial population was acclimated to biphenyl and PCBs and might have been more efficient at using biphenyl than the electron donors provided.

Another variable investigated in the anaerobic phase was the ferrous iron concentration. If iron reduction were occurring, the concentration of ferrous iron would be expected to increase. Since the soil was iron rich, iron reduction seemed possible and if it occurred could interfere with dechlorination of the PCBs. In agreement with the results described in Chapter 5, no production of ferrous iron was observed from wk 22 to wk 60. In fact, a decrease in ferrous iron concentration by an order of magnitude (approximately 1.5 mg/L to 0.20 mg/L) was observed during this period. The concentration of ferrous iron in the sterile controls was always higher than that observed in the non-sterile microcosms at all sampling times. These ferrous iron concentration results reinforce the hypothesis that iron may be oxidized in the microcosms; a decrease in ferrous iron content would thus be observed from the oxidation of the ferric iron.

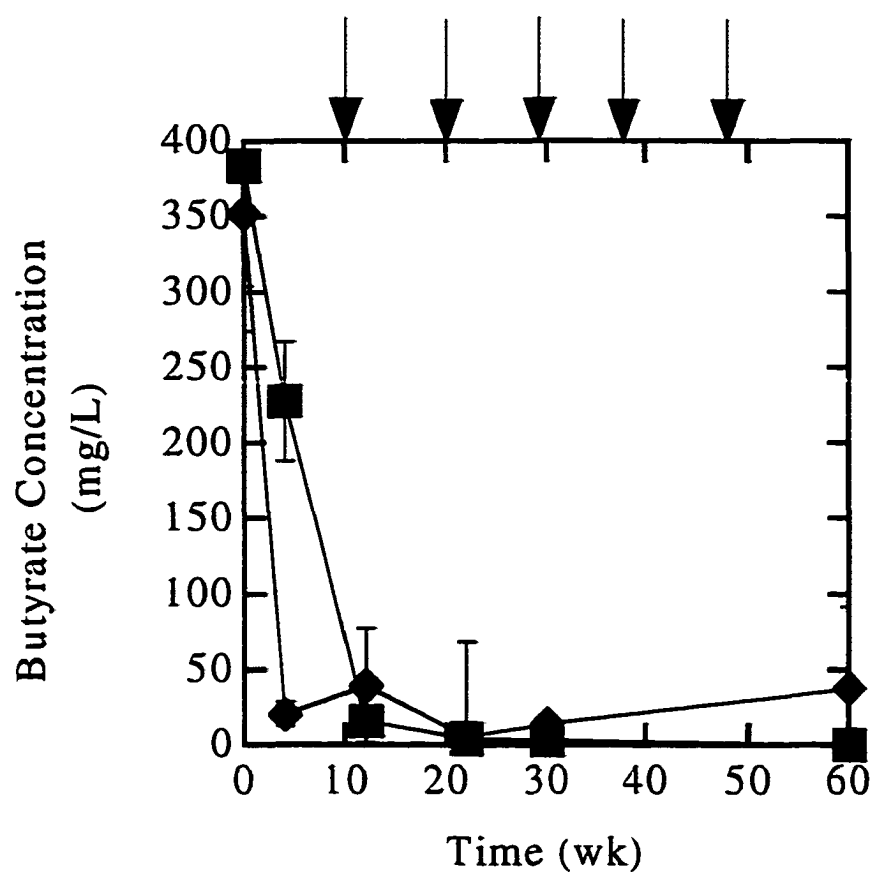


Figure 8.10 Utilization of butyrate in anaerobic phase microcosms. Arrows indicate additional dosing times. $\blacklozenge$ - butyrate-amended microcosms, $\blacksquare$ - biphenyl+butyrate amended microcosms. Error bars represent the standard deviation between duplicate samples.

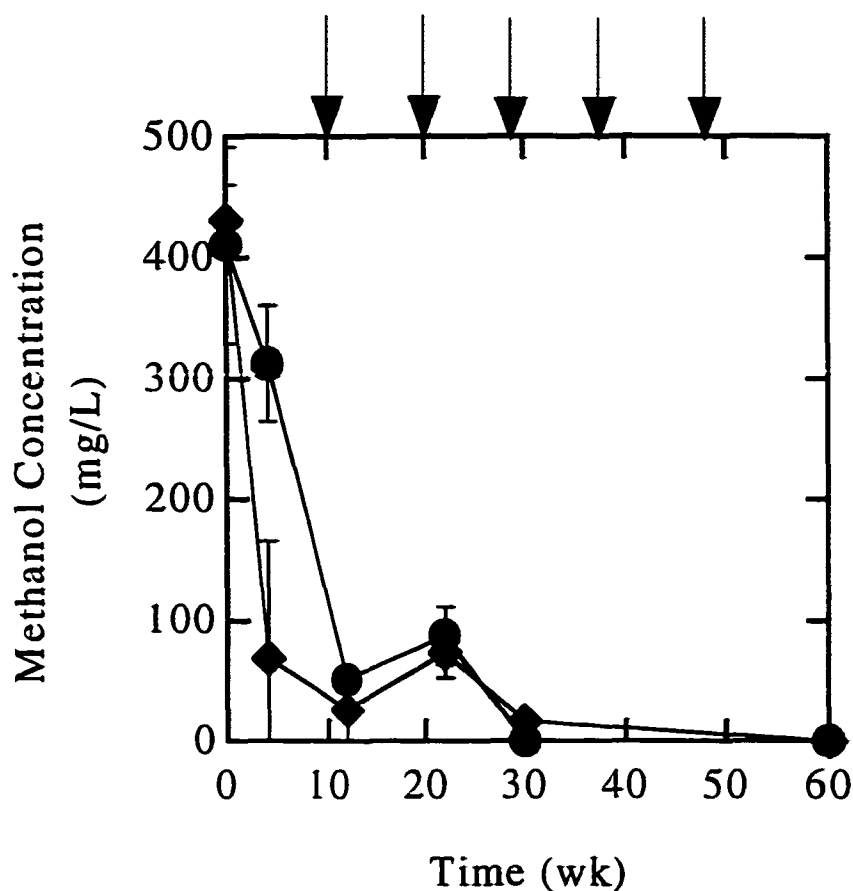


Figure 8.11 Utilization of methanol in anaerobic phase microcosms. Arrows indicate additional dosing times. ♦ - methanol-amended microcosms, ● - methanol+biphenyl amended microcosms. Error bars represent the standard deviation between duplicate samples.

8.B.3. PLFA/FAME analysis

The PLFA/FAME analysis was used to describe the changes in total biomass and in specific FAMES over the course of the 60 wk anaerobic phase. First, the change in total biomass in each treatment was investigated (Figures 8.12 and 8.13). The concentration of total biomass remained relatively constant throughout the sampling period but increased at wks 30 and 60 for all treatments. The exception to this was the

unamended treatment, which displayed an increase in the total biomass concentration at wk 30 and then a decrease at wk 60. This pattern of total biomass concentrations is similar to that observed in Chapter 5. The microcosms that had been amended with biphenyl had a higher final total biomass concentration than did their corresponding treatments without biphenyl amendment. Biphenyl-amended and unamended microcosms reached nearly the same final biomass concentration, which was lower than any of the other treatments. This seems counter-intuitive as one would expect a higher biomass concentration to correspond with the higher amounts of dechlorination observed in biphenyl-amended and unamended treatments. It may be that the actively dechlorinating population is small in numbers compared to the total number of microorganisms. Thus, the question of whether or not total biomass is a good indicator of active dechlorination is important to investigate.

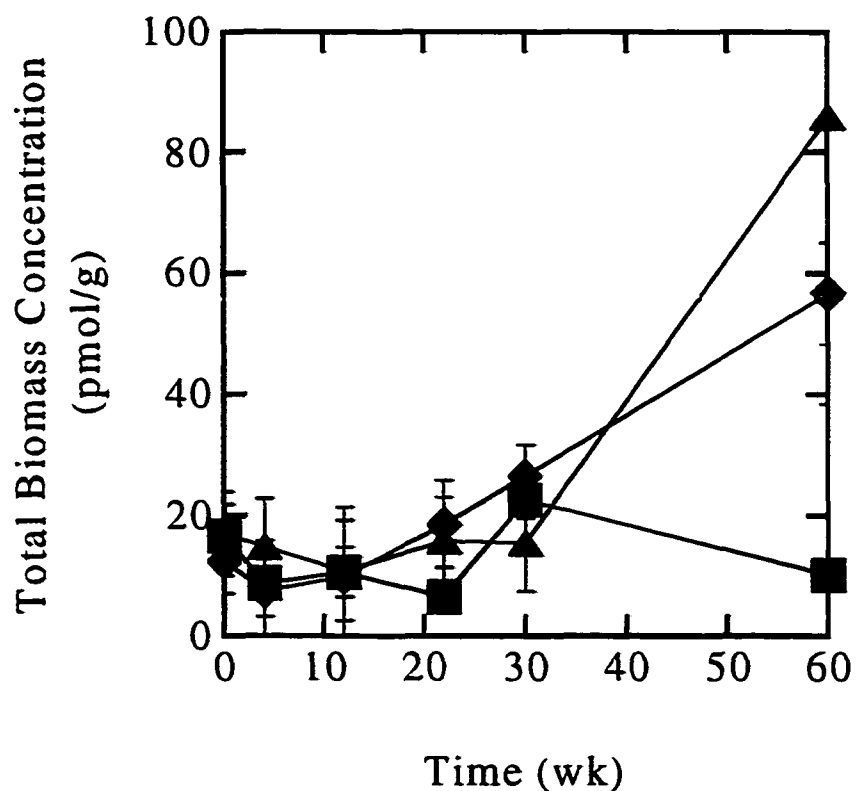


Figure 8.12 Change in total biomass concentration in anaerobic phase microcosms. ■ - unamended microcosms, ♦ - butyrate-amended microcosms, ▲ - methanol-amended microcosms. Error bars represent the standard deviation between duplicate samples.

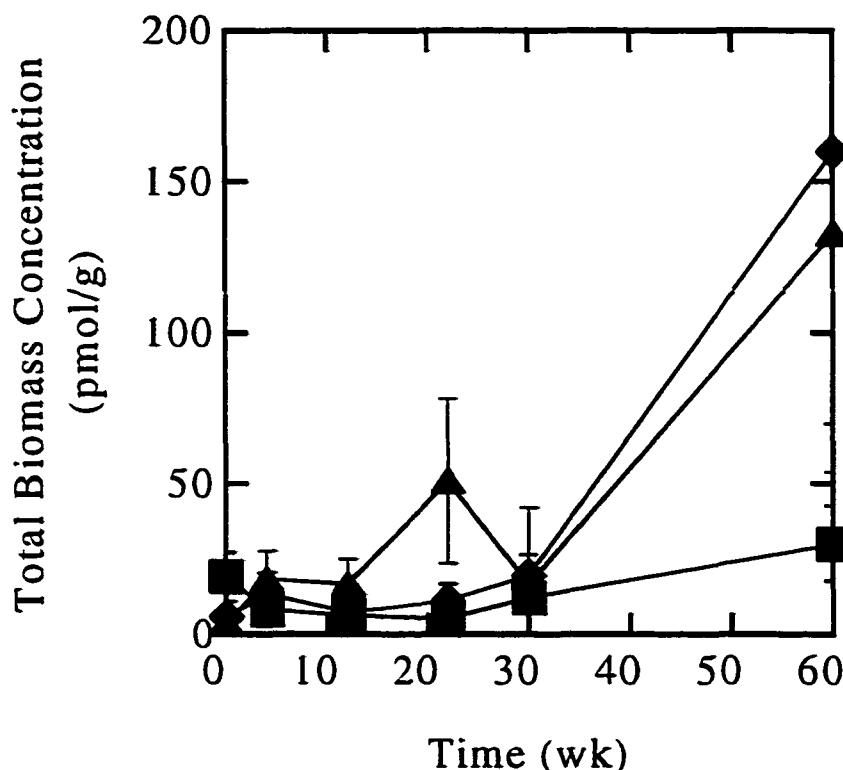


Figure 8.13 Change in total biomass concentration in anaerobic phase microcosms. ■ - biphenyl-amendment, ♦ - butyrate+biphenyl amended, ▲ - methanol+biphenyl amended. Error bars represent the standard deviation between duplicate samples.

In addition to differences in total biomass concentration over the 60 wk sampling period, differences in specific fatty acids between samples were observed. The sum of the FAMES i14:0, i15:0, and i16:0 was used as an indicator of changes in the anaerobic microbial population. In those microcosms not amended with biphenyl, the sum of these FAMES increased throughout the 60 wk anaerobic phase. However, in those microcosms amended with biphenyl, a decrease of these compounds was observed from wk 0 to wk 4 followed by a slow increase to concentrations similar to that observed in unamended

microcosms. Figure 8.14 shows this change in *iso* branched FAMES in biphenyl amended treatments. In addition, an increase in total unsaturated FAMES (16:1 ω 7+18:1 ω 9+18:1 ω 7) in the first 4 wk was observed in only those microcosms amended with biphenyl. These changes are similar to those observed in Chapter 6 in biphenyl-amended microcosms in the aerobic-only experiments and indicate an increase in aerobic, *Pseudomonas*-like species. After 4 wk no significant change was observed in the mole percent of these FAMES.

Finally, a change that was *not* observed in FAME profiles was a decrease in the ratio of the *cis/trans* isomers of 16:1 ω 7 and 18:1 ω 7. This ratio is used as an indicator of stress in aerobic microorganisms. A decrease in this ratio indicates organisms that are undergoing physiological stresses. Because changes in this ratio were not observed, the aerobic microbial population was most likely not experiencing physiological stress and was thus able to survive under the conditions of the anaerobic microcosms. The lack of PLFA stress indicators and the fact that the aerobic biomarkers did not decrease over the 60 wk appeared to be a good indicator that sequential treatment would be successful in this system.

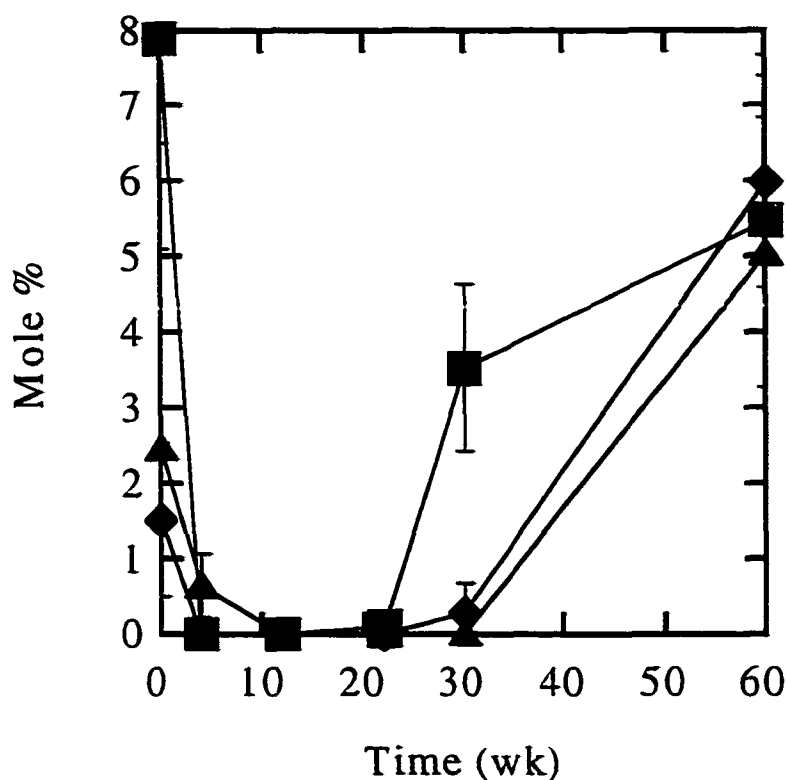


Figure 8.14 Change in mole percent of (i14:0+i15:0+i16:0) in biphenyl-amended anaerobic phase treatments. ■ - biphenyl-amended microcosms, ◆ - butyrate+biphenyl amended microcosms, ▲ - methanol+biphenyl amended microcosms. Error bars represent the standard deviation between duplicate samples.

To compare differences and similarities between overall FAME profiles, principal component analysis was used. Three principal components were used; the total variance explained by the three factors was 73.79%, 10.1%, and 5.4% for factors 1, 2, and 3 respectively. No clustering was apparent in any of the two-factor plots. Thus, during the anaerobic phase, use of PLFA/FAME analysis does not appear to correlate directly with dechlorination. Franzmann and co-workers found that PLFA/FAME analysis gave

limited information into the microbial community structure in an aquifer contaminated with aromatic hydrocarbons (5). In contrast, Knight and co-workers use PLFA/FAME analysis to determine that distinct populations were enriched under iron-reducing, sulfidogenic, or methanogenic conditions in cultures carrying out the dehalogenation of halogenated phenols and benzoic acids (7). These results appear contradictory to those observed in Chapter 5; however, the total biomass concentration was included in the analysis of Chapter 5. This measure was not included here once it was noticed that biomass did not appear to correlate with dechlorination. If the total biomass was excluded from the PCA analysis from Chapter 5, no clustering was observed in agreement with these results.

8.B.4. Conclusions from Anaerobic Phase

During the anaerobic phase of the sequential treatment, six treatments were investigated: 1) unamended, 2) biphenyl-amended, 3) butyrate-amended, 4) butyrate+biphenyl amended, 5) methanol-amended, and 6) methanol+biphenyl amended. Dechlorination was observed in all treatments, with biphenyl and unamended treatments displaying the largest extent and fastest rates of dechlorination. The same pattern of dechlorination to two pentachlorobiphenyls with a second dechlorination of one of those pentachlorobiphenyls to a tetrachlorobiphenyl was observed. Degradation of 2,3',4'-trichlorobiphenyl and biphenyl was observed in the first 22 wk and first 4 wk of the sampling period, respectively.

Differences in FAME profiles were observed between treatments. Biphenyl-amended treatments had significant shifts during the time that biphenyl was being

degraded (0-4 wk) with a decrease in anaerobic markers and an increase in aerobic, *Pseudomonas* like markers. After this time point, anaerobic markers increased in all treatments while aerobic markers remained constant. Despite these differences, PCA of overall FAME profiles showed that overall; the samples at wk 60 were not significantly different than any of the other data points.

8.C. Aerobic Phase

After 60 wk under anaerobic conditions, hydrogen peroxide was added to the microcosms to initiate the aerobic phase of treatment. The middle level of hydrogen peroxide tested in Chapter 7 (0.25% H₂O₂) was chosen for oxygenation. Since there was little apparent inhibition of the microorganisms due to the hydrogen peroxide treatment, we chose a higher treatment level since there was some evidence in the oxygenation experiments that higher rates of DO utilization occurred with higher hydrogen peroxide doses. In addition to oxygenation, biphenyl was re-added to those microcosms with biphenyl amendment, as the biphenyl had been nearly depleted during the first 4 wk of the anaerobic phase.

8.C.I. PCB Concentration

Figures 8.16 and 8.17 show the degradation of the trichlorobiphenyl during the aerobic phase of the sequential treatment. Also shown are model predictions of the degradation of 2,3',4'-PCB utilizing the kinetic parameters determined in Chapter 6. In unamended and biphenyl-amended treatments, degradation occurred more slowly than observed in the aerobic-only microcosms. In those treatments amended with butyrate or

methanol, there was no significant difference between the aerobic-only degradation and that observed after the prolonged anaerobic phase. However, the difference between the model predictions and actual data values was less in biphenyl-amended treatments. Degradation of biphenyl was also observed in each of the biphenyl amended microcosms (Figure 8.18).

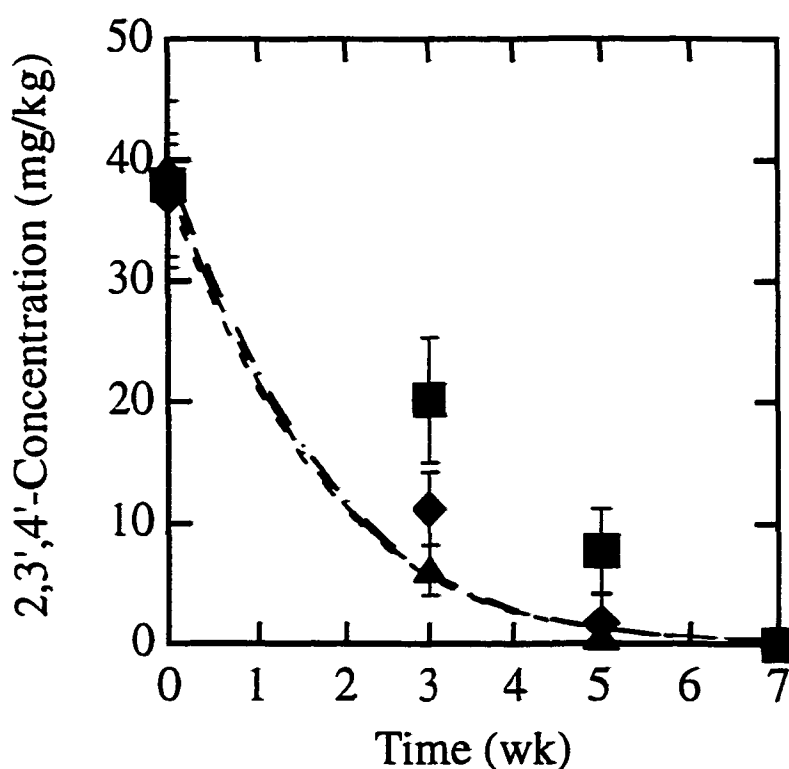


Figure 8.16 Degradation of 2,3',4'-PCB in aerobic phase microcosms. ■ - unamended microcosms, ♦ - butyrate-amended microcosms, ▲ - methanol-amended microcosms. Lines represent model predictions using the kinetic parameters in Table 6.1. Error bars represent the standard deviation between duplicate samples.

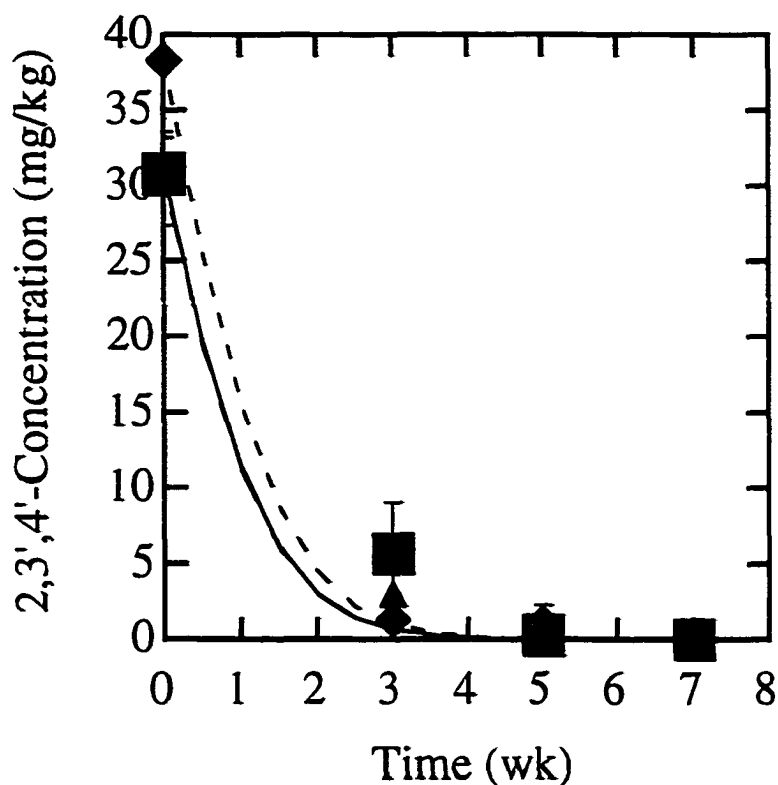


Figure 8.17 Degradation of 2,3',4'-PCB in aerobic phase microcosms. ■ - biphenyl amended microcosms, ♦ - butyrate+biphenyl amended microcosms, ▲ - methanol+biphenyl amended microcosms. Lines represent model predictions using the kinetic parameters in Table 6.1. Error bars represent the standard deviation between duplicate samples.

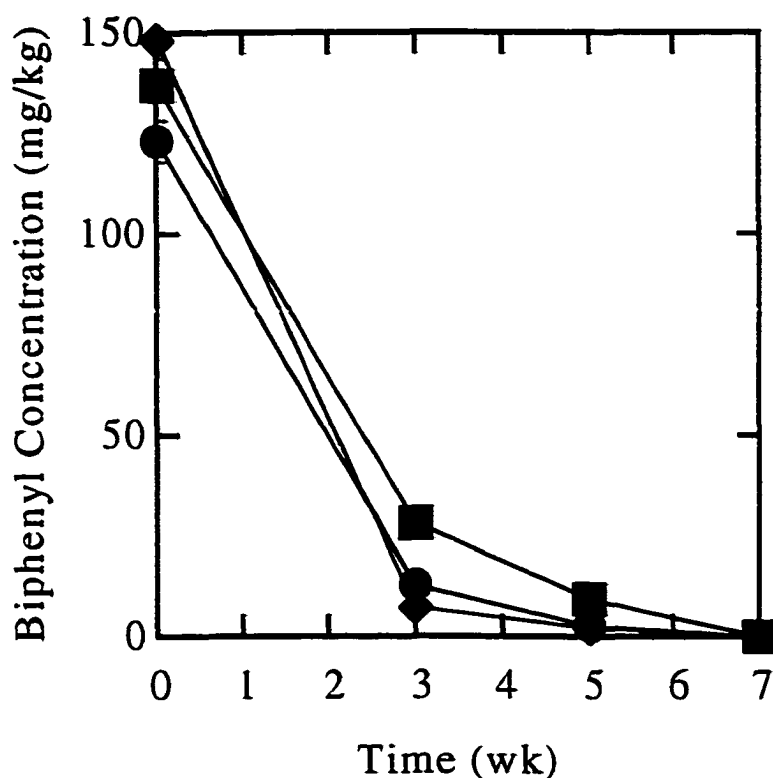


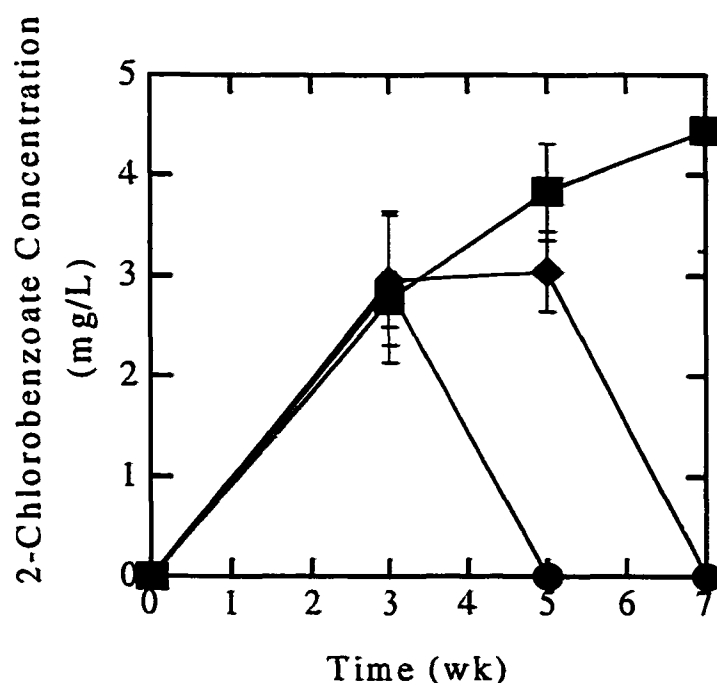
Figure 8.18 Degradation of biphenyl in aerobic phase microcosms. ■ - biphenyl microcosms, ▲ - methanol+biphenyl amended microcosms, ● - butyrate+biphenyl amended microcosms. Error bars represent the standard deviation between duplicate samples.

No additional degradation or dechlorination of the hexachlorobiphenyl or 3,3',4,4'-tetrachlorobiphenyl was observed in any of the microcosms. This lack of dechlorination of the 3,3',4,4'-PCB is unusual as most researchers have reported anywhere from 30-95% dechlorination of this congener by indigenous organisms (3, 6, 8, 9). Degradation of the tetrachlorobiphenyl produced by dechlorination of the hexachlorobiphenyl did occur. In fact, this congener, 2,2',5,5'-PCB, was totally

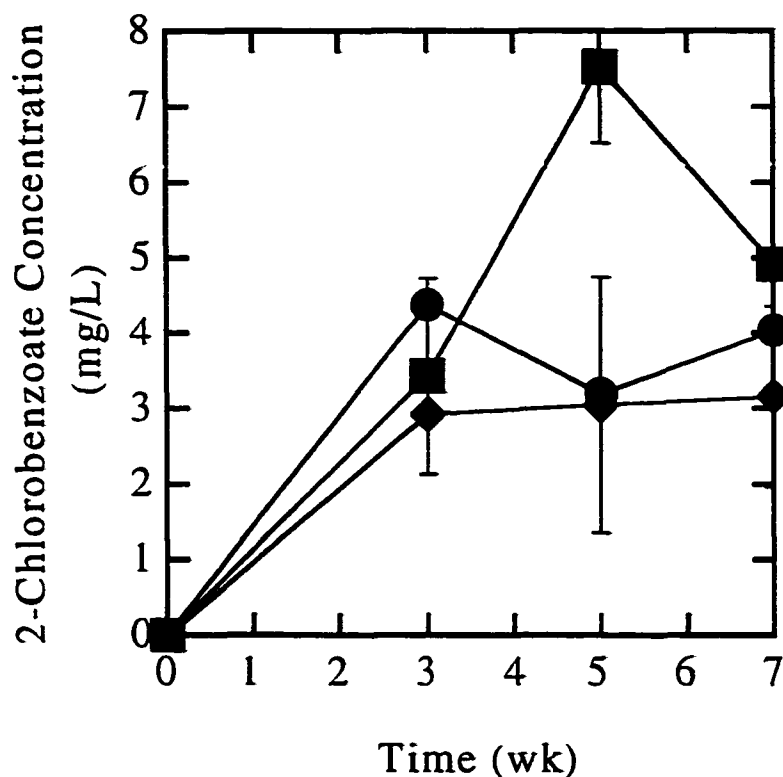
degraded by wk 7 in all treatments. The indigenous microorganisms had been shown to degrade this compound in preliminary aerobic studies (Chapter 4) albeit at much higher concentrations.

8.C.2. Detection of Aerobic Degradation Intermediates

As was observed in the oxygenation experiment, production of chlorobenzoic acids occurred in all treatments. Unlike the results presented in Chapter 7, only one of the chlorobenzoic acids from the degradation of 2,3',4'-PCB was detected using HPLC analysis (2-chlorobenzoic acid). The concentration of this degradation intermediate during the 7-wk aerobic period is shown in Figures 8.19 and 8.20.



8.19 Concentration of aerobic degradation intermediate, 2-chlorobenzoate, in aerobic phase microcosms. ■ - unamended microcosms, ▲ - methanol-amended microcosms, ● - butyrate-amended microcosms. Error bars represent the standard deviation between duplicate samples.



8.20 Concentration of aerobic degradation intermediate, 2-chlorobenzoate, in aerobic phase microcosms. ■ - biphenyl-amended microcosms, ▲ - methanol+biphenyl amended microcosms, ● - butyrate+biphenyl amended microcosms. Error bars represent the standard deviation between duplicate samples.

In methanol- and butyrate-amended microcosms, the concentration of 2-chlorobenzoate increased and then decreased. In all other treatments, the 2-chlorobenzoate had not been degraded by the end of the 7 wk. The reason for this persistence could either be that microorganisms able to degrade the chlorobenzoates did

not survive the anaerobic conditions or that because the degradation of 2,3',4'-PCB was slower than observed in Chapter 6, the production and subsequent depletion of the chlorobenzoate was also delayed. In addition to the production of 2-CBA, detection of low concentrations of 2,5-CBA was observed. Production of this intermediate occurs via a 3,4-dioxygenase attack on the 2,2',5,5'-tetrachlorobiphenyl. As with the products observed in Chapter 7, lower concentrations of chlorobenzoic acids were detected than would theoretically be produced from the amount of tri- and tetrachlorobiphenyls degraded. Also, no production of catechol was observed in any treatments, as was observed in the air treated microcosms.

8.C.3. PLFA/FAME Analysis

After complete degradation of the trichlorobiphenyl and biphenyl were observed in 7 wk, samples were analyzed using PLFA/FAME analysis to determine if changes occurred in the microbial population during this time period, and if those changes differed between treatments. Significant changes in the total biomass concentration were observed in all treatments (Figure 8.21 and 8.22). An initial decrease in total biomass concentration was observed immediately after addition of the hydrogen peroxide for initiation of aerobic conditions. In unamended, butyrate- and methanol-amended microcosms, the microbial concentrations remained constant for the remainder of the sampling time. In contrast, the concentration of microorganisms in those microcosms amended with biphenyl had begun to increase by wk 5 and had increased further by wk 7. The results from microcosms not amended with biphenyl is consistent with that observed in Chapter 7 in that biomass levels did not increase. Unlike the oxygenation experiment,

complete degradation of the trichlorobiphenyl was observed in 7 wk in all treatments. A possible explanation is that the concentration of the trichlorobiphenyl was roughly half that dosed during the oxygenation experiments since some had been degraded in the early part of the anaerobic phase. The addition of biphenyl either during the anaerobic phase, the aerobic phase, or both appeared to have had a protective effect on the microbial population. The reason for this is not clear, but could be due to an enrichment of organisms resistant to hydrogen peroxide toxicity or simply due to an increase in the number of total organisms.

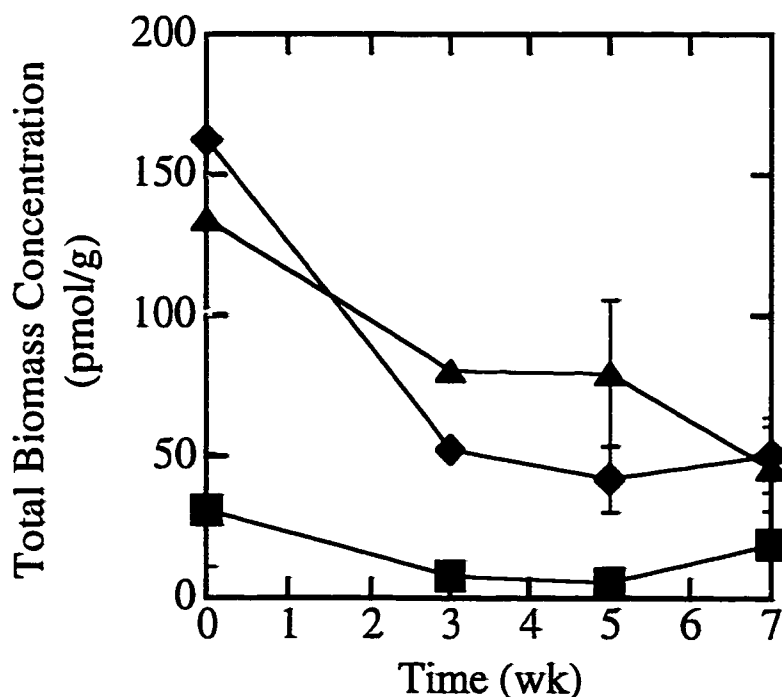


Figure 8.21 Changes in total biomass concentration (pmol/g) in aerobic phase microcosms. ■ - unamended microcosms, ♦ - butyrate-amended microcosms, ▲ - methanol-amended microcosms. Error bars represent the standard deviation between duplicate samples.

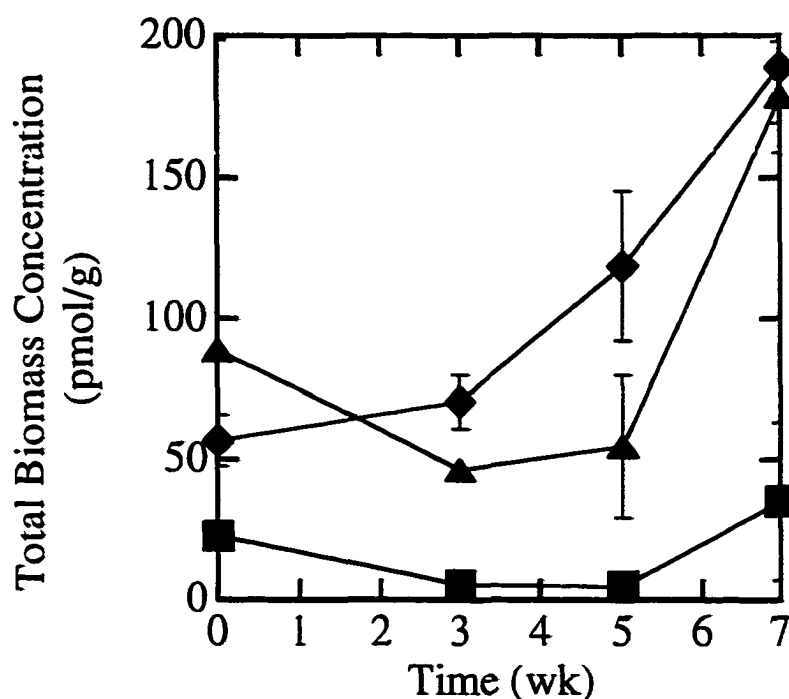


Figure 8.22 Changes in total biomass concentration in aerobic phase microcosms amended with biphenyl. ■ - biphenyl-amended microcosms, ◆ - butyrate+biphenyl amended microcosms, ▲ - methanol+biphenyl amended microcosms. Error bars represent the standard deviation between duplicate samples.

Principal component analysis of FAME profiles revealed three significant components of variation within the data set. Principal Components 1, 2, and 3 accounted for 65.7, 13.4, and 5.6% of the variance within the data set. The combination of Principal Components 1 and 2 mapped the data into three distinct clusters (Figure 8.23). Data from samples at wks 5 and 7 (single box) mapped together while data from wks 0 and 3

(double box) mapped together. The exceptions were the cluster of samples from butyrate or butyrate+biphenyl amended microcosms at wk 0 and wk 3 (thick box).

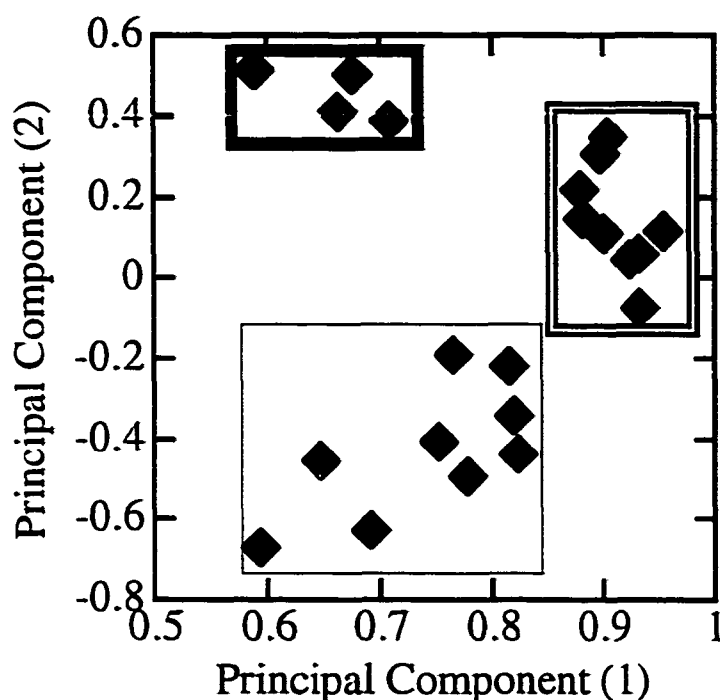


Figure 8.23 Principal component analysis of aerobic phase microcosm FAME profiles, Factor 1 by Factor 2.

Samples with eigenvalues for Principal Component 1 less than 0.8 were enriched in the monounsaturated fatty acids 16:1 ω 7c, 18:1 ω 9c, and 18:1 ω 7c, while those with eigenvalues greater than 0.8 were enriched in *iso* branched fatty acids (i14:0, i15:0, and i16:0). The monounsaturated fatty acids are indicative of aerobic gram-negative bacteria while *iso*-branched fatty acids are typical of either *Bacillus*-type organisms or anaerobic gram-negative bacteria. Similar increases in the monounsaturates and decreases of *iso*-

branched fatty acids were observed in biphenyl-amended microcosms in the aerobic-only experiments (Chapter 6). The data in Figure 8.23 indicate that the same changes in FAME profiles occurred in those microcosms not amended with biphenyl during this phase of treatment. A significant effect on the PCA analysis is the decrease in iso-branched fatty acids. If this were due to a decrease in anaerobic microorganisms, then it would be reasonable to observe this change in all treatments with the addition of hydrogen peroxide. In aerobic only microcosms, the only selective pressure for a decrease in the percent of anaerobic microorganisms compared to aerobic microorganisms was biphenyl enrichment of aerobes. Here, there is an enrichment of aerobes, but also a decrease in anaerobes due to the hydrogen peroxide.

Graphical analysis indicated that Principal Components 2 and 3 mapped the samples into two distinct groups (Figure 8.24). The two clusters were divided solely by sampling time. Samples from wks 5 and 7 predominantly mapped together while those from wks 0 and 3 were mapped together. Those samples with negative eigenvalues for principal component two were enriched in cyclopropane fatty acids *cyl*17:0 and *cyl*19:0 while those with positive eigenvalues were enriched in *anteiso* branched fatty acids (*al*15:0 and *al*17:0). The fatty acids *cyl*17:0 and *cyl*19:0 are found in aerobic microorganisms and are often thought to be indicators of physiological stress in those organisms. If stress were caused by the addition of hydrogen peroxide to the samples, a sharp increase in these fatty acids would have been expected at wk 3. This was not the case; these fatty acids gradually increased over the 7-wk period. This indicates that the stress was caused by another mechanism, perhaps the buildup of metabolic by-products unable to be used by the microbial population. Although the production and

disappearance of chlorobenzoic acids was observed, a mass balance could not be made leaving open the possibility of undetected metabolites that were not degraded in this system.

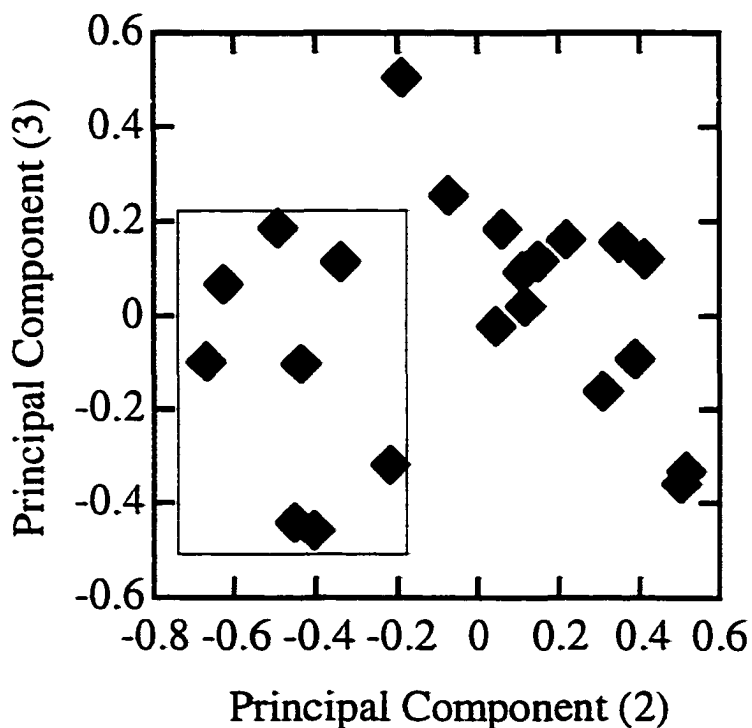


Figure 8.24 Principal component analysis of aerobic phase microcosms FAME profiles, Factor 2 by Factor 3.

8.D. Overall Treatment

The overarching goal of this research was to evaluate the sequential anaerobic/aerobic treatment of PCBs in soil. Once the anaerobic and aerobic phases had been described separately, the overall reduction in PCBs and changes in community

composition over the whole experiment were determined to evaluate the effectiveness of the sequential treatment.

8.D.1. PCB Concentration

The change in concentration of each PCB homolog over the course of the sequential treatment is shown in Figure 8.25. In all treatments, the sequential process significantly decreased the total concentration of PCBs in the soil compared to the anaerobic treatment alone with an average decrease of 50.5% for sequential treatment and 25% for anaerobic treatment alone. A maximum decrease in PCB concentration was observed in sequentially treated biphenyl-amended microcosms (57.5%). In those treatments that also corresponded to an aerobic phase alone (unamended, biphenyl-amended) the decrease in PCB concentration observed in the sequential process was also significantly higher than the aerobic process alone. Differences in the overall degradation in microcosms were tested using Tukey's HSD for significance. The final PCB concentrations in unamended and biphenyl-amended microcosms were found to be significantly different from the other treatments at a significance level of $p=0.1$ but not from each other. None of the remaining treatments were significantly different from each other.

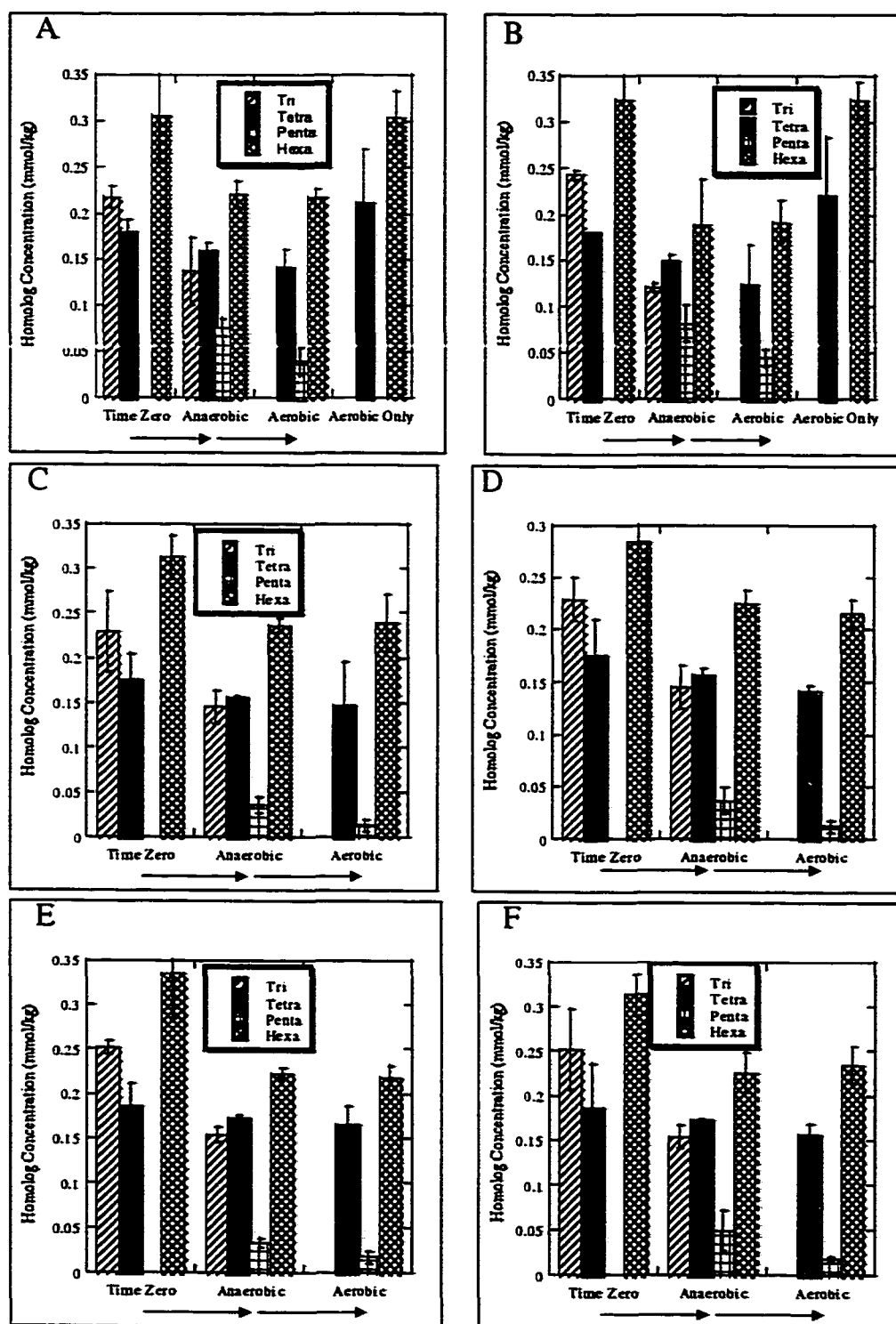


Figure 8.25 Change in homolog concentration (mmol/kg) in sequential treatment microcosms. A – unamended microcosms, B – biphenyl amendment, C – butyrate amendment, D – butyrate+biphenyl amendment, E – methanol amendment, and E – methanol+biphenyl amendment. Error bars represent the standard deviation between duplicate samples.

Evans and co-workers observed a 70% reduction of PCBs in anaerobic-aerobic treatment of Hudson River sediments incubated anaerobically for 60 wk and aerobically for an additional 19 wk (4). No additional electron donor was added to these sediments; the natural organic matter was sufficient to provide reducing conditions after 1 wk incubation. Anid et al. observed a reduction similar to that observed in this work; 40% reduction of 300 mg/kg Aroclor 1242 in Hudson River sediments (1). Evans et al. attributed the differences between their work and Anid et al.'s work to their use of a superior aerobic PCB degrader and thus loss of more trichlorobiphenyl. The significant difference between these works and the work presented here is that the extent of dechlorination observed in this work is less than that observed in either of the other studies. Both Evans et al. and Anid et al. utilized Hudson River sediments that are known to contain active, abundant dechlorinating populations and whose dechlorinating characteristics and microbial requirements are better understood than this work.

8.D.2. PLFA/FAME Analysis

The entire data set of FAME profiles was used for principal components analysis of the overall sequential anaerobic/aerobic treatment process. This was used to determine if the microbial populations had changed significantly from time zero to the final aerobic sampling point. Three principal components were sufficient to describe over 86% of the variation in the data; Principal Components 1, 2, and 3 described 72.3%, 8.5%, and 5.3% of the total variation, respectively. Two-factor plot of the principal component analysis are shown in Figures 8.26 – 8.28. The clusters mapped by the principal components analysis were similar to those in Section 8.C.3 (aerobic phase). Three distinct clusters

were mapped by Principal Components 1 and 2. The first cluster, differentiated by the double box, contained samples from wk 5 and wk 7 from all of the treatments. The second cluster contained samples from butyrate and butyrate+biphenyl amended microcosms at wk 30 and wk 60 from the anaerobic phase as well as from butyrate-amended microcosms at wk 22 and is shown in the box with thick borders. The remaining samples clustered together. It seems contradictory that those microcosms amended with butyrate under anaerobic conditions did not cluster with the rest of the anaerobic samples as was observed in the anaerobic-only PCA analysis. However, this can be explained by the addition of all of the samples together. The larger the number of samples, the higher the power with which comparisons can be made using multivariate statistics. Thus, differences unable to be picked up with the anaerobic samples alone can be detected looking at the entire sample set.

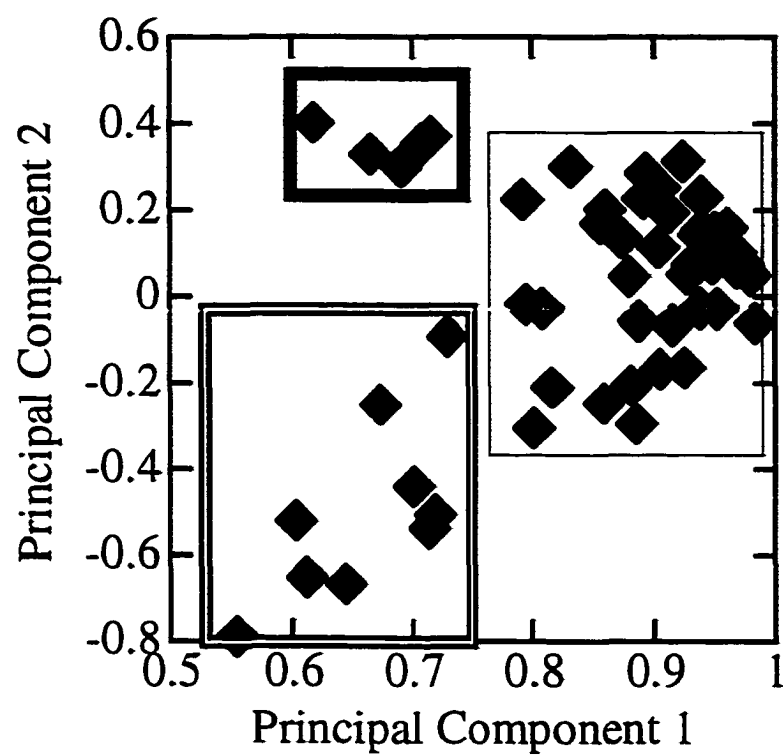


Figure 8.26 Principal components analysis of overall sequential treatment FAME profiles, Factor 1 by Factor 2.

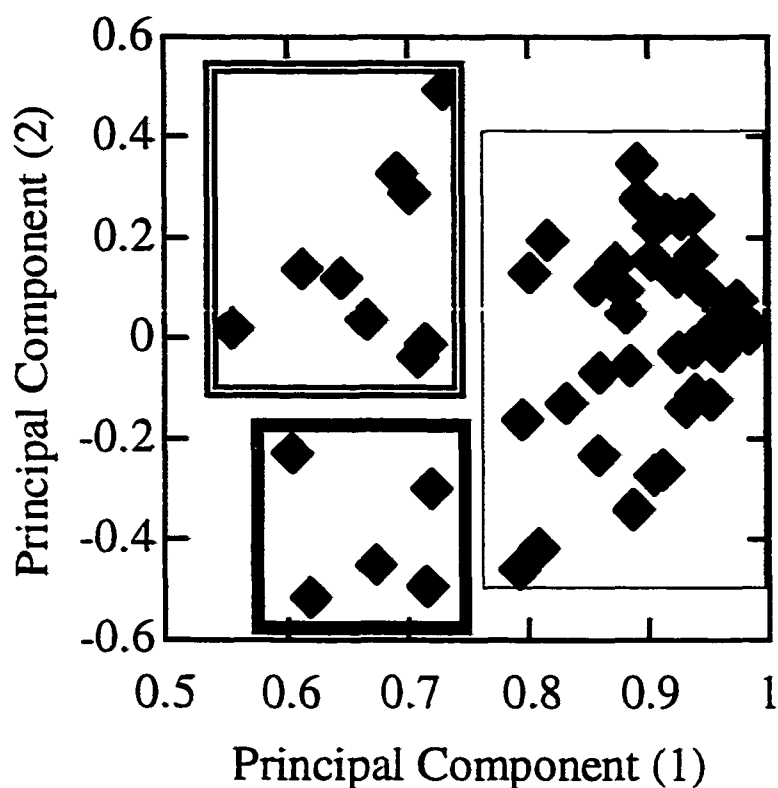


Figure 8.27 Principal components analysis of overall sequential FAME profiles, Factor 1 by Factor 3.

Samples with eigenvalues less than zero for Principal Component 2 were enriched in *cy17:0* and *18:1 ω 7c*, both indicative of aerobic microorganisms and observed in the PCA for aerobic phase analysis. The clusters mapped by Principal Components 1 and 3 were nearly identical to those mapped by Principal Components 1 and 2, with the exception that those samples that had positive eigenvalues for Principal Component 2 had negative eigenvalues for Principal Component 3 and vice versa (Figure 8.27). The data in both graphs indicate that the factor having the greatest effect on microbial community composition as determined by PLFA/FAME analysis was not different treatments (e.g.

biphenyl amended, methanol amended) but the switch from anaerobic to aerobic conditions. The samples from anaerobic sampling points mapped with those from time zero; thus, the anaerobic phase conditions did not significantly alter the overall microbial community structure. Butyrate amendment appeared to have some effect on the microbial community after long periods under anaerobic conditions, but this effect was less important than the switch to aerobic conditions for final determination of community composition.

Principal Components 2 and 3 did not map any clusters within the data (Figure 8.28) and thus did not provide any additional information about community structure.

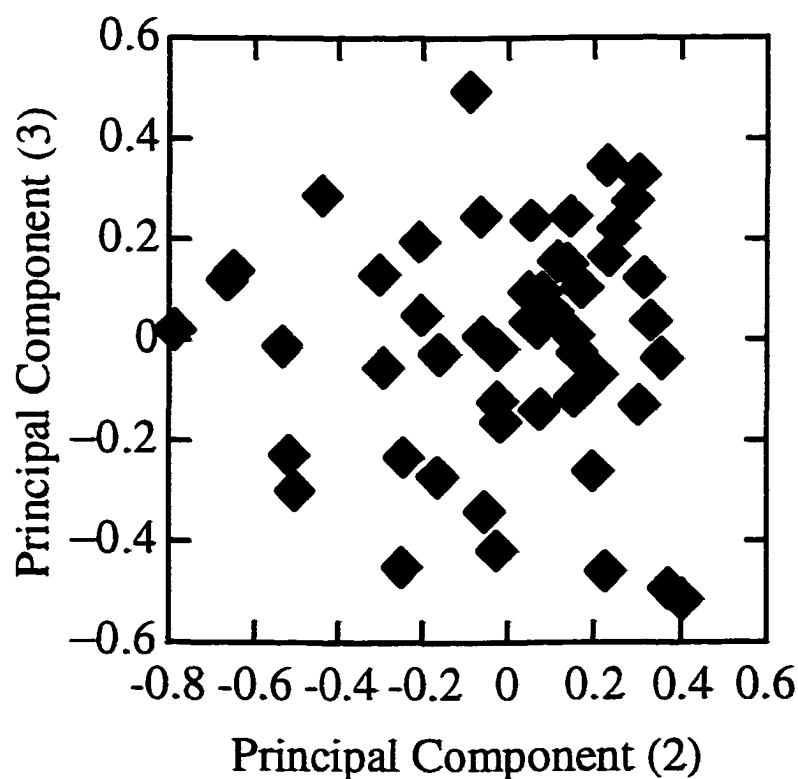


Figure 8.28 Principal components analysis of overall sequential FAME profiles, Factor 2 by Factor 3.

8.E. Conclusions

A sequential treatment process was evaluated for the overall reduction of PCB concentration. A 60-wk anaerobic phase was followed by 7 wk under aerobic conditions. Hydrogen peroxide (0.25%) was used to initiate the switch from anaerobic conditions to aerobic conditions. Similar results were obtained for anaerobic dechlorination patterns and extents of dechlorination in unamended, butyrate- and methanol-amended microcosms. In addition to the original three treatments, three more treatments were investigated, biphenyl-amended, butyrate+biphenyl, and methanol+biphenyl amended. In each of these, biphenyl was intended to act as an inducer of degradation under the aerobic phase, but was almost completely degraded within the first 4 wk of the anaerobic phase and thus served as an additional treatment variable for both phases. As was observed in the anaerobic-only treatment, significant degradation of the trichlorobiphenyl occurred in the first 12-22 wk of the anaerobic phase until reducing conditions were obtained, at which point the degradation of trichlorobiphenyl ceased. Biphenyl-amended microcosms displayed the highest extent of dechlorination, but biphenyl was not believed to be serving as an electron donor. It is possible that the same electron donor acting in unamended microcosms, hypothesized to be an inorganic electron donor or the natural organic matter, was also being utilized in biphenyl-amended microcosms. Butyrate and methanol were completely degraded in the system, but did not appear to be enriching for dechlorinating populations.

PLFA/FAME analysis of the anaerobic phase microcosm samples showed that anaerobic fatty acid biomarkers increased while those that are markers for aerobic microorganisms remained constant. This was an indication that an increase in the

anaerobic population occurred, but that the aerobic population was able to survive during the prolonged anaerobic phase. Principal components analysis showed no clustering of different samples, indicating that despite the changes in mole percent of certain fatty acids, overall the microbial community composition did not change significantly from time zero samples over the 60 wk anaerobic phase.

After 60 wk under anaerobic conditions, hydrogen peroxide was added and aerobic conditions were initiated. In the aerobic phase, complete degradation of the remaining trichlorobiphenyl occurred as well as degradation of re-dosed biphenyl. In addition, degradation of the dechlorination product 2,2',5,5'-tetrachlorobiphenyl occurred. Degradation of the trichlorobiphenyl occurred more slowly than was observed in the aerobic-only treatment, indicating that either the hydrogen peroxide addition or the prolonged time under anaerobic conditions had affected the aerobic microbial population. PLFA/FAME analysis detected a significant decrease in microbial community concentration directly after addition of hydrogen peroxide. In those microcosms amended with biphenyl, the total biomass concentrations then increased at wk 5 and wk 7. In those microcosms, not amended with biphenyl, total biomass concentrations remained constant for the remainder of the 7 wk indicating that biphenyl was able to either enrich for microorganisms less susceptible to hydrogen peroxide toxicity or had increased the concentration of microorganisms enough that they were able to rebound better than in non biphenyl-amended microcosms. PCA analysis showed that samples from wk 5 and wk 7 from all treatments were more similar than those taken from wk 0 and wk 3.

The sequential anaerobic/aerobic treatment achieved a decrease in the overall PCB concentration that was significantly higher than that achieved by either anaerobic or aerobic treatment alone in all treatments. The highest overall degradation occurred in biphenyl-amended treatments with 57.5% degradation of the original PCBs dosed being degraded. This amount of degradation was not significantly higher than in unamended treatments, but was significantly higher at a significance level of $p=0.1$ than all other treatments. Although differences in degradation were observed, few differences in microbial community composition as determined by PLFA/FAME analysis between treatments was observed. The differences observed were based on changes in community composition during the aerobic phase, with samples from wks 5 and 7 clustering together. This agrees with the DGGE analysis run on samples from the anaerobic-only treatments at wk 30. The microbial community composition was nearly the same in each treatment. This indicates that the same dechlorinating population was being used for each treatment, but this population was either enhanced or inhibited by different treatments.

8.F. References

1. Anid, P. J., L. Nies, and T. M. Vogel. 1991. Sequential anaerobic-aerobic biodegradation of PCBs in the river model, p. 428-436. *In* R. E. Hinchee and R. F. Olfenbuttel (ed.), *On-site Bioreclamation Processes for Xenobiotic and Hydrocarbon Treatment*. Butterworth-Heinemann, Columbus.
2. Boyle, A. W., C. J. Silvin, J. P. Hassett, J. P. Nakas, and S. W. Tanenbaum. 1992. Bacterial PCB biodegradation. *Biodegradation*. 3:285-298.

3. **Brown, J. F., and R. E. Wagner.** 1990. PCB movement, dechlorination, and detoxification in the Acushnet estuary. *Environmental Toxicology and Chemistry*. **9**:1215-1233.
4. **Evans, B. S., C. A. Dudley, and K. T. Klasson.** 1996. Sequential anaerobic-aerobic biodegradation of PCBs in soil slurry microcosms. *Applied Biochemistry and Biotechnology*. **57/58**:885-894.
5. **Franzmann, P. D., B. M. Patterson, T. R. Power, P. D. Nichols, and G. B. Davis.** 1996. Microbial biomass in a shallow, urban aquifer contaminated with aromatic hydrocarbons: analysis by phospholipid fatty acid content and composition. *Journal of Applied Bacteriology*. **80**:617-625.
6. **Kim, J., and G.-Y. Rhee.** 1997. Population dynamics of polychlorinated biphenyl-dechlorinating microorganisms in contaminated sediments. *Applied and Environmental Microbiology*. **63**:1771-1776.
7. **Knight, V. K., L. J. Kerkhof, and M. M. Haggblom.** 1998. Presented at the American Society for Microbiology, San Francisco.
8. **Nies, L., and T. M. Vogel.** 1990. Effects of organic substrates on dechlorination of Aroclor 1242 in anaerobic sediments. *Applied and Environmental Microbiology*. **56**:2612-2617.
9. **Quensen III, J. F., S. A. Boyd, and J. M. Tiedje.** 1990. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. *Applied and Environmental Engineering*. **56**:2360-2369.

10. **Rhee, G.-Y., R. C. Sokol, B. Bush, and C. M. Bethoney.** 1993. Long-term study of the anaerobic dechlorination of Aroclor 1254 with and without biphenyl enrichment. *Environmental Science and Technology*. **27**:714-719.

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

9.A. *Introduction*

This research investigated the bioremediation of polychlorinated biphenyls in soil microcosms. Polychlorinated biphenyls are common contaminants in sediments and soils throughout the world. Microbial degradation of PCBs is occurring naturally at many sites, but little is understood about how to enhance this degradation. While improvement and optimization of PCB bioremediation strategies is an important goal, this work has also focused on the microbial fundamentals of the degradation process in the hope that the results would be useful for developing bioremediation strategies for other pollutant mixtures with similar characteristics (e.g., chlorinated phenols).

Two soils previously contaminated with low levels of PCBs were tested in preliminary experiments and the indigenous microbial populations of both demonstrated potential for anaerobic dechlorination of PCBs. Only one was able to aerobically degrade less chlorinated congeners, and was thus chosen for use in the remainder of the work. Soil-slurry microcosms were utilized for all phases of work to allow for simultaneous testing of different treatment conditions. In addition to determining the change in PCB concentration, three non-culture based microbiological techniques were utilized to describe the microbial population. One of

these methods (PLFA/FAME) provided a description of overall community structure, e.g., the proportion of anaerobes to aerobes. The other two provided a more specific analysis based on the DNA sequences of organisms in the microbial community. The results from these methods were used to provide insight into the microbial community and the types of microorganisms carrying out the PCB degradation.

9.B. Anaerobic Dechlorination

Dechlorination was observed in all treatments but not in sterile controls. Unamended microcosms and those amended with biphenyl (which was degraded within 4 wks) showed significantly higher levels of dechlorination than any of the other treatments investigated. The higher rate of dechlorination for these two treatments was unexpected. The level of organic matter in the soil was very low, which suggests that there was insufficient organic matter to serve as an electron donor for PCB dechlorination. This result, coupled with the results from the DGGE and sequencing analysis, indicated that utilization of an inorganic electron donor might be occurring. Originally, ferrous iron appeared to be a possible candidate, but a balance of the reduction-oxidation reactions shows that this is not thermodynamically favorable ($\Delta G^\circ = 39.3$ kcal/mol). Unfortunately, this does not explain what mechanism and electron donor are used for the dechlorination.

Pulliam Holoman et al. found evidence of iron-respiring microorganisms in a 2,3,5,6-tetrachlorobiphenyl dechlorinating microbial community using 16S rDNA sequencing, although the PCB-dechlorinating cultures were methanogenic (6). In this work, levels of ferrous iron did not increase during the anaerobic phase. In fact, they decreased by an order of magnitude from wk 22 to wk 60, again supporting the hypothesis that ferrous iron was being

utilized as an electron donor. Ferrous iron-oxidation occurs at redox levels near zero and thus could have been occurring in the microcosms but could have been unrelated to the observed dechlorination.

The conditions of dechlorination were also unusual. To date, only highly reducing conditions that can support the growth of methanogens and/or sulfate-reducers have been shown to support PCB dechlorination. The conditions in this study were only slightly reducing; redox levels never dropped below -90 mV, and thus facultative anaerobes may have played a role in the dechlorination observed. DGGE analysis revealed the presence of a sequence type similar to one such facultative anaerobe, *Dechlorimonas* sp., which suggests that this organism may play a role in the reductive dechlorination of the PCBs in this system. Besides this organism, no other sequence types were identified that matched with anaerobic microorganisms. In fact, nearly all were representative of aerobic microorganisms from genera that contain known PCB degraders. Only a small fraction of the total clones were sequenced; thus it is possible that anaerobes carrying out the dechlorination were not identified. Because methanogens and sulfate-reducers have been implicated in PCB dechlorination studies by other researchers, a PCR primer set specific to each of these microbial groups was utilized to test for their presence in our system. Using the PCR method, neither type of microorganism was found to be present in the anaerobic microcosms.

PLFA/FAME analysis indicated that there was an increase in the concentration of anaerobic microorganisms over the course of the 60-wk sampling period. A decrease in aerobic fatty acid biomarkers was not observed; these markers remained constant throughout the sampling period. Thus the results of the PLFA/FAME analysis and DGGE/sequencing

analysis concur that aerobic organisms were able to survive under the conditions of the anaerobic phase. This was promising for implementation of the sequential treatment.

9.C. Aerobic Degradation

Aerobic degradation of both biphenyl and the dosed trichlorobiphenyl was observed in all treatments with complete degradation of both by 5 wk. No degradation of 3,3',4,4'-PCB or 2,2',4,4',5,5'-PCB was observed in any of the aerobic microcosms. Degradation in biphenyl-amended microcosms occurred at a higher rate than in unamended microcosms. Conflicting reports have been described on the effect of biphenyl on PCB degradation (2, 4, 5). In this work, biphenyl does not appear to be required for degradation of less chlorinated congeners, but does appear to enhance that degradation. PLFA/FAME analysis showed significant changes in microbial community composition between the two aerobic treatments. In biphenyl-amended microcosms, markers for anaerobic microorganisms decreased while those indicative of aerobes increased. PCA analysis showed that two clusters were mapped by Principal Components 1 and 2. In one cluster were unamended treatments at wk 0, 1, and 7. The other cluster contained all of the biphenyl-amended microcosms as well as unamended treatment data at wk 2 and 5. Biphenyl and 2,3',4'-trichlorobiphenyl were both completely degraded by wk 5. This indicates that while biphenyl addition had a significant effect on community composition, actively degrading populations in unamended microcosms were similar to those in biphenyl-amended microcosms. By wk 7 however, the microbial community composition in unamended microcosms had begun to return to their original state whereas those in biphenyl-amended microcosms had not.

DGGE combined with sequencing analysis of 16S rDNAs showed the presence of many sequence types similar to organisms known to degrade PCBs. Several *Comamonas* species, a *Pseudomonas* species and several *Acinetobacter* species were identified. One of the most characterized PCB degraders is *Acinetobacter* P6. In addition to the sequencing, a PCR primer set developed to amplify a region of the *bphC* gene was tested on DNA extracted from aerobic samples. This primer set was able to detect sequences in time zero samples, but not in any samples from later time points. This is an indication that a microbial population with more divergent genes for PCB degradation was enriched for in both unamended and biphenyl-amended treatments.

9.D. Oxygenation

A set of experiments were performed to evaluate the effect that hydrogen peroxide or air addition for initiation of aerobic conditions after the anaerobic phase would have on the microbial population and its ability to aerobically degrade PCBs. Little difference was observed in either PCB-degrading ability or in microbial community composition between air treated and hydrogen peroxide treated microcosms at all levels. A slight increase in DO utilization rates was observed in those microcosms amended with higher concentrations of hydrogen peroxide. The rate and extent of degradation of 2,3',4'-PCB was less than that observed in the aerobic phase alone for all treatments. Because this phenomenon occurred in air-treated microcosms also, the cause could not be ascribed to the addition of hydrogen peroxide. The decrease in degradation rates and extent was probably due to a decrease in the viability of aerobic microorganisms caused by the week under anaerobic conditions. Although no difference in PCB degradation was observed between treatments, the middle concentration

of hydrogen peroxide was chosen for use in the sequential treatment process because of the higher rate of DO utilization observed.

9.E. Sequential Results

The sequential anaerobic/aerobic treatment achieved a greater decrease of total PCB concentration than did either the anaerobic or aerobic phase alone. The highest degradation observed was 57.5% for sequential treatment, 25% for anaerobic treatment, and 27% for aerobic treatment in biphenyl-amended microcosms. Unamended and biphenyl-amended microcosms had significantly higher amounts of degradation than did the other treatments, which were not significantly different from each other. This higher amount of degradation was based on higher extents of dechlorination, as the trichlorobiphenyl was completely degraded in all treatments by the end of the sequential treatment. The tetrachlorobiphenyl added to the soil, 3,3',4,4' was not degraded to a significant extent in either aerobic or anaerobic phase treatment. Biphenyl amendment enhanced both the anaerobic and aerobic phases of treatment and thus appears to be important for further design considerations. A benefit due to electron donor amendment was not apparent; in fact, addition of either methanol or butyrate decreased the extent and rate of dechlorination of PCBs. This may have been caused by either an increase in non-PCB degrading organisms or utilization of these compounds by PCB-degrading organisms to the exclusion of dechlorination. However, since dechlorination had not completely leveled off, it remains to be seen if the butyrate- or methanol-amended microcosms would reach the extent of dechlorination achieved in the unamended and biphenyl amended microcosms. During the aerobic phase, degradation of the trichlorobiphenyl and biphenyl were slower than that observed in the aerobic-only microcosms, but complete degradation of both was observed.

Changes in microbial community composition were determined by PLFA/FAME analysis and showed that significant changes in the microbial community occurred primarily during aerobic degradation. Unlike the results observed in the aerobic phase only, samples from both biphenyl-amended and unamended microcosms at wk 5 and 7 clustered together indicating few differences in overall microbial community composition between the two. The aerobic population was able to survive under conditions in the anaerobic phase while the anaerobic population appeared to decrease during the final weeks of the aerobic phase. To date, nearly all of the sequential treatment studies for PCBs have involved inoculation with a known aerobic degrading microorganisms at the time aerobic conditions were initiated (1, 3). This work is one of the first to demonstrate the presence and viability of both anaerobic and aerobic indigenous organisms able to carry out the sequential treatment of PCBs.

9.F. Recommendations for Future Work

This work has demonstrated that sequential anaerobic/aerobic treatment carried out by indigenous organisms was successful in this particular soil. Dechlorination of the hexachlorobiphenyl was achieved, but no degradation of the tetrachlorobiphenyl occurred. While some hypotheses could be made about treatment conditions, for instance the use of biphenyl as an inducer of both the anaerobic dechlorination and aerobic degradation of the PCBs, questions remain. If answered even more information for optimization of the sequential treatment process would be provided. In particular, we were unable to identify the specific microbial population carrying out the dechlorination. As sulfate-reducers and methanogens were ruled out, the dechlorination observed could be due to a previously unreported mode of metabolism coupled with dechlorination that could be utilized in other remediation systems.

PLFA/FAME analysis provided important information on the fate of large groups of organisms, i.e. aerobic survival, anaerobic concentration, and the presence of eukaryotes. However, in this system, it was less effective as an indicator of which samples were undergoing PCB dechlorination. To understand the anaerobic phase more completely, efforts should be centered on further analysis of the anaerobic population using DGGE and sequencing to identify potential dechlorinating microorganisms. The development of primers specific to those potential candidates could be used with quantitative PCR or another quantitative method to observe changes in that population's concentration over the course of the anaerobic phase. In addition, other experiments should be carried out to test for iron-oxidation in the anaerobic soil microcosms.

Even longer-term microcosm studies than used in this work would be useful in determining whether a plateau had indeed been reached for the anaerobic phase or if further dechlorination would have occurred. These studies could focus on the use of either no electron donor amendment or biphenyl amendment. Ferrous iron and ferric iron concentrations should be monitored from time zero to better understand the role that iron plays in this system. Once more was understood about the microbial population, studies with more extensive numbers of congeners or with Aroclor mixtures could assess the PCB degradative ability of the population. In addition, once the dechlorinating population was identified, this information could be used to enrich this population to achieve greater extents of dechlorination, as this step appears to be the rate-limiting step in the process.

9.G. Significance

While important pollutants, polychlorinated biphenyls are not the only contaminants that are potential candidates for sequential anaerobic/aerobic treatment. The same design issues apply for many chlorinated contaminants, such as what electron donor, if any, is ideal for the anaerobic dechlorination phase and whether or not cometabolism is important for the aerobic phase. This study has demonstrated the feasibility of inducing anaerobiosis and aerobiosis in sequence in an environment that simulates the unsaturated zone. Many contaminated waste sites exist naturally under anaerobic conditions. If substantially reducing conditions exist, the existence of aerobic populations able to survive under those conditions is questionable. There is still substantial resistance towards bioaugmentation to remediate environmental sites. Even if bioaugmentation was chosen as a treatment method, questions remain about the survival and viability of the introduced populations. Thus, the ability to induce aerobic degrading microorganisms for sequential treatment without augmentation is highly desired. This study has shown the feasibility of using PLFA/FAME analysis to determine if aerobic microbial populations are present at a certain anaerobic site. This information could help in decision making to determine if sequential treatment is feasible at a particular site.

The indigenous microorganisms used in this study were better able to carry out dechlorination in those microcosms not amended with an additional electron donor. Much focus has been geared toward determining the best electron donor for stimulation of dechlorination. However, in this work, the microorganisms may have already been adapted to use the resources available in the soil and addition of electron donor decreased the microbial dechlorination observed. Thus, it appears as though the electron donor concentration was not initially limiting in this system. After 30-wk in the unamended treatments, dechlorination had

leveled off, perhaps indicating that the electron donor had become limiting. Addition of the electron donors methanol and butyrate decreased the rate and extent of PCB dechlorination. It is possible that these additions did not enhance PCB dechlorination due to a lack of fermenters able to produce H_2 from their use. However, both of these compounds were degraded completely in the microcosms. This suggests that specific attention must be paid in the determination of the rate-limiting step to pollutant dechlorination and degradation. Another example of this importance of the rate-limiting step was observed in the oxygenation treatments. Different levels of either air or hydrogen peroxide treatment did cause differences in either population composition or in PCB degradation. This indicates that oxygen was not determining the rate at which the PCBs were being degraded aerobically.

The use of microbial ecology methods (DGGE/sequencing) may allow for identification of “microbial markers” that can be used in future feasibility studies. For example, the development of competitor sequences for cPCR can be used to test for the presence of indigenous dechlorinating organisms at waste sites contaminated with chlorinated compounds. The sequences identified in this work are particularly interesting as they may provide information about a new group of PCB dechlorinating bacteria that carry out dechlorination at redox conditions that are not highly reducing. This testing procedure could alleviate the need for extensive feasibility studies required to begin *in situ* treatment. These “microbial markers” could also be used during a bioremediation treatment as a type of process control to monitor the course of the biodegradation and provide feedback that could then be used to optimize the process.

9.H. References

1. **Anid, P. J., L. Nies, and T. M. Vogel.** 1991. Sequential anaerobic-aerobic biodegradation of PCBs in the river model, p. 428-436. *In* R. E. Hinchee and R. F. Olfenbittel (ed.), *On-site Bioreclamation Processes for Xenobiotic and Hydrocarbon Treatment*. Butterworth-Heinemann, Columbus.
2. **Brunner, W., F. H. Sutherland, and D. D. Focht.** 1985. Enhanced biodegradation of polychlorinated biphenyls in soil by analog enrichment and bacterial inoculation. *Journal of Environmental Quality*. **14**:324-328.
3. **Evans, B. S., C. A. Dudley, and K. T. Klasson.** 1996. Sequential anaerobic-aerobic biodegradation of PCBs in soil slurry microcosms. *Applied Biochemistry and Biotechnology*. **57/58**:885-894.
4. **Focht, D. D., and W. Brunner.** 1985. Kinetics of biphenyl and polychlorinated biphenyl metabolism in soil. *Applied and Environmental Microbiology*. **50**:1058-1063.
5. **Mohn, W. W., K. Westerberg, W. R. Cullen, and K. J. Reimer.** 1997. Aerobic biodegradation of biphenyl and polychlorinated biphenyls by arctic soil microorganisms. *Applied and Environmental Microbiology*. **63**:3378-3384.
6. **Pulliam Holoman, T. R., M. A. Elbersen, L. A. Cutter, H. D. May, and K. R. Sowers.** 1998. Characterization of a defined 2,3,5,6-tetrachlorobiphenyl-*ortho*-dechlorinating microbial community by comparative sequence analysis of genes coding for 16S rRNA. *Applied and Environmental Microbiology*. **64**:3359-3367.

APPENDIX I

MEDIA AND SOLUTIONS

DNA Extraction Solutions:

200 mM Phosphate Buffer

to make 200 mL:

NaH₂PO₄ 0.1473 g

Na₂HPO₄ 5.08 g

Bring pH to 8.0 with HCl and autoclave 20 min, store at room temperature

Polymerase Chain Reaction Solutions:

TE

0.01 M Trizma Base, pH 8.0 (Sigma)

0.005 M EDTA, pH 8.0 (National Diagnostics)

10X PCR Amplification Buffer

500 mM	KCl
100 mM	Tris- Cl (pH 8.3 at room temperature)
15 mM	MgCl ₂
0.1%	gelatin

1X PCR Amplification Buffer

10%	10X PCR Amplification Buffer
1%	dinucleotide triphosphates (dNTP's)
in ddH ₂ O	

10X TBS

60.85 g	Tris Base
87.66 g	NaCitrate
add ddH ₂ O to 2 L, bring to pH 7.5	

Lipid Analysis Extraction Solutions:**20 mM Ammonium Acetate**

0.154 g	Ammonium Acetate
100 mL	Methanol

50 mM Phosphate Buffer

1.74 g K_2HPO_4

Dissolve in 200 mL ddH₂O, bring pH to 7.4 with 1 N HCl

0.2 N KOH

2 pellets KOH

16 mL methanol

1 N Acetic Acid

570 μL glacial acetic acid

Dilute to 10 mL with ddH₂O

Serum Bottle Experiments:**PAS media**

PA Concentrate: to make 1 L

KH_2PO_4 21.94 g

K_2HPO_4 56.77 g

NH_4Cl 27.61 g

PAS Salt 100X: to make 1 L

MgSO_4 19.5 g

MnSO₄-H₂O 5 g

FeSO₄-7H₂O 1 g

CaCl₂-2H₂O 0.3 g

3-4 drops conc. H₂SO₄

to prepare PAS for plates: for 20 plates, autoclave 500 mL in a 1 L flask for 20 min

38.5 mL PA concentrate

5 mL PAS 100X salts

455 mL ddH₂O

7.5 g bacto-agar

Anaerobic Mineral Media

100X Stock Solution: in 1 L ddH₂O add

ZnCl ₂	.019 g	NiSO ₄ -6H ₂ O	0.02 g
FeSO ₄ -7H ₂ O	0.1 g	AlCl ₃ -6H ₂ O	0.04 g
H ₃ BO ₃	0.038 g	CoCl ₂ -6H ₂ O	0.15 g
CuCl ₂ -2H ₂ O	0.02 g	Na ₂ MoO ₄ -H ₂ O	0.023 g

1X Media: in 1.5 L add 15 mL of 100X stock solution to 1000 mL ddH₂O

$(\text{NH}_4)_2\text{HPO}_4$	0.12 g	NH_4Cl	1.5 g
K_2HPO_4	0.3 g	NaCl	0.015 g
CaCl_2	0.015 g	MgCl_2	0.075 g

Stir until all salts are dissolved, bring volume to 1.5 L, autoclave for 20 min

Denaturing Gradient Gel Electrophoresis Solutions:

40% Acrylamide

37.5 g	acrylamide
10 g	bis

Dissolve in 962.5 mL ddH₂O, store in refrigerator

10X TAE Buffer

242.2 g	Tris
82.05 g	Sodium Acetate
100 mL	EDTA (0.5 M)

Dissolve in 4.5 L ddH₂O, bring volume to 5 L, store at room temperature

Cleaning Solution for Gel Plate

10 M	NaOH
0.2 M	Sodium Dodecyl Sulfate (keep as emulsion)

0% Denaturing Solution

18.8 mL 40% acrylamide solution

10 mL 10X TAE

79.2 mL ddH₂O

Stir for several hours, filter solution and store in refrigerator, covered

50% Denaturing Solution

18.8 mL 40% acrylamide

10 mL 10X TAE

20 mL formamide (deionized)

21 g urea

Bring to 100 mL using ddH₂O, stir for several hours, filter solution and store in refrigerator, covered

80% Denaturing Solution

18.8 mL 40% acrylamide

10 mL 10X TAE

32 mL formamide (deionized)

33.6 g urea

Bring to 100 mL using ddH₂O, stir for several hours, filter solution and store in refrigerator, covered

100% Denaturing Solution

18.8 mL	40% acrylamide
10 mL	10X TAE
40 mL	formamide (deionized)
42 g	urea

Bring to 100 mL using ddH₂O, stir for several hours, filter solution and store in refrigerator, covered

APS Solution

0.1 g	ammonium persulfate
500 mL	ddH ₂ O

Dissolve immediately prior to use

Single-Strand Polymorphism Conformation Gel:

Denaturing Loading Mixture (DLM)

0.005 g	xylene cyanol blue
0.005 g	bromophenol blue
9 mL	(deionized) Formamide

0.2 mL 1 M NaOH

0.8 mL ddH₂O

Combine in a 15 mL conical Falcon tube. Aliquot in 1 mL amounts into 1.5 mL microcentrifuge tubes. Store in freezer.

Cloning

SOC Medium

2% tryptone

0.5% yeast extract

10.0 mM NaCl

2.4 mM KCl

10.0 mM MgCl₂·6H₂O

20.0 mM glucose

For 1 liter, dissolve 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 950 mL ddH₂O. Prepare a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 mL ddH₂O. Add 10 mL of KCl stock to the first solution. Adjust the pH to 7.0 with 5 M NaOH, then bring the volume to 980 mL with ddH₂O. Prepare a 1 M solution of MgCl₂ (20.33g in 100 mL ddH₂O). Autoclave both solutions for 20 min. Filter sterilize a 2 M solution of glucose (36 g glucose in 100 mL ddH₂O). After

autoclaved solutions have cooled, add 10 mL of the filter-sterilized 2 M glucose solution and 10 mL MgCl_2 . Store at room temperature.

APPENDIX II

GC ACQUISITION PROGRAMS AND CALIBRATION CURVES

The following is a summary of the GC-MS method called "PCB".

ACQUISITION PARAMETERS

General Information:

Inlet	GC
Tune File	ATUNE.U
Acquisition mode	Scan

MS Information:

Solvent delay:	7 min
EM Absolute:	TRUE

Scan Parameters:

Low Mass:	50
High Mass:	500
Threshold:	550
Sampling #:	2

GC Inlet Information (inlet B):

Inlet B Pressure Program Information:

Constant Flow:	Off
Constant Flow Pressure:	0.0 psi
Constant Flow Temp:	50 C
Initial Pressure:	45 psi
Initial Time:	0.25 min

<u>Level</u>	<u>Rate (psi/min)</u>	<u>Final Pres. (psi)</u>	<u>Final time(min)</u>
1	99.0	25.0	0.0
2	0		

Inlet B Flow Settings:

Column length: 50 m
Column diameter: 0.2 mm
Gas: He (99.998% pure)
Vacuum compensation: on
Pressure: 45.0 psi
Flow: 1.89 mL/min
Linear velocity: 38.3 cm/sec

GC Temperature Information:**GC Zone Temperatures:**

Injection B: 250 C
Detector B: 280 C

Oven Parameters:

Oven Equilibration time: 0.0 min
Oven maximum: 325 C
Oven: on
Cryo: off
Ambient: 20 C
Cryo blast: off

Oven Program:

Initial temp: 50 C
Initial time: 2 min

<u>Level</u>	<u>Rate (C/min)</u>	<u>Final temp (C)</u>	<u>Final time (min)</u>
1	20.0	160	0.0
2	5.0	294	7.0
3	0.0	50	0.0

Run time: 41.3 min

Injector Information:

Injection source: auto
Injection location: rear
Sample washes: 1
Sample pumps: 6
Injection volume: 1 microliter
Syringe size: 10 microliter
On column: off
Solvent A washes: 2
Solvent B washes: 2
Viscosity delay: 0 sec
Plunger speed: fast

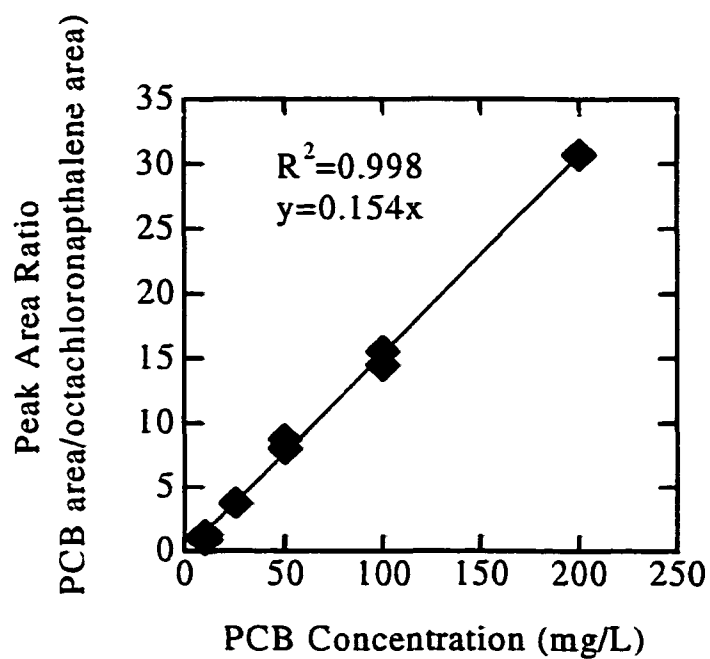


Figure II.1 Sample calibration curve for 2,3',4'-trichlorobiphenyl.

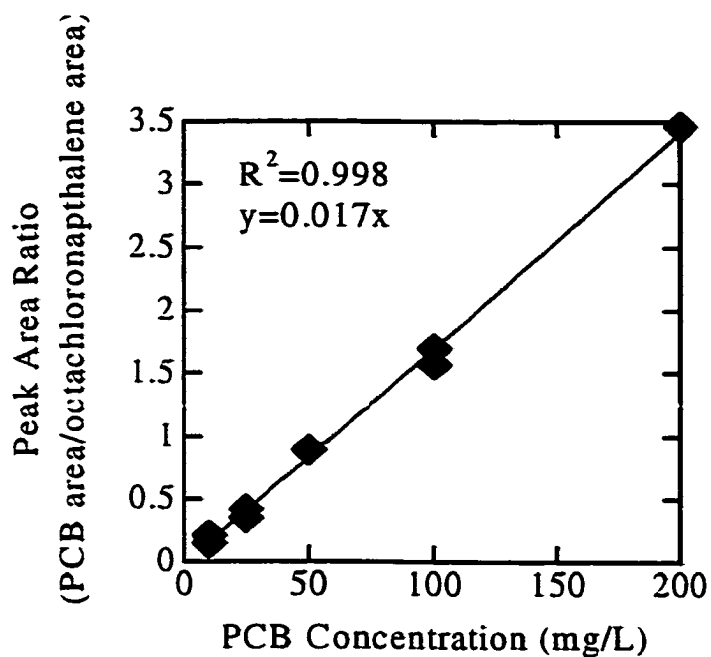


Figure II.2. Sample calibration curve for 3,3',4,4'-tetrachlorobiphenyl.

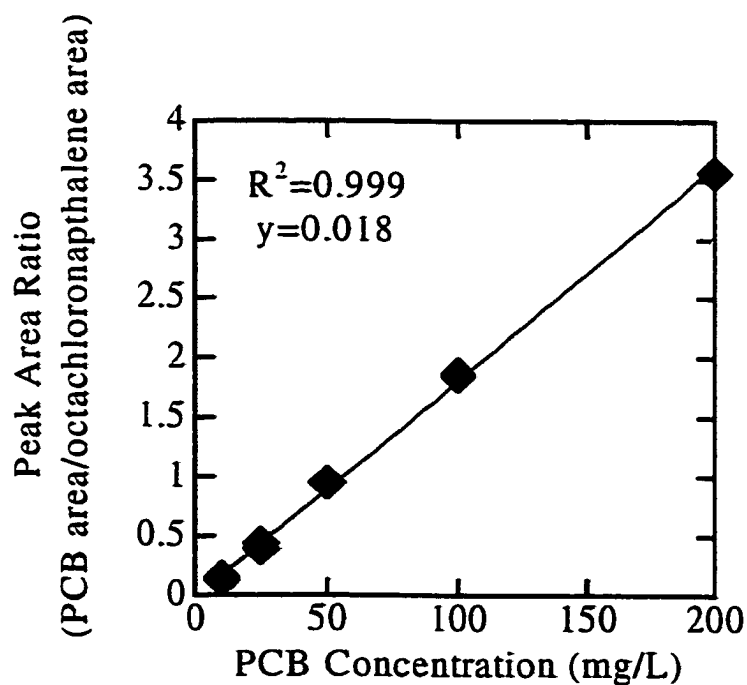


Figure II.3. Sample calibration curve for 2,2',4,4',5,5'-hexachlorobiphenyl.

"FAME" Method:

ACQUISITION PARAMETERS

General Information:

Inlet	GC
Tune File	ATUNE.U
Acquisition mode	Scan

MS Information:

Solvent delay:	11 min
EM Absolute:	TRUE

Scan Parameters:

Low Mass:	50
High Mass:	500
Threshold:	550
Sampling #:	2

GC Inlet Information (inlet B):

Inlet B Pressure Program Information:

Constant Flow:	Off
Constant Flow Pressure:	0.0 psi
Constant Flow Temp:	50 C
Initial Pressure:	45 psi
Initial Time:	0.25 min

Level	Rate (psi/min)	Final Pres. (psi)	Final time(min)
1	99.0	25.0	0.0
3	0		

Inlet B Flow Settings:

Column length:	50 m
Column diameter:	0.2 mm
Gas:	He (99.998% pure)
Vacuum compensation:	on
Pressure:	45.0 psi
Flow:	1.89 mL/min
Linear velocity:	38.3 cm/sec

GC Temperature Information:

GC Zone Temperatures:

Injection B:	270 C
Detector B:	290 C

Oven Parameters:

Oven Equilibration time:	0.0 min
Oven maximum:	325 C
Oven:	on

Cryo: off
Ambient: 20 C
Cryo blast: off

Oven Program:
Initial temp: 50 C
Initial time: 2 min

<u>Level</u>	<u>Rate (C/min)</u>	<u>Final temp (C)</u>	<u>Final time (min)</u>
1	10.0	160	0.0
2	4.0	294	7.0
3	0.0	50	0.0

Run time: 41.3 min

Injector Information:

Injection source: auto
Injection location: rear
Sample washes: 1
Sample pumps: 6
Injection volume: 1 microliter
Syringe size: 10 microliter
On column: off
Solvent A washes: 2
Solvent B washes: 2
Viscosity delay: 0 sec
Plunger speed: fast

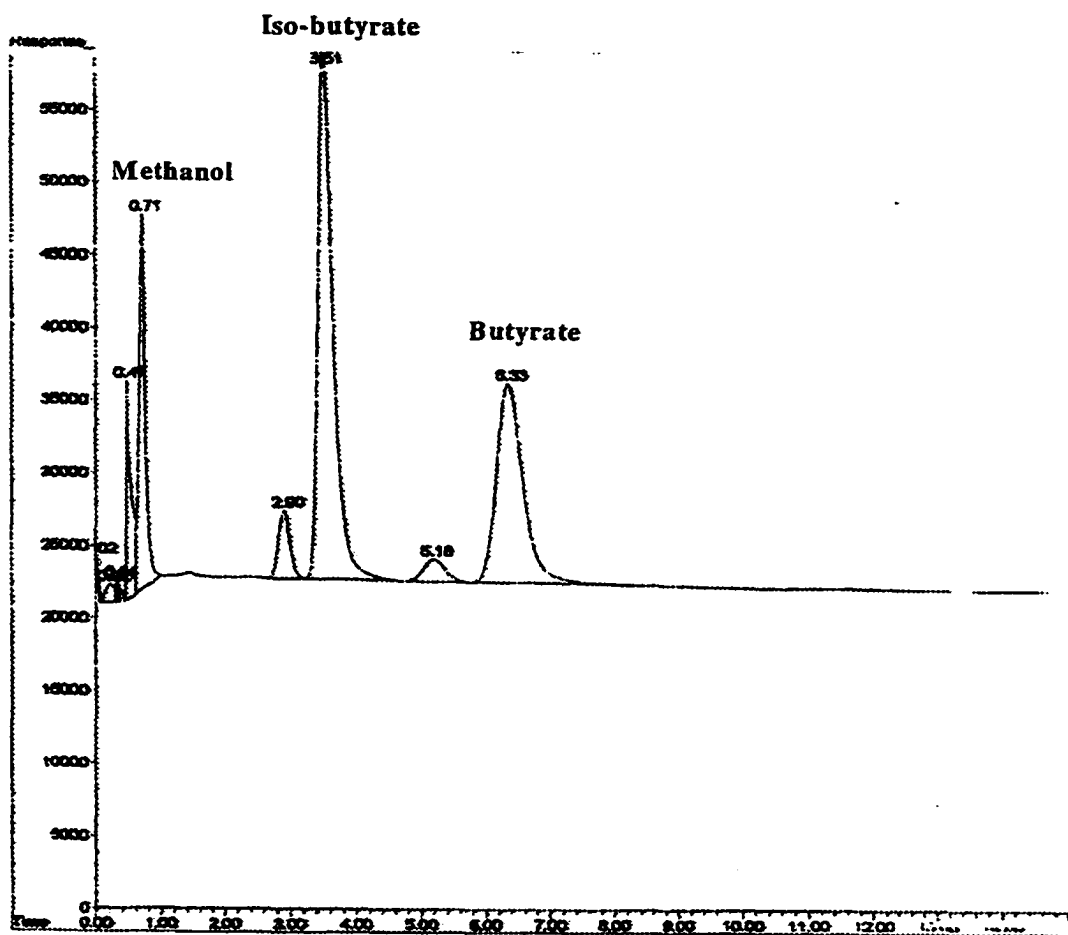


Figure II.4 Typical standard chromatogram from GC-FID analysis of methanol and butyrate. A 100 mg/L standard solution is shown.

APPENDIX III

PRIMER SEQUENCES AND PCR PROGRAMS

Primer Sequences utilized for this study

Primer Name	Sequence (5' to 3')	Reference
16s-341f	Ctccctcctctctcttctttctttttcacggggggg	[Muyzer, 1993 #307]
16s-534r	Attaccgaggctgctgg	[Muyzer, 1993 #307]
ME1	GCMATGCARATHGGWATGTC	[Hales, 1996 #111]
ME2	TCATKGCRTAGTTDGGRTAGT	[Hales, 1996 #111]
SRB	CCCGGGATCCTGACGCAGCIAC GCCG	[Amman, 1992 #183]
UNTIV907R	TCTAGAAGCTTCCCCGTCAATT CCTTTGAGTTT	[Amman, 1992 #183]
P51D	CGGGATCCCGGGTTACCTCGGG TTGACCGT	[Erb, 1993 #89]
P52U	CCCAAGCTTGGGTAGAAGGTC AGCGCCTGGTC	[Erb, 1993 #89]
16S-63fr	CAGGCCTAACACATGCAAGTC	[Marchesi, 1998 #271]
16S-1387r	GGGCGG(W)GTGTACAAGGC	[Marchesi, 1998 #271]

In these sequences, M=A/C, R=A/g, W=A/T, S=C/g, Y=C/T, K=g/T, V=A/C/g,

H=A/C/T, D=A/g/T, B=C/g/T, N=A/C/g/T

PCR Temperature Programs

DGGE (16S-341f & 16S-534r)

Step	Temperature (°C)	Time (min)
1	95	5
2	80	10

3	95	0.5
4	65	1
5	72	3
6	94	1
7	increase 0.5/cyc	
8	72	3
9	18 times to step 5	
10	94	1
11	55	1
12	72	3
13	8 times to step 9	
14	94	1
15	55	1
16	72	10

Methanogen (ME1 & ME2)

<u>Step</u>	<u>Temperature (°C)</u>	<u>Time (min)</u>
1	95	5
2	80	10
3	95	0.6
4	54	1.5

5	72	3
6	30 times to step 3	
7	72	10

16S-F (16S-63f & 16S-1387r)

<u>Step</u>	<u>Temperature (°C)</u>	<u>Time (min)</u>
1	95	5
2	80	10
3	95	0.5
4	50	0.5
5	72	1
6	29 time to step 3	
7	72	5

HotFang

<u>Step</u>	<u>Temperature (°C)</u>	<u>Time (min)</u>
1	95	5
2	80	10
3	92	1
4	48	1
5	72	2

6	10 times to step 3	
7	92	1
8	54	0.5
9	72	2
10	30 times to step 7	
11	72	7

Biphenyl Dioxygenase (P51D & P52U)

<u>Step</u>	<u>Temperature (°C)</u>	<u>Time (min)</u>
1	95	5
2	80	10
3	95	0.5
4	35	1
5	72	3
6	34 times to step 3	
7	72	10

Sulfate-reducing bacteria

<u>Step</u>	<u>Temperature (°C)</u>	<u>Time (min)</u>
1	95	5
2	80	10

3	95	3
4	58	2
5	72	3
6	30 times to step 3	
7	72	5

APPENDIX IV

PLFA/FAME ANALYSIS DATA

Treatment #1 – Aerobic Phase, Unamended

PLFA	0 wk	1 wk	2 wk	3 wk	5 wk	7 wk
pmol/g	264.6 ±94.9	282.9 ± 18.7	154.2 ± 69.7	295.2 ± 64.8	204.8 ± 55.2	322.1 ± 97.5
<i>i</i> 14:0	3.6 ± 1.5	3.3 ± 1.0	0.9 ± 0.4	0.4 ± 0.2	1.0 ± 0.5	1.4 ± 0.9
14:0	3.2 ± 0.8	1.8 ± 0.5	0.5 ± 0.3	3.5 ± 1.2	0.7 ± 0.2	3.0 ± 1.3
<i>i</i> 15:0	1.6 ± 0.9	1.7 ± 0.1	0.4 ± 0.3	1.9 ± 0.8	1.5 ± 0.5	2.3 ± 1.1
<i>a</i> 15:0	2.9 ± 0.4	2.2 ± 0.3	0.9 ± 0.5	2.2 ± 0.7	0.7 ± 0.1	2.6 ± 1.1
15:0	3.5 ± 0.4	1.7 ± 1.0	1.6 ± 0.8	2.1 ± 0.5	0.7 ± 0.5	1.2 ± 0.6
<i>i</i> 16:0	14.3 ± 2.5	7.8 ± 3.1	4.2 ± 1.7	6.7 ± 1.4	3.2 ± 0.9	21.9 ± 5.6
16:1ω7c	5.6 ± 1.8	3.1 ± 0.7	2.0 ± 0.8	2.8 ± 1.1	1.9 ± 0.6	8.7 ± 2.2
16:1ω7t	0.0	0.0	0.0	1.8 ± 0.8	0.0	0.6 ± 0.2
16:0	18.4 ± 10.2	45.9 ± 9.7	50.1 ± 3.1	48.8 ± 4.6	51.2 ± 8.3	32.7 ± 5.1
<i>cy</i> 17:0	0.0	2.7 ± 0.2	2.9 ± 2.2	4.8 ± 1.3	1.3 ± 0.9	1.1 ± 0.5
<i>i</i> 17:0	1.1 ± 0.8	0.0	0.5 ± 0.1	0.3 ± 0.2	0.0	0.4 ± 0.1
<i>a</i> 17:0	3.4 ± 0.5	2.1 ± 0.4	0.9 ± 0.2	1.0 ± 0.3	0.3 ± 0.1	1.9 ± 1.1
17:0	5.7 ± 0.9	1.0 ± 0.5	0.9 ± 0.1	1.4 ± 0.5	1.8 ± 0.3	1.6 ± 0.7
18:1ω9c	5.8 ± 1.4	9.8 ± 2.0	8.9 ± 1.1	8.7 ± 2.1	10.4 ± 2.5	4.7 ± 1.2
18:1ω7c	2.7 ± 1.0	4.2 ± 1.2	4.9 ± 0.6	7.0 ± 3.2	4.2 ± 1.1	1.3 ± 0.6
18:1ω7t	0.0	0.2 ± 0.1	0.0	0.0	0.0	0.0
18:0	8.1 ± 1.3	9.3 ± 2.5	16.1 ± 1.8	2.8 ± 3.2	20.4 ± 5.4	6.8 ± 3.6
18:2ω6,9	19.1 ± 6.2	3.4 ± 2.8	3.2 ± 1.9	2.8 ± 3.1	0.7 ± 0.1	5.2 ± 4.3
<i>cy</i> 19:0	3.0 ± 0.3	0.0	1.1 ± 1.0	1.0 ± 0.5	0.0	2.6 ± 0.1

Treatment #2, Aerobic Phase – Biphenyl-Amended

PLFA	0 wk	1 wk	2 wk	3 wk	5 wk	7 wk
pmol/g	264.6 ± 94.9	319.7 ± 110.1	183.3	195.3 ± 25.8	343.8	207.4 ± 24.1
<i>i</i> 14:0	3.6 ± 1.5	0.0	0.0	0.0	0.0	0.0
14:0	2.2 ± 0.8	2.4 ± 1.2	2.8	2.1 ± 0.7	3.7	2.0 ± 1.6
<i>i</i> 15:0	1.6 ± 0.9	2.5 ± 0.2	0.2	0.5 ± 0.2	0.0	0.0
<i>a</i> 15:0	1.9 ± 0.4	1.8 ± 0.6	4.0	1.9 ± 0.4	5.8	3.4 ± 1.8
15:0	1.5 ± 0.4	1.4 ± 0.8	1.1	1.6 ± 1.3	2.1	2.9 ± 1.4
<i>i</i> 16:0	14.3 ± 2.5	9.8 ± 2.7	1.3	6.5 ± 2.1	1.6	4.6 ± 1.3
16:1 ω 7c	5.6 ± 1.8	8.5 ± 1.1	9.2	7.3 ± 1.8	5.4	7.3 ± 1.3
16:1 ω 7t	0.0	0.0	0.0	0.0	0.0	0.0
16:0	30.5 ± 10.2	43.1 ± 4.8	47.9	39.5 ± 2.8	44.1	44.1 ± 9.9
cyl7:0	0.0	3.9 ± 1.1		3.2 ± 1.7	0.5	2.4 ± 1.5
<i>i</i> 17:0	1.1 ± 0.8	0.0	0.5	0.4 ± 0.1	0.0	0.0
<i>a</i> 17:0	2.3 ± 0.5	2.9 ± 0.6	4.8	1.3 ± 0.8	4.2	3.2 ± 1.8
17:0	1.7 ± 0.9	1.0 ± 0.6	0.7	1.7 ± 1.1	1.2	1.6 ± 1.3
18:1 ω 9c	5.8 ± 1.4	7.8 ± 0.9	8.2	11.0 ± 2.3	13.4	12.2 ± 2.5
18:1 ω 7c	2.7 ± 1.0	5.3 ± 0.7	6.3	7.1 ± 1.1	5.6	6.9 ± 0.9
18:1 ω 7t	0.0	0.0	0.0	0.0	0.0	0.0
18:0	5.1 ± 1.3	7.9 ± 2.3	11.5	13.7 ± 1.9	12.4	9.0 ± 2.8
18:2 ω 6,9	19.1 ± 6.2	0.0	0.0	0.0	0.0	0.0
cyl9:0	1.0 ± 0.3	1.7 ± 0.9	1.5	2.2 ± 1.3	0.0	0.4 ± 0.1

Treatment #4, Anaerobic Only Phase – Unamended

PLFA	0 wk	4 wk	12 wk	22 wk	30 wk	40 wk	50 wk	60 wk
pmol/g	5.8 ± 2.2	7.4 ± 1.3	8.2 ± 0.8	2.8 ± 0.5	6.3 ± 2.1	23.7 ± 5.1	3.9 ± 1.1	8.3 ± 1.3
<i>i</i> 14:0	0.0	0.2 ± 0.1	0.4 ± 0.2	0.0	0.0	0.4 ± 0.1	0.6 ± 0.2	1.1 ± 0.3
14:0	0.0	1.1 ± 0.5	10.6 ± 2.1	0.0	1.3 ± 0.4	0.0	0.7 ± 0.1	0.0
<i>i</i> 15:0	0.4 ± 0.1	1.2 ± 0.3	1.5 ± 0.9	1.7 ± 0.5	0.7 ± 0.1	0.7 ± 0.3	0.6 ± 0.1	1.8 ± 0.2
<i>a</i> 15:0	0.1 ± 0.1	1.1 ± 0.6	0.0	0.0	0.5 ± 0.2	1.4 ± 0.6	0.0	0.5 ± 0.3
15:0	0.3 ± 0.1	1.4 ± 0.7	0.0	0.0	0.0	3.2 ± 1.2	0.7 ± 0.3	1.3 ± 0.7
<i>i</i> 16:0	4.1 ± 0.4	8.0 ± 0.8	3.3 ± 0.6	4.4 ± 1.6	6.4 ± 0.6	5.7 ± 0.4	7.1 ± 1.2	9.5 ± 0.9
16:1ω7c	1.1 ± 0.5	3.6 ± 1.2	0.0	6.2 ± 2.1	6.1 ± 2.0	14.9 ± 5.3	1.2 ± 1.0	1.3 ± 0.3
16:1ω7t	0.1 ± 0.1	0.2 ± 0.1	0.0	0.0	0.7 ± 0.3	1.1 ± 0.4	0.7 ± 0.2	0.0
16:0	29.3 ± 6.4	27.1 ± 5.6	28.8 ± 8.5	60.0 ± 9.3	28.1 ± 6.1	36.5 ± 7.1	34.4 ± 5.2	38.8 ± 6.2
<i>cyl</i> 17:0	3.4 ± 1.0	12.6 ± 3.2	8.3 ± 1.3	4.2	5.2 1.3	5.2 ± 2.1	0.6 ± 0.1	3.3 ± 1.1
<i>i</i> 17:0	1.0 ± 0.6	0.7 ± 0.2	0.0	0.5 ± 0.1	0.3 ± 0.1	0.9 ± 0.2	1.1 ± 0.3	0.8 ± 0.4
<i>a</i> 17:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
17:0	2.0 ± 1.3	1.1 ± 0.4	7.8 ± 2.7	25.5 ± 4.3	5.9 ± 1.8	1.1 ± 0.4	1.2 ± 0.3	1.4 ± 0.3
18:1ω9c	19.4 ± 7.2	17.2 ± 2.3	9.3 ± 4.1	4.6 ± 1.1	14.5 ± 2.5	11.5 ± 4.1	16.0 ± 3.4	14.3 ± 2.5
18:1ω7c	13.1 ± 3.2	10.9 ± 1.6	3.4 ± 1.2	8.8 ± 2.3	10.5 ± 1.3	3.8 ± 1.7	9.4 ± 2.1	11.3 ± 3.7
18:1ω7t	1.1 ± 0.8	0.8 ± 0.4	0.0	0.0	0.0	0.4 ± 0.2	2.8 ± 1.1	1.0 ± 0.4
18:0	8.1 ± 2.4	13.1 ± 4.8	15.3 ± 3.8	0.0	17.8 ± 6.1	11.9 ± 3.3	22.0 ± 4.2	13.1 ± 5.2
18:2ω6,9	8.2 ± 2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>cyl</i> 19:0	8.3 ± 1.8	4.6 ± 2.2	3.1 ± 0.8	5.7 ± 1.3	2.0 ± 1.2	1.3 ± 0.8	0.9 ± 0.3	1.5 ± 0.4

Treatment #5, Anaerobic Only – Butyrate-Amended

PLFA	0 wk	4 wk	12 wk	22 wk	30 wk	40 wk	50 wk	60 wk
pmol/g	4.3 ± 1.2	15.0 ± 4.1	6.8 ±1.0	3.7 ± 0.9	5.3 ± 1.5	8.5 ± 3.1	31.1 ± 5.3	84.9 ±10.2
<i>i</i> 14:0	0.0	0.0	0.2 ± 0.1	0.0	0.0	0.3 ± 0.1	0.5 ± 0.3	1.0 ± 0.2
14:0	0.0	2.8 ± 1.1	2.1 ± 0.9	1.4 ± 0.5	0.0	1.8 ± 0.8	1.8 ± 1.1	0.0
<i>i</i> 15:0	0.0	1.1 ± 0.1	2.3 ± 0.5	0.0	0.7 ± 0.1	2.3 ± 0.2	1.8 ± 0.1	3.1 ± 0.5
<i>a</i> 15:0	0.0	0.6 ± 0.2	0.2 ± 0.1	0.0	0.0	0.6 ± 0.2	0.0	1.1 ± 0.6
15:0	0.7 ± 0.2	1.3 ± 0.4	0.0	0.8 ± 0.3	0.1 ± 0.1	0.0	0.0	2.4 ± 1.1
<i>i</i> 16:0	2.9 ± 0.8	3.5 ± 0.6	2.7 ± 1.0	2.8 ± 1.1	5.6 ± 0.5	2.6 ± 0.5	4.4 ± 0.5	9.0 ± 0.7
16:1ω7c	1.2 ± 0.3	1.5 ± 0.5	2.3 ± 1.1	3.5 ± 1.7	2.8 ± 0.9	3.9 ± 1.2	5.3 ± 1.8	4.7 ± 2.1
16:1ω7t	0.0	0.7 ± 0.2	0.0	0.0	0.4 ± 0.1	0.0	0.7 ± 0.2	0.0
16:0	31.4 ± 5.6	29.1 ± 8.5	29.2 ± 6.1	50.1 ± 10.2	42.7 ± 8.3	35.1 ± 5.1	37.7 ± 6.3	36.9 ± 9.2
<i>cyl</i> 17:0	3.7 ± 1.1	5.1 ± 2.1	8.3 ± 2.6	0.0	3.8 ± 1.7	6.7 ± 1.5	2.0 ± 0.7	4.8 ± 2.1
<i>i</i> 17:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>a</i> 17:0	0.0	0.9 ± 0.2	0.0	0.0	0.0	0.0	0.6 ± 0.2	0.0
17:0	1.5 ± 0.8	1.7 ± 0.7	1.8 ± 0.4	1.8 ± 0.3	0.0	4.7 ± 2.3	1.7 ± 1.0	0.0
18:1ω9c	14.3 ± 6.3	19.4 ± 5.9	19.9 ± 7.5	18.2 ± 5.7	11.9 ± 5.4	15.8 ± 6.1	18.1 ± 5.7	12.3 ± 3.9
18:1ω7c	12.9 ± 3.7	9.9 ± 3.5	10.7 ± 3.5	9.7 ± 3.2	10.7 ± 2.8	9.5 ± 2.5	14.3 ± 4.8	14.2 ± 4.1
18:1ω7t	3.6 ± 1.2	2.4 ± 1.1	1.2 ± 0.6	0.0	0.0	2.8 ± 0.9	0.0	0.0
18:0	17.1 ± 8.2	12.1 ± 4.9	15.9 ± 4.8	7.3 ± 2.1	18.1 ± 4.5	10.2 ± 2.7	7.5 ± 2.3	7.7 ± 1.3
18:2ω6,9	6.2 ± 2.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>cyl</i> 19:0	4.5 ± 1.9	7.9 ± 1.3	3.2 ± 1.2	4.8 ± 1.8	3.2 ± 1.0	3.7 ± 1.1	3.6 ± 1.1	2.8 ± 0.8

Treatment #7, Anaerobic Only – Methanol-Amended

PLFA	0 wk	4 wk	12 wk	22 wk	30 wk	40 wk	50 wk	60 wk
pmol/g	5.3 ± 0.9	19.5 ± 3.2	18.1 ± 5.1	2.1 ± 0.3	17.8 ± 6.5	16.3 ± 3.7	12.1 ± 3.4	50.5 ± 2.3
<i>i</i> 14:0	0.1 ± 0.1	0.9 ± 0.3	0.5 ± 0.1	0.0	0.6 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	2.6 ± 0.2
14:0	0.0	0.0	0.0	0.0	0.2 ± 0.1	0.0	0.0	0.0
<i>i</i> 15:0	1.2 ± 0.2	1.6 ± 0.4	1.8 ± 0.3	0.2 ± 0.1	1.9 ± 0.2	1.7 ± 0.2	2.1 ± 0.4	3.2 ± 0.3
<i>a</i> 15:0	0.0	0.2 ± 0.1	1.8 ± 0.3	2.5 ± 0.6	0.0	0.1 ± 0.1	0.4 ± 0.1	1.0 ± 0.2
15:0	0.0	0.5 ± 0.1	3.4 ± 0.2	0.0	0.2 ± 0.2	1.2 ± 0.2	0.6 ± 0.1	2.1 ± 0.3
<i>i</i> 16:0	1.7 ± 0.2	2.5 ± 0.5	2.8 ± 0.5	3.6 ± 0.7	4.0 ± 0.4	2.8 ± 0.4	3.8 ± 0.8	7.3 ± 0.3
16:1 ω 7c	0.0	2.3 ± 0.4	10.8 ± 1.3	0.0	1.1 ± 0.2	6.4 ± 0.3	0.4 ± 0.2	0.3 ± 0.1
16:1 ω 7t	0.0	0.0	0.9 ± 0.1	0.0	0.0	0.4 ± 0.1	1.1 ± 0.3	0.1 ± 0.1
16:0	32.7 ± 4.3	24.5 ± 3.2	39.4 ± 4.2	33.2 ± 5.6	32.8 ± 6.2	33.4 ± 3.9	20.9 ± 5.1	39.0 ± 2.3
<i>cyl</i> 17:0	2.4 ± 0.5	5.7 ± 0.6	2.7 ± 1.0	1.9 ± 0.2	1.1 ± 0.3	8.0 ± 2.0	9.6 ± 1.2	3.2 ± 1.0
<i>i</i> 17:0	0.0	0.0	0.0	0.5 ± 0.1	0.0	0.0	0.0	0.0
<i>a</i> 17:0	0.0	0.0	0.3 ± 0.1	0.0	0.0	0.0	0.0	0.0
17:0	3.9 ± 1.1	3.5 ± 1.1	1.7 ± 0.5	3.4 ± 0.6	0.0	0.5 ± 0.1	3.2 ± 0.6	1.7 ± 0.4
18:1 ω 9c	1.1 ± 0.3	10.4 ± 1.7	14.2 ± 2.1	12.8 ± 2.3	13.8 ± 3.2	9.6 ± 1.3	14.7 ± 3.7	12.3 ± 2.6
18:1 ω 7c	16.9 ± 5.2	9.1 ± 0.8	3.9 ± 0.6	4.2 ± 1.1	9.3 ± 0.9	2.8 ± 0.8	6.7 ± 1.4	6.3 ± 1.1
18:1 ω 7t	6.7 ± 0.9	11.4 ± 2.0	0.6 ± 0.1	11.3 ± 2.6	5.0 ± 1.0	0.0	4.9 ± 0.6	0.7 ± 0.2
18:0	23.9 ± 4.8	22.0 ± 3.9	13.6 ± 0.9	21.3 ± 2.1	33.7 ± 3.7	20.1 ± 5.2	23.4 ± 2.5	16.5 ± 4.1
18:2 ω 6,9	5.6 ± 0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>cyl</i> 19:0	4.8 ± 1.0	5.4 ± 0.7	1.6 ± 0.2	5.1 ± 0.4	6.3 ± 1.2	12.2 ± 4.1	7.4 ± 1.3	3.7 ± 0.3

Treatment #9 – Sequential Treatment – Unamended

PLFA	0 wk	4 wk	12 wk	22 wk	30 wk	60 wk	2 wk	5 wk	7 wk
pmol/g	24.8 ± 3.4	8.5 ± 1.3	6.5 ± 0.6	5.5 ± 0.9	12.2 ± 3.2	31.1 ± 2.8	7.9 ± 1.1	5.5 ± 1.0	18.9 ± 2.5
<i>i</i> 14:0	2.4 ± 0.5	0.6 ± 0.1	0.0	0.0	0.1 ± 0.1	5.1 ± 1.1	9.5 ± 0.8	0.0	0.0
14:0	0.7 ± 0.1	0.4 ± 0.2	0.0	1.3 ± 0.3	0.0	2.3 ± 0.5	2.8 ± 0.2	2.9 ± 0.7	0.6 ± 0.1
<i>i</i> 15:0	1.5 ± 0.7	0.4 ± 0.1	0.9 ± 0.2	0.7 ± 0.1	0.9 ± 0.2	1.4 ± 0.3	0.0	0.0	0.0
<i>a</i> 15:0	0.8 ± 0.2	0.0	0.5 ± 0.1	0.2 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	0.0	0.0	2.9 ± 1.1
15:0	0.5 ± 0.1	1.1 ± 0.5	1.3 ± 0.5	0.4 ± 0.2	0.9 ± 0.1	3.4 ± 1.2	0.3 ± 0.1	1.1 ± 0.4	4.2 ± 0.7
<i>i</i> 16:0	3.8 ± 1.1	3.9 ± 1.4	12.5 ± 3.1	6.6 ± 0.2	4.0 ± 1.5	4.8 ± 1.7	0.6 ± 0.2	0.0	2.4 ± 0.5
16:1ω7c	3.1 ± 0.9	1.9 ± 1.1	2.1 ± 0.8	0.3 ± 0.1	3.5 ± 0.9	1.1 ± 0.4	3.4 ± 1.6	0.1 ± 0.1	4.9 ± 1.2
16:1ω7t	0.5 ± 0.1	1.7 ± 0.4	0.0	0.8 ± 0.3	0.2 ± 0.1	0.8 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.0
16:0	28.8 ± 5.2	29.8 ± 5.4	41.0 ± 5.2	38.5 ± 4.3	31.9 ± 3.8	25.9 ± 6.1	35.5 ± 4.6	15.5 ± 3.6	18.2 ± 5.4
<i>cyl</i> 17:0	10.4 ± 1.4	6.0 ± 1.9	7.9 ± 1.3	6.8 ± 1.5	2.7 ± 1.1	4.5 ± 0.3	0.6 ± 0.2	1.9 ± 0.3	12.4 ± 1.3
<i>i</i> 17:0	0.0	0.0	0.5 ± 0.2	0.0	0.0	0.0	0.3 ± 0.1	0.0	0.0
<i>a</i> 17:0	3.5 ± 1.0	0.0	1.9 ± 0.8	0.0	0.0	0.0	0.0	0.0	0.0
17:0	0.0	1.1 ± 0.5	1.4 ± 0.8	0.9 ± 0.2	1.4 ± 0.6	1.7 ± 0.2	1.9 ± 0.5	0.0	3.0 ± 0.8
18:1ω9c	12.3 ± 3.4	8.5 ± 2.1	9.2 ± 3.6	10.7 ± 3.1	9.9 ± 1.6	10.9 ± 2.1	1.4 ± 0.2	12.7 ± 4.1	3.6 ± 1.0
18:1ω7c	10.2 ± 2.6	11.9 ± 0.8	10.3 ± 2.8	10.9 ± 2.8	6.4 ± 2.1	19.8 ± 3.0	12.8 ± 3.6	4.2 ± 0.4	9.4 ± 2.1
18:1ω7t	1.3 ± 0.5	7.2 ± 1.4	0.0	6.2 ± 1.4	0.7 ± 0.1	2.0 ± 0.5	5.2 ± 2.1	5.0 ± 2.1	2.5 ± 0.8
18:0	8.5 ± 1.5	18.9 ± 2.5	7.3 ± 2.1	13.9 ± 3.0	33.3 ± 4.8	10.3 ± 2.0	25.3 ± 5.5	22.6 ± 3.6	25.5 ± 4.3
18:2ω6,9	3.2 ± 1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>cyl</i> 19:0	8.5 ± 1.6	6.6 ± 1.6	3.2 ± 1.0	1.8 ± 0.3	3.3 ± 1.0	4.9 ± 0.6	0.2 ± 0.1	33.7 ± 4.3	10.4 ± 2.7

Treatment #10 – Sequential Treatment, Biphenyl-Amended

PLFA	0 wk	4 wk	12 wk	22 wk	30 wk	60 wk	2 wk	5 wk	7 wk
pmol/g	19.1 ± 2.6	17.2 ± 3.1	11.5 ± 2.8	10.7 ± 2.4	6.6 ± 0.2	22.6 ± 4.3	5.6 ± 0.4	5.0 ± 0.8	135.1 ± 15.2
<i>i</i> 14:0	1.3 ± 0.3	2.8 ± 0.2	0.0	0.0	0.5 ± 0.1	16.5 ± 2.5	10.4 ± 2.3	0.0	0.0
14:0	0.9 ± 0.1	0.0	18.9 ± 4.6	0.0	1.2 ± 0.3	0.4 ± 0.1	9.7 ± 0.6	2.1 ± 0.3	3.9 ± 1.0
<i>i</i> 15:0	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	1.5 ± 0.3	0.7 ± 0.2	0.4 ± 0.2	0.0	0.0	1.9 ± 0.7
<i>a</i> 15:0	0.5 ± 0.2	0.8 ± 0.3	6.9 ± 0.9	0.6 ± 0.1	0.6 ± 0.3	0.4 ± 0.1	0.0	0.0	3.3 ± 0.3
15:0	1.3 ± 0.3	1.1 ± 0.4	0.3 ± 0.1	0.7 ± 0.2	1.6 ± 1.1	2.1 ± 0.7	0.7 ± 0.2	1.1 ± 0.4	2.9 ± 0.9
<i>i</i> 16:0	3.5 ± 1.1	6.7 ± 1.6	3.4 ± 1.1	9.5 ± 2.3	7.8 ± 0.4	1.8 ± 1.0	0.6 ± 0.1	0.4 ± 0.1	3.9 ± 1.3
16:1ω7c	2.0 ± 0.9	0.9 ± 0.2	1.2 ± 0.5	1.9 ± 0.6	2.1 ± 1.1	0.4 ± 0.1	2.3 ± 0.6	1.6 ± 0.5	2.3 ± 0.6
16:1ω7t	0.7 ± 0.1	2.6 ± 0.8	0.0	0.7 ± 0.2	0.0	0.1 ± 0.1	0.2 ± 0.1	0.6 ± 0.1	1.9 ± 0.3
16:0	32.5 ± 2.3	10.2 ± 2.4	38.7 ± 2.1	32.7 ± 5.2	33.3 ± 5.1	32.2 ± 4.8	34.2 ± 6.2	16.7 ± 2.1	17.8 ± 4.5
<i>cy</i> 17:0	4.3 ± 1.0	6.2 ± 1.1	3.2 ± 0.7	6.9 ± 0.3	7.5 ± 0.9	1.8 ± 0.4	0.9 ± 0.2	27.9 ± 4.3	17.7 ± 2.7
<i>i</i> 17:0	0.0	0.0	0.0	0.3 ± 0.1	0.0	0.0	0.4 ± 0.1	0.0	0.0
<i>a</i> 17:0	0.0	0.2 ± 0.1	0.0	0.7 ± 0.2	0.0	0.0	0.0	0.0	2.6 ± 0.5
17:0	2.2 ± 0.8	3.8 ± 0.9	0.6 ± 0.2	1.9 ± 0.5	0.7 ± 0.1	1.2 ± 0.3	2.9 ± 1.0	0.0	2.7 ± 0.8
18:1ω9c	12.3 ± 2.7	22.3 ± 1.5	8.1 ± 2.1	12.6 ± 2.1	12.4 ± 2.3	6.8 ± 1.6	6.4 ± 1.8	2.1 ± 0.3	8.8 ± 2.5
18:1ω7c	8.3 ± 0.4	9.2 ± 1.1	5.8 ± 1.2	10.1 ± 2.5	11.2 ± 1.2	17.4 ± 4.1	8.7 ± 2.1	8.5 ± 2.8	2.0 ± 0.4
18:1ω7t	3.1 ± 0.2	5.1 ± 0.8	0.0	0.5 ± 0.1	0.5 ± 0.1	0.9 ± 0.2	3.0 ± 0.8	0.9 ± 0.3	4.3 ± 1.1
18:0	15.4 ± 1.6	14.5 ± 2.5	5.2 ± 0.7	12.0 ± 3.5	15.9 ± 3.5	14.9 ± 3.5	18.3 ± 2.2	24.0 ± 3.6	11.1 ± 3.2
18:2ω6,9	5.6 ± 0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>cy</i> 19:0	5.8 ± 1.1	13.1 ± 2.3	2.1 ± 0.3	7.4 ± 1.4	4.0 ± 0.5	2.7 ± 0.7	1.3 ± 0.3	14.1 ± 3.2	12.9 ± 2.4

Treatment #11, Sequential Treatment, Butyrate-Amended

PLFA	0 wk	4 wk	12 wk	22 wk	30 wk	60 wk	2 wk	5 wk	7 wk
pmol/g	6.2 ± 1.0	13.5 ± 3.2	7.4 ± 1.2	11.4 ± 2.5	19.8 ± 3.4	162.9 ± 4.5	52.7 ± 5.1	42.3 ± 4.3	50.8 ± 3.6
<i>i</i> 14:0	1.5 ± 0.6	0.0	0.0	0.0	0.3 ± 0.1	5.9 ± 1.2	0.0	0.0	0.0
14:0	0.0	0.0	0.0	6.4 ± 1.8	0.4 ± 0.2	0.7 ± 0.3	0.0	0.4 ± 0.1	1.5 ± 0.5
<i>i</i> 15:0	0.0	0.6 ± 0.2	0.1 ± 0.1	1.9 ± 0.7	1.2 ± 0.3	0.9 ± 0.1	0.0	0.0	0.9 ± 0.3
<i>a</i> 15:0	0.0	0.6 ± 0.1	0.2 ± 0.1	0.9 ± 0.3	1.7 ± 0.5	2.1 ± 0.5	0.0	0.0	0.3 ± 0.1
15:0	0.3 ± 0.1	0.8 ± 0.3	0.0	0.3 ± 0.1	2.6 ± 0.9	2.7 ± 0.4	0.0	0.4 ± 0.1	3.1 ± 1.1
<i>i</i> 16:0	3.7 ± 0.7	2.9 ± 1.2	3.2 ± 0.5	7.5 ± 2.0	6.6 ± 1.0	2.7 ± 1.1	0.0	0.7 ± 0.2	4.6 ± 0.8
16:1 ω 7c	1.4 ± 0.4	1.9 ± 0.5	0.5 ± 0.2	2.7 ± 0.9	7.5 ± 2.3	1.8 ± 0.7	0.0	0.3 ± 0.1	2.8 ± 1.2
16:1 ω 7t	0.7 ± 0.2	0.9 ± 0.3	1.7 ± 0.5	0.3 ± 0.1	0.5 ± 0.1	0.8 ± 0.3	0.0	1.4 ± 0.3	0.0
16:0	26.9 ± 3.1	25.9 ± 5.1	25.1 ± 6.1	26.8 ± 2.1	39.1 ± 4.6	28.4 ± 5.1	30.3 ± 3.5	57.2 ± 4.2	22.6 ± 3.2
<i>cy</i> 17:0	5.3 ± 1.4	4.1 ± 1.2	3.1 ± 0.8	7.5 ± 1.3	3.6 ± 1.3	2.1 ± 0.7	0.0	2.3 ± 1.0	6.2 ± 1.5
<i>i</i> 17:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>a</i> 17:0	0.9 ± 0.2	0.0	0.0	1.7 ± 0.4	0.8 ± 0.2	0.0	0.0	0.0	0.0
17:0	0.0	2.4 ± 0.7	3.7 ± 1.1	0.6 ± 0.2	0.6 ± 0.1	2.2 ± 0.9	0.0	0.0	7.5 ± 1.0
18:1 ω 9c	8.4 ± 1.0	12.2 ± 2.5	9.3 ± 2.1	12.6 ± 1.5	11.9 ± 2.4	27.5 ± 4.3	15.8 ± 3.4	6.0 ± 1.3	9.1 ± 0.7
18:1 ω 7c	14.5 ± 3.5	7.3 ± 1.6	17.5 ± 3.3	12.5 ± 2.3	4.6 ± 1.2	9.8 ± 1.5	53.9 ± 5.1	5.1 ± 1.2	10.4 ± 2.5
18:1 ω 7t	10.9 ± 2.0	2.2 ± 0.3	0.6 ± 0.1	3.1 ± 0.9	5.9 ± 1.6	1.1 ± 0.6	0.0	2.4 ± 0.8	5.7 ± 1.0
18:0	13.6 ± 1.4	30.7 ± 2.5	18.1 ± 2.4	9.3 ± 1.3	8.6 ± 0.8	8.5 ± 1.6	0.0	11.5 ± 3.4	13.9 ± 2.4
18:2 ω 6,9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>cy</i> 19:0	11.7 ± 1.5	7.3 ± 1.0	16.9 ± 2.3	5.8 ± 2.0	1.9 ± 0.2	2.7 ± 0.9	0.0	12.4 ± 2.7	5.6 ± 1.3

Treatment #12 – Sequential Treatment, Methanol-Amended

PLFA	0 wk	4 wk	12 wk	22 wk	30 wk	60 wk	2 wk	5 wk	7 wk
pmol/g	3.9 ± 0.6	18.5 ± 3.2	16.9 ± 2.6	51.1 ± 4.3	16.9 ± 3.3	134.1 ± 6.2	80.5 ± 4.7	73.6 ± 4.2	45.9 ± 3.6
<i>i</i> 14:0	7.9 ± 1.2	0.0	0.0	0.1 ± 0.1	3.5 ± 0.9	5.5 ± 1.2	1.0 ± 0.4	0.0	0.0
14:0	1.0 ± 0.2	3.7 ± 1.1	0.8 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	0.0	1.5 ± 0.8	1.2 ± 0.3	1.5 ± 0.3
<i>i</i> 15:0	0.0	0.7 ± 0.1	0.9 ± 0.2	0.4 ± 0.1	0.4 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	0.0	0.0
<i>a</i> 15:0	0.0	0.5 ± 0.2	1.4 ± 0.6	0.0	0.1 ± 0.1	0.3 ± 0.2	0.0	0.0	0.4 ± 0.1
15:0	1.8 ± 0.5	0.9 ± 0.4	0.8 ± 0.1	0.8 ± 0.2	0.4 ± 0.1	1.3 ± 0.5	0.2 ± 0.1	2.7 ± 1.2	0.9 ± 0.2
<i>i</i> 16:0	2.8 ± 0.9	2.2 ± 0.9	6.6 ± 1.2	3.9 ± 1.0	0.3 ± 0.2	1.6 ± 0.5	0.9 ± 0.3	1.3 ± 0.2	0.8 ± 0.1
16:1 ω 7c	0.0	1.3 ± 0.4	7.6 ± 1.5	1.2 ± 0.5	0.7 ± 0.3	0.5 ± 0.1	0.2 ± 0.2	0.8 ± 0.1	1.3 ± 0.3
16:1 ω 7t	0.0	0.2 ± 0.1	0.0	1.7 ± 0.4	0.9 ± 0.2	0.1 ± 0.1	0.0	3.0 ± 0.3	0.0
16:0	23.4 ± 3.1	28.9 ± 4.2	48.8 ± 4.7	24.9 ± 5.1	15.5 ± 3.1	45.4 ± 3.5	45.1 ± 3.7	27.1 ± 4.5	37.4 ± 2.7
<i>cyl</i> 17:0	4.1 ± 1.2	3.5 ± 0.9	5.8 ± 1.4	22.5 ± 3.8	24.7 ± 5.2	0.9 ± 0.2	31.3 ± 2.8	2.1 ± 0.7	12.8 ± 1.1
<i>i</i> 17:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>a</i> 17:0	0.0	0.0	0.4 ± 0.2	0.0	0.0	0.0	0.2 ± 0.1	0.0	0.0
17:0	1.5 ± 0.6	1.8 ± 0.4	1.1 ± 0.3	2.1 ± 0.7	3.4 ± 1.0	1.5 ± 0.6	0.7 ± 0.2	0.0	1.6 ± 0.3
18:1 ω 9c	6.9 ± 1.4	12.9 ± 2.6	9.1 ± 1.8	10.2 ± 2.5	8.5 ± 1.9	17.6 ± 2.4	0.0	5.2 ± 1.2	5.9 ± 2.1
18:1 ω 7c	23.5 ± 3.5	8.9 ± 1.5	3.8 ± 0.9	8.0 ± 2.0	1.7 ± 0.7	8.9 ± 1.7	2.1 ± 1.0	10.9 ± 2.0	6.8 ± 0.7
18:1 ω 7t	0.0	2.3 ± 0.8	0.0	4.2 ± 1.0	0.1 ± 0.1	4.6 ± 1.0	0.3 ± 0.1	3.1 ± 0.7	5.9 ± 1.2
18:0	12.7 ± 2.6	27.5 ± 4.1	10.5 ± 1.7	7.6 ± 0.9	34.9 ± 3.8	10.1 ± 3.7	3.7 ± 1.1	26.7 ± 4.1	18.8 ± 3.1
18:2 ω 6,9	1.3 ± 0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>cyl</i> 19:0	13.3 ± 2.8	4.7 ± 1.7	2.4 ± 0.9	11.7 ± 2.3	4.6 ± 1.4	1.5 ± 0.8	12.6 ± 2.6	15.9 ± 3.2	5.9 ± 0.3

Treatment #13 – Sequential Treatment, Butyrate and Biphenyl-Amended

PLFA	0 wk	4 wk	12 wk	22 wk	30 wk	60 wk	2 wk	5 wk	7 wk
pmol/g	13.4 ± 2.1	7.6 ± 1.2	9.9 ± 1.8	18.6 ± 3.1	26.6 ± 2.5	56.7 ± 6.3	70.4 ± 5.2	118.8 ± 10.2	188.9 ± 9.8
<i>i</i> 14:0	7.2 ± 1.0	0.0	0.0	0.0	1.9 ± 0.2	21.7 ± 3.4	0.6 ± 0.2	0.0	0.0
14:0	0.5 ± 0.1	0.0	0.5 ± 0.1	0.0	1.1 ± 0.3	0.0	0.5 ± 0.1	5.1 ± 1.2	3.6 ± 1.0
<i>i</i> 15:0	0.0	0.1 ± 0.1	0.5 ± 0.2	0.8 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.0	0.0	1.9 ± 0.6
<i>a</i> 15:0	0.0	0.1 ± 0.1	0.5 ± 0.1	1.1 ± 0.5	0.0	1.2 ± 0.3	0.0	0.0	1.8 ± 1.0
15:0	0.8 ± 0.1	0.0	0.7 ± 0.1	1.7 ± 0.2	3.2 ± 0.6	0.7 ± 0.1	3.4 ± 1.2	5.6 ± 1.3	6.4 ± 0.6
<i>i</i> 16:0	5.7 ± 2.3	1.6 ± 0.3	4.8 ± 1.3	3.4 ± 1.4	4.9 ± 1.2	2.0 ± 0.4	1.7 ± 0.3	3.4 ± 1.0	3.6 ± 0.5
16:1 ω 7c	2.0 ± 0.8	0.8 ± 0.2	1.8 ± 0.8	6.1 ± 3.1	3.7 ± 0.4	0.4 ± 0.1	0.1 ± 0.1	2.7 ± 0.6	2.2 ± 0.4
16:1 ω 7t	0.7 ± 0.2	0.8 ± 0.1	1.2 ± 0.6	1.4 ± 0.7	5.5 ± 1.3	0.0	1.9 ± 0.5	7.1 ± 0.9	1.8 ± 0.5
16:0	29.5 ± 3.1	20.0 ± 2.5	32.7 ± 4.3	21.6 ± 2.0	22.9 ± 5.1	26.8 ± 3.4	23.9 ± 4.2	34.8 ± 3.5	16.9 ± 2.1
<i>cyl</i> 17:0	5.2 ± 0.3	4.5 ± 0.6	4.9 ± 1.3	3.4 ± 0.5	4.6 ± 0.4	0.9 ± 0.1	1.2 ± 0.5	0.3 ± 0.1	15.6 ± 3.0
<i>i</i> 17:0	0.3 ± 0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>a</i> 17:0	1.2 ± 0.4	0.0	0.3 ± 0.1	0.0	2.1 ± 0.3	0.0	2.4 ± 0.6	0.0	2.6 ± 0.3
17:0	0.9 ± 0.2	2.6 ± 0.8	2.0 ± 0.5	2.3 ± 0.7	0.0	1.1 ± 0.4	2.1 ± 0.5	0.0	2.1 ± 0.2
18:1 ω 9c	8.3 ± 1.1	10.9 ± 2.3	8.1 ± 3.4	13.0 ± 6.2	19.6 ± 3.5	21.6 ± 3.5	25.4 ± 3.3	5.7 ± 1.4	7.4 ± 1.0
18:1 ω 7c	14.0 ± 2.5	12.4 ± 1.8	12.3 ± 2.8	28.0 ± 8.1	7.8 ± 1.2	2.5 ± 0.1	30.7 ± 5.3	7.6 ± 3.1	7.7 ± 1.5
18:1 ω 7t	6.4 ± 0.9	14.8 ± 2.2	7.9 ± 1.0	3.9 ± 0.2	16.6 ± 4.1	6.3 ± 2.1	0.0	5.2 ± 1.2	7.9 ± 2.1
18:0	10.8 ± 0.5	22.6 ± 4.8	16.5 ± 2.6	9.9 ± 1.3	3.7 ± 0.8	5.9 ± 1.2	5.4 ± 1.0	12.5 ± 2.1	9.1 ± 0.6
18:2 ω 6,9	3.1 ± 0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>cyl</i> 19:0	7.0 ± 2.2	8.8 ± 1.2	5.2 ± 0.8	3.5 ± 0.2	1.9 ± 0.5	4.5 ± 0.3	0.7 ± 0.2	9.9 ± 1.3	10.4 ± 2.3

Treatment #15 – Sequential Treatment, Methanol and Biphenyl-Amended

PLFA	0 wk	4 wk	12 wk	22 wk	30 wk	60 wk	2 wk	5 wk	7 wk
pmol/g	18.0 ± 2.1	14.8 ± 3.4	10.9 ± 1.4	15.8 ± 1.2	15.5 ± 1.2	88.8 ± 4.2	46.1 ± 4.8	54.5 ± 5.1	178.5 ± 22.2
<i>i</i> 14:0	5.3 ± 1.0	0.0	0.0	0.0	0.8 ± 0.1	5.7 ± 0.3	1.1 ± 0.3	0.0	0.0
14:0	0.3 ± 0.1	0.0	0.0	5.1 ± 1.5	0.5 ± 0.2	0.0	4.9 ± 1.1	1.5 ± 0.5	2.2 ± 0.6
<i>i</i> 15:0	0.8 ± 0.2	1.2 ± 0.2	0.6 ± 0.1	1.7 ± 0.8	0.2 ± 0.1	0.6 ± 0.1	0.0	0.0	0.2 ± 0.1
<i>a</i> 15:0	0.5 ± 0.1	1.1 ± 0.3	0.6 ± 0.2	1.2 ± 0.4	0.5 ± 0.1	1.1 ± 0.4	10.9 ± 1.2	0.0	0.9 ± 0.3
15:0	0.9 ± 0.3	1.0 ± 0.1	2.1 ± 0.8	1.2 ± 0.2	1.7 ± 0.7	0.7 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	2.9 ± 1.1
<i>i</i> 16:0	5.3 ± 0.9	5.6 ± 1.4	7.9 ± 1.6	7.4 ± 1.5	5.9 ± 1.5	2.9 ± 0.8	1.9 ± 0.8	0.0	1.9 ± 0.9
16:1ω7c	2.1 ± 0.5	0.8 ± 0.2	0.6 ± 0.2	2.3 ± 0.8	7.4 ± 1.9	0.5 ± 0.1	1.7 ± 0.3	0.0	1.6 ± 0.5
16:1ω7t	0.8 ± 0.2	4.1 ± 1.0	2.6 ± 1.0	1.6 ± 0.3	1.7 ± 0.4	0.0	0.9 ± 0.2	0.4 ± 0.1	0.0
16:0	22.5 ± 5.2	19.1 ± 4.5	37.9 ± 6.1	24.3 ± 4.1	27.4 ± 3.8	33.3 ± 5.2	20.2 ± 2.1	30.4 ± 3.2	31.0 ± 6.0
<i>cyl</i> 17:0	4.9 ± 1.1	9.2 ± 2.1	6.8 ± 1.3	3.9 ± 1.0	3.1 ± 1.0	15.7 ± 4.3	1.2 ± 0.1	3.7 ± 1.0	4.4 ± 1.4
<i>i</i> 17:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>a</i> 17:0	1.4 ± 0.6	0.0	0.9 ± 0.2	0.0	0.0	0.0	0.0	0.0	0.0
17:0	0.0	2.6 ± 0.9	1.2 ± 0.5	2.9 ± 0.5	2.4 ± 0.8	1.2 ± 0.5	1.4 ± 0.4	0.0	2.8 ± 0.7
18:1ω9c	15.4 ± 3.3	11.6 ± 1.7	15.5 ± 3.1	16.2 ± 2.5	11.0 ± 3.5	7.9 ± 2.2	9.3 ± 2.1	6.2 ± 0.9	10.1 ± 1.8
18:1ω7c	10.1 ± 2.5	19.6 ± 5.2	7.9 ± 0.6	11.1 ± 2.4	6.5 ± 1.2	6.9 ± 1.5	8.9 ± 1.3	2.7 ± 0.5	11.3 ± 1.8
18:1ω7t	8.6 ± 2.1	3.8 ± 0.8	3.6 ± 0.7	3.7 ± 1.6	10.3 ± 2.7	1.9 ± 0.4	6.6 ± 1.0	1.4 ± 0.8	10.4 ± 2.1
18:0	12.8 ± 3.5	13.1 ± 2.1	9.4 ± 1.6	9.9 ± 2.3	11.9 ± 3.6	9.9 ± 2.0	13.5 ± 2.3	42.2 ± 4.7	15.4 ± 3.5
18:2ω6,9	2.1 ± 0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>cyl</i> 19:0	6.2 ± 1.6	7.2 ± 1.3	2.4 ± 0.2	7.5 ± 2.7	2.2 ± 0.7	11.7 ± 3.1	16.9 ± 4.2	11.3 ± 2.3	4.9 ± 1.6

Treatment #17 - Sequential Treatment, Hexane-only

PLFA	0 wk	4 wk	12 wk	22 wk	30 wk	60 wk	2 wk	5 wk	7 wk
pmol/g	3.6 ± 0.8	10.0 ± 2.1	14.2 ± 1.8	6.2 ± 1.1	8.2 ± 0.9	8.6 ± 1.2	9.3 ± 0.7	10.3 ± 2.3	9.2 ± 0.7
<i>i</i> 14:0	0.0	0.0	0.9 ± 0.2	0.0	0.6 ± 0.1	0.1 ± 0.1	0.0	0.0	0.3 ± 0.1
14:0	0.0	0.4 ± 0.1	0.0	1.5 ± 0.5	0.0	1.3 ± 0.3	0.9 ± 0.2	1.1 ± 0.3	2.1 ± 0.6
<i>i</i> 15:0	0.0	0.0	2.5 ± 0.8	0.6 ± 0.1	0.4 ± 0.1	0.0	0.5 ± 0.5	0.6 ± 0.2	0.7 ± 0.1
<i>a</i> 15:0	0.0	0.0	1.9 ± 0.4	0.4 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	1.3 ± 0.5	1.2 ± 0.3	0.9 ± 0.4
15:0	0.0	2.3 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	1.4 ± 0.3	0.8 ± 0.2	0.2 ± 0.5	0.3 ± 0.5	1.3 ± 0.7
<i>i</i> 16:0	3.5 ± 1.1	5.3 ± 0.7	8.8 ± 1.6	5.9 ± 0.3	8.0 ± 1.0	2.7 ± 0.2	3.2 ± 0.8	4.8 ± 0.5	7.2 ± 1.3
16:1 ω 7c	0.9 ± 0.1	0.0	2.9 ± 0.8	1.4 ± 0.5	6.6 ± 1.2	2.5 ± 0.6	1.1 ± 0.3	2.5 ± 1.1	1.9 ± 0.3
16:1 ω 7t	0.0	3.0 ± 0.7	0.8 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	6.4 ± 1.2	10.2 ± 1.4	2.6 ± 0.9	4.2 ± 1.5
16:0	32.1 ± 2.5	20.6 ± 3.1	32.3 ± 1.3	29.3 ± 4.3	38.8 ± 2.5	21.8 ± 1.1	28.3 ± 0.9	31.1 ± 3.3	26.7 ± 2.7
cyl7:0	4.8 ± 0.9	8.1 ± 1.2	3.6 ± 1.1	6.5 ± 0.8	3.4 ± 0.6	2.8 ± 0.9	5.1 ± 1.0	2.2 ± 1.5	1.9 ± 0.7
<i>i</i> 17:0	0.9 ± 0.1	0.0	0.4 ± 0.1	0.0	0.0	0.0	0.0	0.0	0.0
<i>a</i> 17:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
17:0	0.0	2.9 ± 0.5	2.9 ± 0.2	2.0 ± 0.3	0.6 ± 0.2	5.8 ± 0.6	3.2 ± 0.5	4.5 ± 0.9	5.1 ± 1.6
18:1 ω 9c	16.6 ± 1.8	17.7 ± 2.5	19.0 ± 1.6	20.8 ± 4.1	13.4 ± 1.3	14.7 ± 2.4	15.3 ± 2.4	16.2 ± 2.1	12.1 ± 2.5
18:1 ω 7c	13.8 ± 2.1	13.8 ± 1.3	11.8 ± 1.5	9.9 ± 1.3	8.7 ± 0.7	15.3 ± 4.1	10.2 ± 4.2	11.3 ± 0.6	16.2 ± 0.9
18:1 ω 7t	0.0	5.1 ± 0.8	0.1 ± 0.1	1.4 ± 0.3	0.5 ± 0.1	1.2 ± 0.5	0.8 ± 0.1	0.4 ± 0.1	2.1 ± 0.4
18:0	17.3 ± 1.1	13.9 ± 0.9	8.9 ± 1.3	13.3 ± 0.5	13.9 ± 2.2	15.5 ± 1.4	12.3 ± 2.1	14.2 ± 2.4	10.3 ± 2.1
18:2 ω 6,9	0.0	1.0 ± 0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
cyl9:0	10.1 ± 0.3	5.9 ± 1.1	2.9 ± 0.6	6.0 ± 1.2	2.5 ± 0.9	8.7 ± 1.2	7.4 ± 1.2	7.0 ± 0.3	7.0 ± 2.3