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Anaerobic Microbial Dechlorination of Polychlorinated Biphenyls
(Under the Direction of DR. JUERGEN WIEGEL)

The LCP Superfund site in Brunswick, Georgia, was extensively contaminated with Aroclor 1268, a mixture of highly chlorinated polychlorinated biphenyl (PCB) congeners. This study investigates whether the indigenous anaerobic bacterial communities in PCB-contaminated salt-marsh sediment can dechlorinate weathered Aroclor 1268. Batch cultures of the contaminated estuarine sediment were separately primed for dechlorination with 2,6-dibromobiphenyl and 2,3,4,5,6-pentachlorobiphenyl under anaerobic, sulfate-reducing conditions and incubated at 28^{o}C at different pH values (5.5-7.5) for one year. Dechlorination of primer 2,3,4,5,6-pentachlorobiphenyl occurred via two routes with the loss of (1) *meta* then *para* chlorine atoms and of (2) *para, ortho*, then *meta* chlorine atoms leading to the accumulation of mono-, di- and tri-chlorobiphenyls. Extensive dehalogenation of primer 2,6-dibromobiphenyl resulted in a significant accumulation of biphenyl and a small proportion of mono-bromobiphenyl. Neither of the two primers induced dechlorination of native Aroclor 1268 congeners.

INDEX WORDS: Polychlorinated biphenyls, *Ortho*-dechlorination, Aroclor 1268.

ANAEROBIC MICROBIAL DECHLORINATION OF POLYCHLORINATED BIPHENYLS

by

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INTRODUCTION

Purpose of Study:

PCBs are widespread, persistent contaminants that interfere with marine and estuarine biota processes and accumulate in the environment and animal tissues. Such bioaccumulation can disturb human health and development. The uncommonly manufactured and utilized Aroclor 1268 contains a combination of maximally chlorinated congeners that have yet to be reductively dechlorinated both effectively and consistently. The reasons for this have been inconclusive. The purpose of this study is to investigate whether the indigenous anaerobic bacterial communities in PCB-contaminated salt-marsh sediment can dechlorinate the weathered PCB mixture. Learning to enhance the process of PCB dechlorination in this sediment requires an understanding of the impact metabolic conditions impose on microbial dechlorination and identifying the specific physical and chemical obstacles PCB dechlorinators must overcome. Examinations of the factors involved for the effective degradation of PCBs would provide for the future development and application of bioremediative strategies. This study does not intend to introduce genetically engineered or non-indigenous microorganisms into the decontamination process.

This study focuses on the influence of temperature, pH, metabolic conditions, and examines if manipulating these factors can dechlorinate the weathered Aroclor 1268 congeners. Temperatures representative of the seasonal climate of the Georgia salt-marsh were chosen to incubate batch cultures of the indigenous microorganisms. The pH values chosen were selected as neutral and slightly acidic conditions to test the pH conditions preferred by the mesophilic dechlorinators in the sediment. Batch cultures were examined under sulfidogenic and/or methanogenic conditions. Both sulfate-reducers and methanogens are known to include dehalogenating species.

Nomenclature of Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls, or PCBs, are a class of man-made compounds consisting of two phenyl rings connected by a freely rotating bond of the C1 on each ring. Its 10 remaining carbons contain a hydrogen or chlorine. Each possible form of a PCB with a unique substitution pattern is called a congener. There are 209 congeners of PCBs due to the 10 available positions on the biphenyl that allow a variety of combinations of chlorine substitutions. PCBs are subdivided into homologs consisting of all congeners possessing the same number of chlorines. The congeners of a homolog having a different chlorine substitution positions are called isomers. For example, congeners 206, 207, and 208 belong to the *homolog* of nonachlorobiphenyls in which each *isomer* within the homolog possesses one of three possible unique substitution patterns with 9 chlorines (10).

Properties and Use

Under the trade name of Aroclor, PCBs were synthesized by Monsanto in the U.S. between1929 and 1978. Aroclors were utilized by chemical plants, paper mills, and

electrical industries until they were banned by EPA under the Toxic Substances Control Act for their potential toxicity (22). By the catalytic chlorination of biphenyl, complex mixtures of specified weight percents of chlorine were produced. For example, the 4-digit number of Aroclor 1268 indicates a mix of 12 carbon biphenyl molecules with 68% chlorine by mass (containing 7-10 chlorines per biphenyl). Other individual mixtures included Aroclor 1242 (2-4 Cl), 1248 (4-6 Cl), 1254 (5-7 Cl), and 1260 (6-8 Cl). These mixtures were extremely heat stable, non-reactive, semi-volatile, non- flammable, hydrophobic, and electrically resistant. Thus, Aroclors were ideal as dielectric fluids in transformers, hydraulic fluids, solvent extenders, and capacitors (10). However, during its production of about 1.4 billion pounds, several hundred million pounds of PCB waste have contaminated marine, estuarine, and freshwater ecosystems. PCB contamination is widespread all over the world due to accidental and improper disposal. In addition, its physical stability and hydrophobicity allows for its adsorption to sediment, persistence under natural conditions, and accumulation in biota, thus qualifying PCBs as recalcitrant compounds.

Toxicity:

The toxicity of PCBs is attributed to the different congeners, which differ in their mode of toxicity and potency. PCBs are allegedly linked to birth defects and cancer in laboratory animals. They are suspected to cause cancer or adverse skin and liver effects. Due to their environmental persistence, tendency to biomagnify in the higher trophic levels of the food chain, and potential health risks, EPA banned PCB production in 1979 (22).

There is no conclusive evidence that PCBs are carcinogenic in humans, even to those exposed by occupation (6). PCBs are however considered potential carcinogens because Aroclor 1260 has been shown to increase the incidence of hepatic tumors in rats (34, 47), though lesser chlorinated PCB mixtures were not as potent as Aroclor 1260 (6). Aroclor mixtures such as 1248 through 1260 contain 60 to 90 different PCBs including the "coplanar" congeners, 34-34-CB, 345-34-CB, and 345-345-CB (57, 59). Non-*ortho* chlorinated congeners are called coplanar and are known as the most toxic forms of PCBs (10). They are only present in minute quantities (0.015 to 0.3 weight %) in Aroclors (61). 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most acutely toxic halogenated aromatic compound (57), is used as a reference for toxicological comparisons. Coplanar PCB congeners are similar to the stereoisomers of TCDD in their coplanar conformation and, thus, they exhibit biologic and toxic effects (57). Because human exposure is primarily through food, congeners that show bioaccumulation potential are likely to be important in causing adverse human health effects. Studies report that high consumption of fish contaminated with organohalogens (PCBs, hydroxy-PCBs, DDT, and DDE) did not appear to affect plasma concentration of pituitary, thyroid, or testosterone hormone in male adults (27). Most congeners that accumulate in human tissues are characterized by adjacent *meta* and *para* chlorines (58), a feature that makes them particularly susceptible to at least partial dechlorination.

Still, PCB contamination has varying effects on marine biota. While a recent study reports the relative insensitivity to some of the toxic effects of PCB exposure in *Fundulus heteroclitus* (41), another study indicates that PCBs function as endocrine disrupters that can induce anomalies in regeneration times, morphology, and

developmental mechanisms. These anomalies are evident by the significant dysfunctions in the endocrine mechanisms controlling regenerative development (17). The overall effect of microbial dechlorination of PCBs is to reduce its tendency to bioaccumulate in order to decrease its exposure to organisms and toxic potential and to decrease or remove the coplanar congeners thought to be the most toxic components.

In conclusion, studies investigating the potential toxicological mechanisms of PCBs in reproductive health have been inconclusive. However, the potential for reproductive and developmental toxicity is a concern because the available data support that PCBs mimic thyroid and other steroidal hormones (39). Although, PCBs have antiestrogenic activity, i.e., they antagonize a broad spectrum of estrogen-induced responses in vertebrates; resulting toxicological consequences have not been conclusively demonstrated to date (42). In addition, case studies examining the association of PCBs with endometriosis in infertile women are statistically insignificant (50).

Environmental Fate:

The extensive use and improper disposal of PCBs has resulted in the widespread contamination of air, water, soil, and sediment (23). One-third of the total United States production of PCBs (about 1.4×10^9 lbs) was released accidentally or purposely into the environment (15, 30, 33, 65). EPA estimates that 150 million pounds of PCBs are dispersed throughout the environment, including air and water supplies; an additional 290 million pounds are located in landfills in the U.S. (22). They have been found in nearly all marine plants and animals, fish, mammals, birds, and humans (68). Since the aqueous solubility of PCBs decreases with increasing chlorine content, a majority of PCBs released into the aquatic environment are expected to end up either adsorbed onto

sediment or resting as sludges at the bottoms of river, lakes, and oceans. Subsequently they enter into the food chains mainly via bottom dwelling organisms (46, 70). The lipophilic and persistent nature of PCBs leads to a magnification of PCB concentrations along the food chain. The aqueous solubility and volatility of PCBs decrease considerably with increasing chlorinated substitution (48). In general, highly substituted PCBs are less volatile, less soluble in water, and chemically more stable than less substituted congeners. Octanol/water partition coefficients [K_{ow}], a measure of hydrophobicity, are commonly used in the estimation of bioconcentration (66), toxicity (37), and partitioning between sediments and water (49). Rappaport and Eisenreich (55) reported that the K_{ow}s for 58 PCB congeners range from a log K_{ow} average of 4.5 for one monochlorobiphenyl to a log K_{ow} of 8.1 for a heptachlorobiphenyl.

Decontamination:

Current methods of PCB destruction involve much energy and expense. PCBs can be burned at very high temperatures (1200-1600^{o}C), yielding hydrogen chloride, but there is a risk of generating small quantities of the much more toxic polychlorinated dibenzodioxins and dibenzofurans (31, 40). Although, PCBs can also be destroyed by photolysis (16, 38), the major portion of the compound accumulated in aquatic sediments, strongly adsorbed to particles, thus, shielded them from photolysis (36). Photolytic decomposition may also lead to the formation of polychlorinated dibenzofurans (18, 29). Ionizing radiation was effective in degrading PCBs in micellar solutions (60). Chemical degradation techniques include adsorption, chlorinolysis, catalytic dehydrochlorination, microwave plasma, ozonation, wet air oxidation, reaction with sodium naphthalide, reaction with molten sodium, and reaction with a sodium salt in an amine solvent (23).

Biodegradation:

Biodegradation of PCBs can occur through microbial-mediated mineralization and anaerobic dechlorination, respectively. Aerobic degradation involves the oxidative destruction of PCB molecules through a series of degradation intermediates whereas anaerobic dechlorination involves the removal of chlorine atoms and replacement by hydrogen atoms in the absence of oxygen.

Aerobic Degradation

The major aerobic microbial degradation route of PCBs is a four step process beginning in most instances with a 2,3-dioxygenase attack at carbons 2 and 3 forming cis 2,3-dihydroxybiphenyl and the subsequent metabolism through the *meta*-fission route to their corresponding chlorobenzoic acids (1). Most of the bacterial strains with the ability to degrade mono- through tetrachlorobiphenyls, have been isolated from soils and aquatic sediments. Members of *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Corynebacterium, Norcardia, Pseudomonas,* and *Rodococus* are capable of aerobic cleavage of lightly chlorinated biphenyl ring (usually less than 4 chloro-substituents) and those organisms which are able to grow on biphenyl usually can cometabolize various PCB congeners (7, 8, 26, 35). Studies of *Alcaligenes eutrophus* H850 demonstrated a novel 3,4-dioxygenase attack on PCB congeners (7). PCB structure is critical for aerobic biodegradability such that correlations between the two have been proposed (24).

a) Biphenyls with more than five chlorines are usually resistant to degradation although exceptions of organisms degrading penta-, hexa-, and hepta-chlorobiphenyls exist (7, 8, 11, 25, 26, 62). However, no aerobic microorganisms have been reported to effectively degrade Aroclor 1260 or 1268 mixtures.

b) Dioxygenation occurs on the ring with the least chlorines substituents.

c) Nonchlorinated vicinal *ortho* and *meta* positions favor dioxygenation.

d) PCBs with chlorine substituents on both rings are more recalcitrant than isomers containing an unchlorinated ring.

e) PCBs containing *ortho* position chlorines are recalcitrant though 2-2-CB degradation has been reported (62).

The resulting chlorobenzoic acids produced in aerobic degradation of PCBs can be further mineralized to carbon dioxide, water, chloride, and biomass (28).

Anaerobic Reductive Dehalogenation

The actual mechanism of reductive dehalogenation is still unclear. Reductive dehalogenation of aryl halides is thought to involve two one-electron reduction steps, resulting in the removal of the halogen substituents and the formation of an intermediate aryl halide radical, which abstracts a proton from water to complete the reaction (44) and releases a halide. Depending on the congener, removing chlorines result in the accumulation of mono- and di-chlorinated biphenyls. Generally, anaerobic transformation of halogenated compounds is initiated by the removal of the halogens via reductive dehalogenation. It is a usually rapid, exergonic reaction that removes chlorines rather from the highly chlorinated congeners than the less chlorinated congeners (21, 45). Steric factors have also been shown to affect the dechlorination of certain congeners; generally, the *ortho*-substituted chlorines are removed the slowest. In theory, highly chlorinated PCBs are more susceptible to reductive dechlorination due to the delocalization of electrons associated with the biphenyl rings. In addition, chlorine is highly electronegative and more likely to bond to available protons in its chemical environment.

However, the highly chlorinated PCBs are less soluble and less available for biologically mediated dechlorination.

The rate and occurrence of dehalogenation are strongly dependent on the environmental conditions and the congeners. The presence of specific electron donors (43), electron acceptors (51), temperatures (74), and available substrates influence rates and extents of PCB dechlorination. The most effective substrate (electron donor) is apparently dependent on the microbial population involved rather than on the chemical structure of the compound to be reduced. Electron acceptor conditions affect the population composition of microbial communities and have been correlated with the occurrence of reductive dehalogenation activity. Methanogenic and sulfidogenic conditions are conducive to dechlorination of arylhalides such as PCBs both in the laboratory and in the environment (14, 43, 44, 53, 54). Reductive dehalogenation depends at least in part on the electron donor requirements and the efficiency with which electrons can be directed to dehalogenation. Stimulation of dehalogenating activity has been observed after amendment with 1) additional electron donors (e.g., methanol, acetate), 2) reducing agents or a reduced environment, and 3) elevated concentrations (above 100 mM) of a "priming" halogenated compound such as specific PCB congeners, chlorobenzoates, bromobiphenyls (3).

Dechlorination Processes:

Examples of dechlorination processes described below are a series of reactions that indicates which congeners are substrates, which chlorines are removed from these congeners, and the order in which they are removed. The resulting congener distribution

profile is referred to as the corresponding dechlorination pattern. There are at least six distinct microbial dechlorination processes, which can be identified through careful analysis of the patterns of congener loss and product formation (10). Other processes can be explained as combination of these six. The six processes are characterized by the predominant removal of (question mark denotes reactions which have not been unequivocally established to be part of that process):

i) **Process M:** flanked and unflanked *meta* dechlorination of PCBs with (3-?), 23-, 25-, 34-, 234-, and 236-chlorophenyl groups (e.g., present in Aroclor 1242).

ii) **Process Q:** flanked and unflanked *para* dechlorination of PCBs with 4-, 24-, 34-, (234-?), and 245-chlorophenyl groups and flanked *meta* dechlorination of PCBs with 23-, (234-?), and (236-?) chlorophenyl groups as found e.g., in Aroclor 1242.

iii) **Process H':** flanked and doubly flanked *para* dechlorination of PCBs with 34-, 245-(2345-?), and (23456-?) chlorophenyl groups as found e.g., in Aroclor 1242.

iv) **Process H:** flanked *para* and doubly flanked *para* dechlorination of PCBs with 34-, 234-, 245-, 2345-, (2346-?), and 23456-chlorophenyl groups as found e.g., in Aroclors 1242 and 1260.

v) **Process P:** flanked *para* and doubly flanked *para* dechlorination of PCBs with 34-, 234-, 245-, 2345-, 2346-, and 23456-chlorophenyl groups as found e.g., in Aroclors 1260.

vi) **Process N:** flanked *meta* and doubly flanked *meta* dechlorination of PCBs with 34-, 234-, 236-, 245-, 2345-, 2346-, and 23456-chlorophenyl groups as found e.g., in Aroclors 1254 and 1260.

Process C is the combination of Processes M and Q. Process LP (10) is the removal of unflanked *para* chlorines of PCBs (e.g. 24-24-CB, and 24-26-CB) which are accumulating during Process N. So far, no reductive *ortho* dechlorination of a PCB mixture under anaerobic conditions has been reported though *ortho* dechlorination of single PCB congeners (e.g. 24-CB, 246-CB, and 2356-CB) has been frequently reported (10, 67, 70).

The specificity of microbial dechlorination varies widely even within the same sediment. There are at least five major factors that determine whether a chlorine will be removed from any particular congener in each sediment (10). These include i) the microbial populations present, and ii) their specificity with respect to the position (*ortho, meta, or para*) of the chlorine relative to the opposite phenyl ring, iii) the surrounding chlorine configuration, iv) as well as the incubation conditions (the Aroclor added, temperature, carbon nature, bioavailability, electron acceptors present, salinity, oil, and other contaminants). The incubation conditions affect the growth and activity of the dehalogenating microorganisms and their interacting members in the community.

Factors influencing rate and extent of PCB dechlorination:

Factors that are important to microbial ecology in general and the bioavailability of specific congeners influence PCB dechlorination. The dechlorination rate of PCBs in sediments is influenced by several factors including the distribution and concentration of

PCB congeners, electron acceptors, organic matter, nutrients, and microbial population (2, 4, 9, 56). Environmental factors include oxygen tension, redox level, temperature, pH, salinity, available carbon, trace metals. Non-environmental factors include PCB structure and physico-chemical properties, and co-contaminants (5, 31, 33, 43, 48, 49, 69, 73-76).

PCBs are low water soluble nonionic organic contaminants that tend to sorb to soil and sediment particles. One hypothesis is that dechlorination rates are limited by the rate of PCB desorption from particles into the aqueous phase. Desorption rates are biphasic; a "labile" portion of the PCB desorbs rapidly, but another "nonlabile" portion desorbs much more slowly (19, 20). For soils or sediments that have been contaminated with PCB for years the desorption rate for this nonlabile portion may be so slow that it precludes achieving target levels in a reasonable length of time. The importance of physical separation of contaminant and microorganism, as effected by sorption onto inorganic and organic matrices, may be one of the least well understood yet most significant phenomena in biological treatment processes. Where sorption on to soil material, biomass, or organic colloids maybe characteristic of natural environments (*in situ*), the surfaces of inert organics, such as activated carbon or dead and inactive cells, may be dominant sorption sites in bioreactors. PCB characteristics such as hydrophobicity and semi-volatility provide insight into the relative tendency of the chemical to sequester in environmental compartments. Incorporation into humic material due to chemical coupling reactions with humic components is not expected since PCBs lack reactive hydroxy or carboxy substituents. Highly chlorinated compounds tend to sorb more strongly to organic matter than less chlorinated contaminants. On the other hand, less chlorinated compounds tend to be more volatile (23). Thus, PCBs may

disappear faster than they are biodegraded due to physical removal or may degrade slower than predicted due to bioavailability limitations.

Many PCB contaminated sediments also contain other contaminants that may affect PCB dechlorination, especially oil and heavy metals. However, reports have shown substantial *in situ* dechlorination in sediments containing as much as 6%-10% oil by weight. Oil serves as a sorptive phase for PCBs (according to their K_{ow}) thereby significantly decreasing their aqueous concentrations (13). This could slow dechlorination for the microorganisms requiring access to PCBs in aqueous solution. The presence of high levels of heavy metals inhibited the dechlorination reactions in sediments from the St. Lawrence River (63). Further studies are needed to better understand the fate and biotransformation of PCBs in these and similar sediments.

Under the highly reduced conditions most metals occur as insoluble sulfides and thus would have little impact on the dechlorination process. The significance of other organics including PAHs and halogenated compounds is unknown.

Dechlorination in LCP Superfund sediment

The LCP Chemicals Superfund Site consists of 550 acres, the majority of which is a tidal marsh. An oil refinery, a paint manufacturing company, a power plant, and a chlor-alkali plant have all operated at this site over the last 70 years. Mercury, polychlorinated biphenyls (PCBs), and semi-volatile contamination are prevalent across the plant site soils, in groundwater, and in the biota in the marsh. Since 1919 this site has been occupied by at least five major companies: Atlantic Refining Company (ARCO); Georgia Power Company; Dixie Paints and Varnish Company (currently, the O'Brien

Company); Allied Chemical Inc. (currently, Allied Signal); and, the Hanlin Group Subsidiary, LCP Chemicals-Georgia, Inc.

The contamination of greatest concern at this site is a large dispersion of mercury and PCBs throughout the marshlands from the chemical manufacturing process undertaken by Allied Signal and LCP between 1955 and 1979. Process wastes were discharged into large holding pits near the top of the marsh and also directly into Purvis Creek. EPA estimates that more than 380,000 pounds of mercury were "lost" in the area during this period. In addition to mercury and PCBs, lead, chromium, zinc, PAHs, and phenols have contaminated the 550-acre marshlands area, a 1-mile portion of the Turtle River and the entirety of Purvis Creek. Mercury and PCBs have been detected in aquatic life at levels sufficient to produce a ban on commercial fishing in these areas and a seafood consumption advisory for part of the river and all of the creek portions. Toxicity of porewaters extracted from sediments in the nearby tidal creeks was attributed to PCBs and methylmercury (71). The concentrations of total PCBs in excavation soil, marsh left- and right-transect sediments, and tidal creek sediments were 567, 481, 276, and 9.6 μg/g dry weight, respectively. The greater than 50-fold reduction in PCBs along this relatively short distance (500 m) suggests a high attenuation by the marsh environment. The transport of PCBs away from the highly contaminated areas (on-site soils and near-marsh sediments) by physicochemical processes appears to be limited by their hydrophobicity and resulting high affinity for marsh sediments. The total PCB concentration in invertebrate, avian, reptilian, and fish tissues collected in the LCP Superfund site has been reported to be in the parts per million range (32). However, the chronic effects of these congeners, as in endocrine disruption, are not known.

Soil and marsh PCB concentration were comparable to those found in sediments from other highly contaminated sites such as New Bedford Harbor, MA (52), the Sheboygan River, WI (64), and the Hudson and St. Lawrence Rivers, NY (12, 63).

Upon the plant's closing in February 1994, the State of Georgia asked EPA to take immediate action at the Site; EPA needed to address the threat of chlorine gas release and the contaminant flow into the adjacent saltwater tidal marsh containing endangered species. A removal action by EPA has excavated the vast majority of the on-site soils and waste piles. The removal was completed in the spring of 1999. Over 132,000 tons of Subtitle C RCRA Hazardous Waste (hazardous wastes that EPA stores and disposes until state government can) and over 121,000 tons of Subtitle D soils and sediment (non-hazardous solid wastes that the local government is responsible for cleaning up) have been removed from the LCP Chemicals site. Approximately 13 acres of marsh and marsh channels adjacent to the LCP site have been excavated.

The PCBs in the sediment came from a highly chlorinated PCB mixture, Aroclor 1268, which was applied to electrical equipment employed in the chlor-alkali process at the LCP Chemicals site (32). Aroclor 1268 was used as a plasticizer in rubbers and synthetic resins and as wax extenders (29). Aroclor 1268 is a crystalline white powder in contrast to several other commercial PCB mixtures, which are viscous liquids or sticky resins (29). The mixture is 4.8% by weight deca-, 35% nona-, 45% octa-, 10.1% hepta-, and 4.4% hexachlorobiphenyls. Based on the computation of TCDD equivalents, Aroclor 1268 reflect relatively low toxic potentials in comparison to other PCB formulations (32). The predominant congeners of the mixture in order of highest to lowest concentration, 206 (23456-2345-CB), 199 (23456-235-CB), 201/196 (2345-2356-/2345-2346-CB), 208

(23456-2356-CB), 202 (2356-2356-CB), 209 (23456-23456-CB), 194 (2345-2345-CB), 207 (23456-2346-CB), and 180 (2345-245-CB), may be expected to be less mobile due to their low aqueous solubilities and vapor pressure. So far they are known as highly resistant to biotransformation because of their high degree of chlorine substitution that increases their potential for bioaccumulation and biomagnification in the estuarine food web. However, very few studies on biodegradation on Aroclor 1268 exist.

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CHAPTER 2

DECHLORINATION PATTERNS BY ANAEROBIC CONSORTIA OF AROCLOR 1268 CONTAMINATED ESTUARINE SEDIMENT[*]

Introduction:

Polychlorinated biphenyls (PCBs) are ubiquitous contaminants that remain a public concern because of their recalcitrance, bioaccumulation, and potential toxicity to humans and wildlife (6). Anaerobic microbial reductive dechlorination of PCBs in aquatic sediments is believed to decrease toxicity while increasing the potential for further degradation by aerobic microorganisms (6-8, 11, 35). Dechlorination of PCBs in estuarine sediment is well documented (10, 16, 19, 22, 24, 27, 28). It is proposed that discrete dechlorinating microorganisms harboring dehalogenases with different regiospecificities are responsible for the various established dechlorination processes (6, 35). Maximal chlorine removal appears to require the complementary action of two or more dehalogenation processes (6, 29, 35) and a combination of anaerobic and aerobic dehalogenation.

The extent of dechlorination *in situ* varies considerably among sites (6, 10, 12, 20, 35, 39). This variability may be due to differential growth conditions for PCB

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dechlorinating microorganisms caused by fluctuations of temperature, pH, nutrients, bioavailability, and co-contaminants (2-4, 6, 13, 30, 37-39).

Sediments at the LCP Superfund Site in coastal Brunswick, Georgia are contaminated with process wastes discharged over decades by a series of industrial tenants. Among various types of contaminants including mercury, lead, chromium, zinc, PAHs, and phenolics, the highly chlorinated PCB mixture known as Aroclor 1268 prevails in the salt-marsh sediments (9.6 to 567 μg/g dry wt) (21). The mixture is 4.8% deca-, 35% nona-, 45% octa-, and 10.1% hepta-chlorobiphenyls by weight (21). The predominant congeners in the mixture, IUPAC nos. 206 (23456-2345-CB), 199 (23456-235-CB), 201/196 (2345-2356-/2345-2346-CB), 208 (23456-2356-CB), 202 (2356-2356-CB), 209 (23456-23456-CB), 194 (2345-2345-CB), 207 (23456-2346-CB), and 180 (2345-245-CB), are tightly bound to the sediment due to their low aqueous solubility and vapor pressure. PCB profiles in fish tissue from this site closely matched that of Aroclor 1268. Up to 170μg PCB/g lipid was observed, indicating that Aroclor 1268 bioaccumulates *in situ* (26).

Very little is known about anaerobic dehalogenation of Aroclor 1268. Recently, PCB congeners #195 (23456-234-CB), 196 (2345-2346-CB), 199 (23456-236-CB), 203 (2345-245-CB), 206 (23456-2345-CB), 207 (23456-2346-CB), and 208 (23456-2356-CB) were slightly dehalogenated under anaerobic conditions using Aroclor 1260 supplemented enrichments in artificial medium (23).

This study investigates whether the anaerobic estuarine microorganisms of the contaminated site can dechlorinate Aroclor 1268 under sulfate-reducing and methanogenic conditions typically associated with anoxic salt-marsh sediments.

Microcosms containing anaerobic sediment slurries were monitored for one year for their PCB congener profiles.

Materials and Methods:

Sediment**:**

PCB contaminated sediments were collected from the LCP Superfund Site, Brunswick, GA. The samples were collected near the rhizosphere of *Spartina* sp. and *Juncus* sp. using a shovel and stainless steel scoop pre-rinsed with acetone and hexane. Approximately 100 kg of estuarine sediments (up to 20 cm in depth) contaminated with Aroclor 1268 were transported in portable plastic containers at ambient temperature to Savannah, GA. These sediments were stored for 1 week at 4^{o}C until use.

Microcosm Preparation:

In a glove bag with a nitrogen atmosphere, one volume each of wet sediment and sterilized, filtered tidal creek water from the contaminated site were used to prepare the sediment slurry. Each microcosm consisted of 150 mL slurry in a 250 mL serum bottle sealed with a Teflon-lined butyl rubber stopper and an aluminum crimp cap. Each microcosm was buffered with sodium phosphate at 30 mM, and the pH was adjusted at every sampling time point by the addition of 1 N sodium hydroxide. Microcosms were adjusted to pH 7.5 (7.1-7.7), pH 6.5 (6.6-6.9), and pH 5.5 (5.6-6.3), respectively. Acetate and lactate were added at final concentrations of 10 mM. For the first 180 days of incubation, all microcosms were fed iron sulfate at a final concentration of 25 mM at every sampling time point. PCB primers 2,3,4,5,6-pentachlorobiphenyl (23456-CB) (150 μM) and 2,6-dibromobiphenyl (26-BB) (167 μM) were added in an acetone solvent carrier (230 μL per microcosm). After vigorously shaking by hand for 1 minute, samples

were allowed to stand in the chamber overnight. Sterile controls consisting of contaminated sediment slurry, buffer, and substrates were autoclaved once a day for 1 hour at 121^{o}C on each of three consecutive days before the primer was added. Additional, controls lacking buffer, substrates, or primers were prepared in duplicate. Experimental microcosms were prepared in triplicate. All microcosms were incubated in the dark without shaking at 13^{o}C or 28^{o}C in an environmental incubator.

Sample Extraction and Analysis of PCBs:

Slurries were sampled for PCB analysis at intervals of 0, 30, 90, 180, and 360 days. After vigorously shaking each bottle, 5 mL of slurry was poured into a graduated 30 mL glass vial in the anaerobic chamber. Dibromooctafluorobiphenyl was then added as an internal (recovery) standard. PCBs were extracted from the slurry with 8 mL hexane and 8 mL acetone on a shaker table at 200 strokes per minute for 24 hours. After shaking, the samples were left undisturbed for 1 hour for phase separation. The organic phase was then transferred into a 100 mL separatory funnel and back-extracted with hexane-washed water to remove residual acetone. Repeated concentrated sulfuric acid and copper applications to the hexane extract removed hydrolyzable impurities and elemental sulfur, respectively. This final extract was concentrated to 1 mL under gently flowing nitrogen gas and transferred to 2 mL borosilicate glass GC vials.

For verification of trace levels of PCB products, a Varian 3400CX gas chromatograph equipped with a Saturn 3 ion trap mass spectrometer and an 8200 autosampler was used for congener-specific identification of PCBs. The column oven temperature program for GC-MS started at 60^{o}C (2 min hold), increased to150^{o}C at of 20^{o}C/min (4.5 min hold), and then ramped to 260^{o}C at 2^{o}C/min (13.5 min hold). The

injector was programmed as follows: 60°C (1 min hold), ramped to 280°C at 200°C/min (15 min hold). The ion trap and transfer line were maintained at 240°C and 280°C, respectively. Nine standard solutions containing all 209 PCB congeners (Accustandard, New Haven, CT) (1 μg/ml) were used to calibrate the GC-MS. Congener distributions for each sample were calculated and reported in units of mole percent.

Results:

Primer Dehalogenation—routes:

2,6-Dibromobiphenyl:

The dehalogenation of 26-BB involved the sequential removal of *ortho*-substituted bromines to produce 2-monobromobiphenyl and subsequently biphenyl.

2,3,4,5,6-Pentachlorobiphenyl:

The molar distributions of dechlorination products versus time were used to construct a proposed dehalogenation pathway network for 23456-CB (Figure 2.1). This network is divided into two branches, a "*meta*-initiated branch and a "*para*-initiated" branch. For some of the steps two possibilities exist which could not be differentiated by the experiments described here (white block arrows in Figure 2.1). 23456-CB was first dehalogenated to the coeluting products, 2346-CB and 2356-CB (3.6-17.7 mol %) by *meta*- and *para*-dechlorination, respectively. Subsequently, 2346-CB was *meta*- or *para*-dechlorinated producing 246-CB or 236-CB, respectively. The latter product could also be derived from 2356-CB by *meta*-dehalogenation. For the trichlorobiphenyl products 246-CB and 236-CB, *para*- and *meta*-dechlorination, respectively, occurred to form the dead-end product 26-CB. 2356-CB of the *para*-initiated branch was *ortho*-dechlorinated

to 235-CB. Subsequently, 235-CB was *meta*- and *ortho*-dechlorinated to 25-CB and 35-CB, respectively. Both of these dichlorobiphenyls were further *ortho*- or *meta*-dechlorinated to 3-CB and trace levels of biphenyl.

The processes following the *meta*-initiated dechlorination branch did not remove *ortho* chlorines except for a possible alternate *meta*-initiated route, 23456-CB→2346-CB→236-CB→25-CB, in which 236-CB *ortho*-dechlorinates to 25-CB. The processes initiated by *para*-dechlorination removed all *ortho* and *meta* chlorines. The extent of dechlorination of the *meta*-initiated branch was limited in contrast to the complete dechlorination of 2346-CB in the *para*-initiated branch. The latter branch is apparently dominant as indicated by the majority of 35-CB at pH 7.5 (53.0 mol %). At pH 6.5 and 5.5, no dominance of either branch was obvious. At both pH 6.5 and 7.5, all the chlorinated congeners were observed but in different proportions. At pH 5.5, intermediate 3-CB was not detected. Only at pH 7.5, trace amounts of biphenyl were detected.

Incubation Temperature:

A comparison of two average estuarine seasonal temperatures shows a clear effect on dehalogenation of 26-BB (Figure 2.2A). Dehalogenation was evident at 28^{o}C (summer average) but not at 13^{o}C (winter average). Similar to the temperature effects on the dehalogenation of 26-BB, dehalogenation of 23456-CB was evident at 28^{o}C but not at 13^{o}C (Figure 2.2B).

Incubation pH:

2,6-Dibromobiphenyl:

Dehalogenation of 26-BB in sediments at pH 6.5-7.5 occurred faster than in sediments at pH 5.5 which exhibited an extended lag time (Figure 2.3A). 2-bromobiphenyl (2-BB) does not accumulate as a final product and biphenyl is the major product in all cultures buffered at pH 7.5 (6.7-7.7) (Figure 2.4). At pH 6.5 (6.5-7.0), 2-BB accumulated to 30-60 mol % within 90 days and was subsequently dehalogenated completely to biphenyl (Figure 2.4). In the cultures incubated at pH 5.5 (5.8-6.2), replicate B showed no evidence of dehalogenation. Data for replicates B at pH 6.5 and pH 5.5 were discarded due to erroneous analysis. However, upon investigation, the microorganisms in Replicate B exhibited methane production (as well as in the dehalogenating Replicates A and C) indicating that the culture was alive.

No attempt was made to maintain the pH in replicate unbuffered controls of batch cultures primed with 26-BB (Figure 2.5). Interestingly, complete dehalogenation of 26-BB occurred within 90 days in both unbuffered control replicates (pH 7.4) whereas the pH buffered cultures at pH 7.5 achieved only 60-70 mol % 26-BB dehalogenation within 90 days (Figure 2.4).

2,3,4,5,6-Pentachlorobiphenyl:

The removal of the first chlorine from 23456-CB over 360 days was similar at pH 6.5 and 7.5 (Figure 2.3B). At pH 5.5, the removal of the first chlorine occurred much later because of an extended dechlorination lagtime. Further dehalogenation of tetrachlorinated biphenyl differed with pH of the incubations (Figures 2.6-2.8). At pH 7.5 (6.9-7.7) (Figure 2.6), the appearance and disappearance of intermediates were not

observed. At 30 days only traces of 2346/2356-CB had appeared and at 90 days most of the observed dehalogenation had occurred. At pH 6.5 (6.6-6.9) (Figure 2.7), the sequential appearance of dechlorinated products was observed. Within 90 days, the coeluting congener pair 2346-CB and 2345-CB appeared to be the major products. 246-CB, 35-CB, and 3-CB are the final major products with average mole percentages of 49.6 mol %, 16.6 mol %, and 25.5 mol %, respectively. 35-CB increased sharply after 180 days then disappeared as 3-monochlorobiphenyl emerged in replicates A and B. Data for replicate C was discarded due to erroneous analysis.

The most striking difference among the pH conditions was the longer dechlorination lag time at pH 5.5 (5.6-6.3) (Figure 2.4B). Cultures showed an extended lag time (~60 days) before the onset of dehalogenation of the primer (Figure 2.8). All the major end products were different among the triplicate microcosms, i.e. replicate A accumulated 35-CB, replicate B produced 246-CB, and replicate C produced 2346/2356-CB. The replicate controls (A and B) primed with 23456-CB were not maintained at any particular pH. Data for replicate B was discarded due to erroneous analysis (Figure 2.9). Excluding a buffer control system, however, did not prevent dechlorination from occurring. It is assumed that the pH changes that occurred in the culture (final pH 7.5-7.7 after 90-360 days) determined what dechlorinators were active and responsible for the difference when compared to the controlled pH incubations. This control replicate appeared similar to replicate A at pH 7.5 (Figure 2.6), except for the lack of dechlorination to 35-CB. However, replicate B (pH 7.7) of the unbuffered controls demonstrated a less extensive dechlorination of primer 23456-CB. Much of the primer remained in the microcosm for the remainder of the experiment.

After 360 days (last time point) 57 mol % of the total amount of chlorines at pH 7.5 was removed. At pH 6.5, 62 mol % of the total amount of chlorines was removed. At pH 5.5, 44 mol % of the total amount of chlorines was removed. Although, a trace level of biphenyl was evident at pH 7.5 (complete dechlorination of 23456-CB), the most extensive removal of chlorines was actually at pH 6.5.

Sulfate Addition:

2,6-Dibromobiphenyl:

When incubated at 28^{o}C and pH 7.5 almost all of the supplemented 26-BB (167 μM) was *ortho*-dehalogenated to 58 mol % biphenyl by 90 days and 89 mol % after 1 year. The microbial communities in the control lacking the iron sulfate amendments completely dehalogenated 26-BB to an average of 93 mol % biphenyl within 90 days of incubation and 98 mol % after 1 year; data for replicate A was discarded due to erroneous analysis (Figure 2.10). Ceasing the sulfate amendments after 180 days did not impact the progress of dehalogenation in general. The overall, dehalogenation profiles of 26-BB with and without sulfate amendments were similar, but 26-BB commenced earlier in the control without sulfate supplement.

2,3,4,5,6-Pentachlorobiphenyl:

The experimental microcosms amended with 23456-CB (150 μM) and sulfate exhibited extensive dehalogenation at pH 7.5 (6.9-7.5). Overall, this treatment transformed 93.3 mol % of the primer to 53.0 mol % 35-CB, 17.6 mol % 246-CB, 10.0 mol % 25-CB (average mole percents of major products). In the controls lacking sulfate amendments, about 85 mol % of the primer 23456-CB was converted to an average of 38.8 mol % 35-CB and 47.8 mol % 246-CB within 90 days, with only small changes after

90 days (Figure 2.11). Since the majority of dehalogenation had already occurred after 90 days, ceasing the sulfate amendments after 180 days (Figure 2.8) was too late to show a significant effect. Incubations with or without sulfate supplements produced 35-CB and 246-CB, which are the major products in most of the experimental microcosms at pH 6.5 and pH 7.5. However, at pH 7.5 the products of the *para*-initiated branch are more predominant with sulfate addition than without sulfate. Without sulfate amendments, about 42 mol % of the total amount of chlorines was removed. With sulfate amendments, 57 mol % of the total amount of chlorines was removed. The presence of sulfate appears to increase the extent of dehalogenation of 23456-CB.

Dehalogenation of Aroclor 1268:

There was no measurable decrease in the concentration of hexa- through deca-chlorinated Aroclor 1268 congeners over the period of 12 months in any of the incubations.

Discussion:

The indigenous sediment microorganisms exhibited the ability to biotransform supplemented halogenated biphenyls, 26-BB and 23456-CB. In contrast to the results of Wu et al., (1997), with freshwater samples from Woods Pond (MA) and Sandy Creek Nature Park (GA), the dehalogenation of the primers did not result in the dehalogenation of native PCBs, in this case Aroclor 1268 congeners. The relatively fast dehalogenation of the primer could not be used to predict the dehalogenation of the weathered Aroclor 1268 mixture.

Diversity of Routes:

As indicated by the presence of a *meta*- and a *para*-initiated branch (Figure 2.1), at least two consortia were active. Based on the accumulation of products after 1 year of incubation at pH 7.5, the *para*-initiated branch dominated over the *meta*-initiated branch, indicating a more active microbial consortium for this route. A microbial preference for the initial dechlorination products of 23456-CB (2346-CB and 2356-CB) may determine which route predominates. Although initial reactions were consistent with the expected order of chlorine removal (first *meta* or *para*, then *ortho*), the *para*-initiated branch also exhibited the most *ortho*-dechlorination. Thus, the *para*-initiated branch exhibited the most extensive dehalogenation, producing significant amounts of tri- and di-chlorobiphenyls, and trace amounts of biphenyl. In contrast, the *meta-meta-para* route lead to the dead-end product 26-BB.

Steric factors have been shown to affect the dechlorination of certain PCBs (17, 18); generally, *ortho*-substituted congeners exhibit the largest resistance. *Ortho*-dechlorination probably occurred in the *para*-initiated route because the *ortho* chlorines were flanked, while the *ortho* chlorines were unflanked in the *meta*-initiated route. The *meta*-initiated branch could not produce any *ortho*-dechlorinated intermediates despite subsequent *para* chlorine removal.

Many of the dechlorination steps of 23456-CB in our study are consistent with known processes as delineated by Bedard and Quensen (6) who described dechlorination in freshwater sediment. While previous studies did not elucidate characteristic patterns of *ortho*-dechlorinating activity, others have observed the sequential *ortho*-dechlorination of 2356-CB to 235-CB and subsequently to 35-CB using cultures enriched from estuarine

sediment (15). The working culture in that study, however, exhibited only *ortho*-dechlorinating activity. The occurrence of *ortho*-dechlorination activity is less common in freshwater sediments and is strongly temperature dependent (6, 7, 39, 40). Although little *ortho*-dehalogenation of Aroclor 1260 was observed in Woods Pond sediments (5, 39), *ortho*-dechlorination of the supplemented primer, 23456-CB, was consistently observed at 27^{o}C. At 8-30^{o}C, approximately half of the primer was dehalogenated via 246-CB and *ortho*-dehalogenated to 24-CB and finally to 4-CB in that study (37).

The results of this study further demonstrates that *ortho*-dechlorination can also be significant in estuarine sediment. The dechlorination profile in this Georgia salt-marsh sediment is generally comparable to dechlorination profiles of lower chlorinated PCB mixtures of other investigations in freshwater and estuarine sediments (Table 2.1). A comparison of dechlorination pathways in pristine and contaminated sediments suggests the likelihood that dechlorinating microorganisms are more active in sediments with elevated PCB levels (Table 2.1).

Influence of Temperature:

Dehalogenation of the primers occurred at 28^{o}C but not at 13^{o}C. Because 28^{o}C and 13^{o}C represent average summer and winter temperatures along the southeastern U.S. coastline, respectively, a greater potential for *in situ* dechlorination likely occurs during the warmer months. Temperature affects anaerobic microbial reductive dechlorination as observed by Wu et al. (1997). However, this study also showed that temperature cannot be used to predict dechlorination routes when comparing contaminated northern Woods Pond sediment with non-PCB contaminated sediment in Athens, Georgia (Sandy Creek Nature Park) (36-39). However, in both instances dehalogenation of the primers was

observed below 13°C. Thus, the failure to obtain dehalogenation of the primer at 13°C in the salt-marsh was unexpected. This lack of dehalogenation at 13°C suggests that the microbial communities in estuarine sediment are different than those in freshwater sediment.

Influence of Sulfate Amendments:

The addition of iron sulfate was carried out to activate sulfate-reducing microorganisms (SRBs) in the microcosms, but the results suggest it did not necessarily stimulate the dehalogenating population. The microcosms at pH 7.5 lacking iron sulfate amendments dehalogenated 26-BB to the same extent as those receiving sulfate amendments. The microcosms at pH 7.5 lacking iron sulfate amendments dechlorinated 23456-CB to only 246-CB and 35-CB. In incubations with sulfate supplementation, higher levels of 35-CB were produced at pH 7.5 suggesting that sulfate facilitates the stimulation of the *ortho*- and *para*-dechlorination in this consortium. One possibility to explain this observation is that sulfate-reducers may be greater contributors to the *meta*- and *para*-initiated branches (Figure 2.1). The controls lacking sulfate and the experimental microcosms produced similar major products; perhaps non-sulfidogenic microorganisms are involved in the proposed dechlorination routes. Based on previous studies (42), predominant sulfate-reducing salt-marsh microbial communities could be expected to play a significant role in the dechlorination of PCBs. It was further hypothesized that sulfate-reducing dechlorinating microorganisms, upon depletion of sulfate in the presence of PCBs, would utilize PCBs as the electron acceptor. The termination of the iron sulfate amendments after 180 days did not result in any discernable dechlorination of native Aroclor PCBs. However, the primer dechlorination

within the first month of incubation indicated that the consortium dominated by SRBs was able to dechlorinate primers and subsequent dehalogenation products. Since no dehalogenation of the sediment Aroclor 1268 congeners occurred at all, it is doubtful whether a second amendment of the primer would have yielded a strong dehalogenation as previously observed with Woods Pond samples (34). However, it remains possible that even longer incubations are necessary for dechlorination of Aroclor 1268.

Influence of pH:

Dehalogenation was more extensive under neutral pH conditions (pH 6.5-7.5). This was true for both 23456-CB and 26-BB. Although the desired pH conditions were 7.5, 6.5, and 5.5, actual pH conditions varied beyond these nominal values as buffer was added at each sampling time point to minimize contamination. Since sampling time points were 30, 60, or 90 days apart, buffer additions had a temporary effect on pH conditions within the microcosms. Although differences in the extent of dehalogenation exist between the two branches, the *para*-initiated branch was consistently the most productive route.

In summary, the microbial consortia were able to remove all bromines of 26-BB and most chlorines of 23456-CB under neutral pH conditions. Less dehalogenation was observed under more acidic conditions (i.e. pH 5.5). There was also an extended lag time before dehalogenation occurred in incubations spiked with either 26-BB or 23456-CB. The more acidic environment must be unfavorable to this consortium or may interfere with the enzymes involved in the dechlorination. This is not consistent with a previous study on dehalogenation of PCBs in freshwater (Woods Pond, MA) sediments (13). In this study, the greatest extent of dehalogenation occurred at a pH range of 7.0-7.5 while

no significant dehalogenation appeared at the more acidic pH of 5.5. This indicates that pH 5.5 had adverse effects on the dehalogenation of the double unflanked *para* chlorine ("lonely *para*") of 246-CB, the single flanked *meta* chlorine of 236-CB, and the subsequent dehalogenation of the dichlorobiphenyls. Thus, the *para*-initiated branch exhibited the most complete dechlorination. It is of interest to note that *ortho*- and not *meta*-dehalogenation of 235-CB occurred. A change in pH in the present study's incubations did not inhibit the *para*-inititated dehalogenation branch, however, it did affect the extent of dehalogenation. Interestingly, *ortho*-dehalogenation persisted at pH 5.5 even though *meta*-dehalogenation occurred at insignificant levels or not at all. The processes in the proposed route initiated by *meta*-dechlorination did not remove *ortho* chlorines while the processes initiated by *para*-dechlorination removed all *ortho* chlorines (at pH 5.5, 6.5, and 7.5) and most *meta* chlorines (only at pH 6.5 and 7.5).

The Persistence of Aroclor 1268:

Several possible reasons may have contributed to the lack of Aroclor 1268 dechlorination. These include interfering co-contaminants, limited bioavailability, and ineffective primers (2, 3, 6).

PCB contaminated sites usually contain other inorganic and organic contaminants (1, 4, 5, 9, 21, 31, 34). Petroleum components in sediment appear to provide a sorptive phase that lowers the solution concentrations of PCBs, thus diminishing the bioavailability of PCBs and rate of dechlorination (43). The presence of heavy metals can also affect the outcome of anaerobic bioremediation of aromatic pollutants. In a study, added cadmium, copper, chromium, and mercury affected the metabolism in anaerobic microorganismsl consortia which degraded 2-chlorophenol, 3-chlorobenzoate,

phenol, and benzoate (25). Observable effects included extended acclimation periods, reduced dechlorination or biodegradation rates, and failure to dechlorinate or biodegrade the target compound. Elevated concentrations of mercury (17-25 μg/g dry wt), lead (45-70 μg/g dry wt), and chromium (87-120 μg/g dry wt) in LCP site sediments may have inhibited dehalogenation enzymes, sulfate-reduction, and microbial growth (14, 30, 32, 33).

Because the congeners in Aroclor 1268 are extremely hydrophobic (log $K_{ow} > 7$), their bioavailability in the aqueous phase can be expected to be low. PCBs may be limited in accessibility to microorganisms or extracellular enzymes as they sorb to organic matter and mineral surfaces in the sediment (TOC ~5%). Bioavailability of the highly chlorinated congeners of Aroclor 1268 is further discussed in Chapter 3.

The primers 2,3,4,5,6-pentachlorobiphenyl and 2,6-dibromobiphenyl have been successful in stimulating dechlorination of highly chlorinated congeners of Aroclor 1260 in previous studies (7, 39). These compounds were added individually or as a mixture of congeners to prime dehalogenation (6, 7, 35, 39, 41). Several studies showed no measurable dechlorination of the Aroclor 1260 residue when incubated without primers for up to 1 year (7, 35, 39). However, the primers used in this study may not be specific to stimulate dehalogenases in these particular estuarine microorganisms needed for the dechlorination of the highly chlorinated congeners of Aroclor 1268.

This study demonstrates that the indigenous microorganisms existing in the LCP Superfund sediment can biotransform lower halogenated biphenyls through a variety of routes (i.e. *ortho*- as well as *meta*- and *para*-dehalogenation routes). Specifically, *ortho*-dehalogenation activity was present under most experimental conditions. The fact that

native Aroclor 1268 was not appreciably dechlorinated may be due to the other factors, i.e. bioavailablity, enzyme specificities, and/or inhibition of the corresponding dehalogenating community by the presence of the heavy metals.

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Figure 2.1: Proposed dechlorination routes of primer 23456-CB after 1 year incubation. The average mole percentages of each congener at pH 7.5, 6.5, and 5.5, respectively, are in parentheses. The asterisk indicates that the mole percentages are designated for both 2346-CB and 2356-CB due to coelution. The white block arrows point to intermediates with two possible precursors. The *para-ortho-ortho* sequence resulting in 35-CB accounted for more than 50 mol % of the measured dechlorination products at pH 7.5.

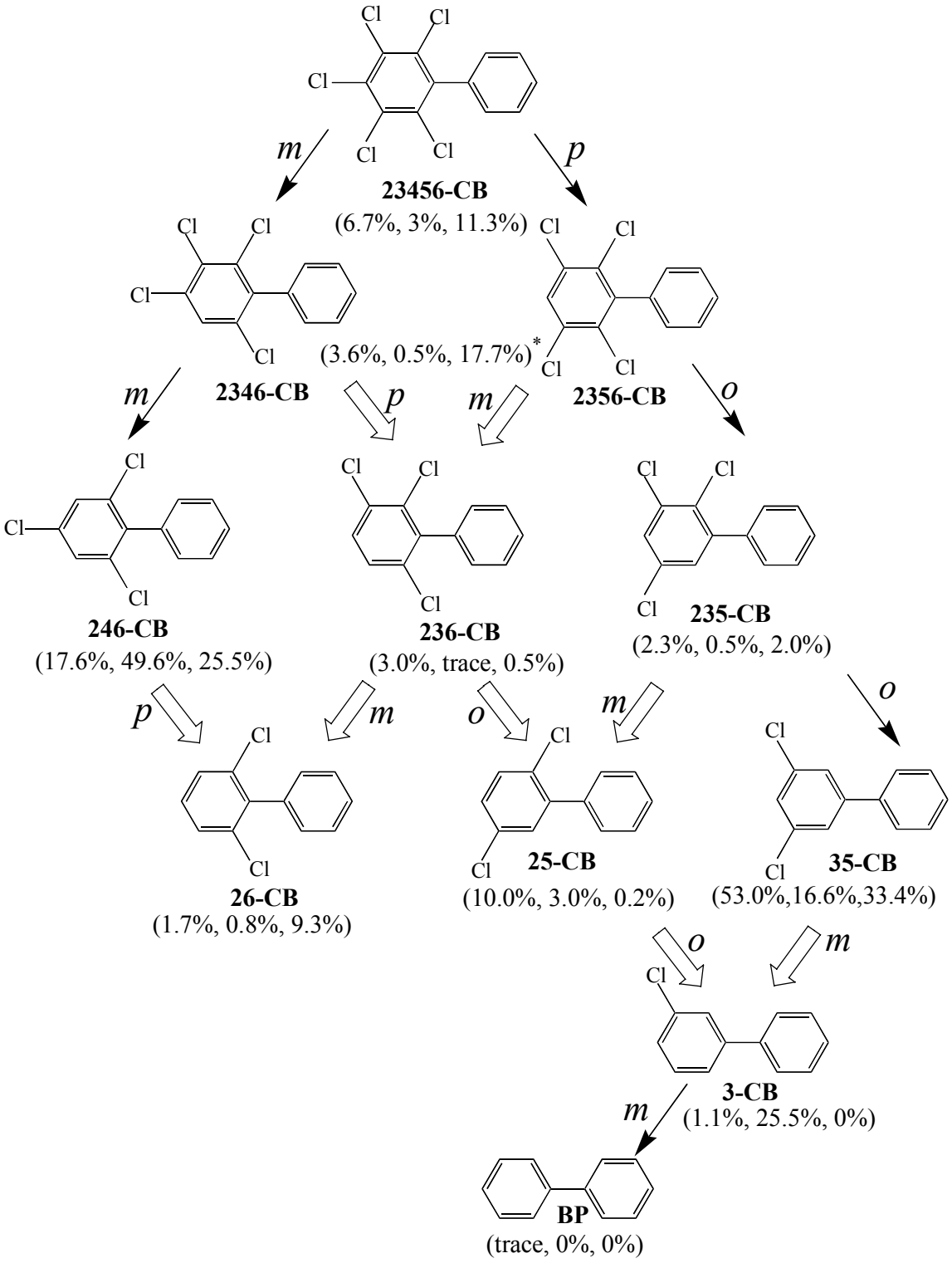
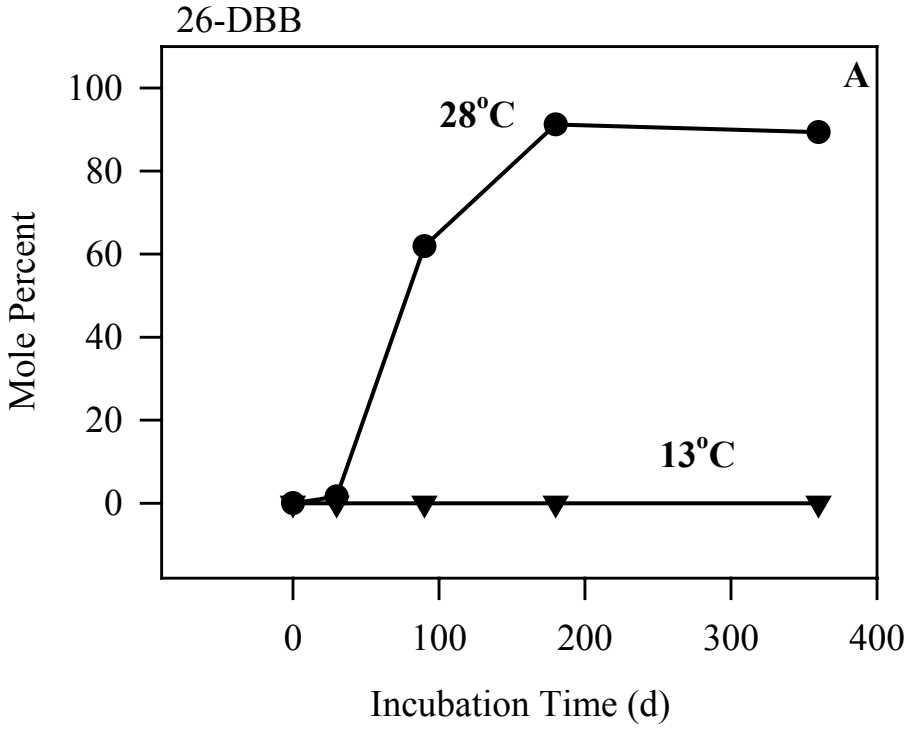

23456-CB
(6.7%, 3%, 11.3%)
m
p
2346-CB
(3.6%, 0.5%, 17.7%)*
2356-CB
246-CB
(17.6%, 49.6%, 25.5%)
236-CB
(3.0%, trace, 0.5%)
235-CB
(2.3%, 0.5%, 2.0%)
o
26-CB
(1.7%, 0.8%, 9.3%)
25-CB
(10.0%, 3.0%, 0.2%)
35-CB
(53.0%,16.6%,33.4%)
3-CB
(1.1%, 25.5%, 0%)
BP
(trace, 0%, 0%)

Figure 2.2AB: Molar abundance of dehalogenation products of (A) 2,6-dibromobiphenyl and (B) 2,3,4,5,6-pentachlorobiphenyl versus incubation time at 13^{o}C and 28^{o}C. Values are the mean of triplicate incubations.


26-DBB
A
28°C
13°C
Mole Percent
100
80
60
40
20
0
0
100
200
300
400
Incubation Time (d)

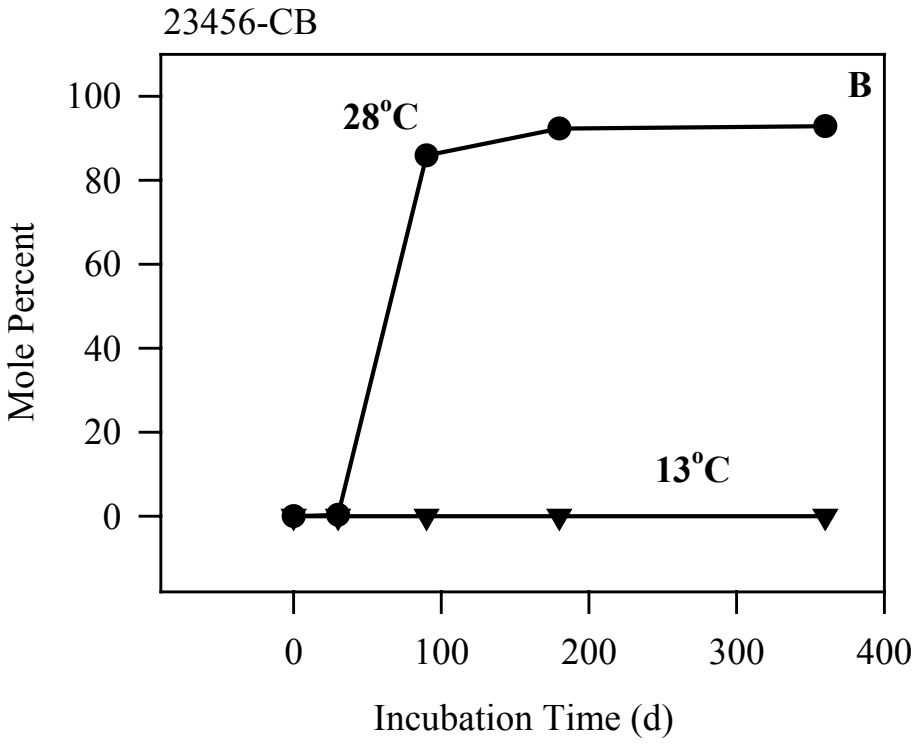
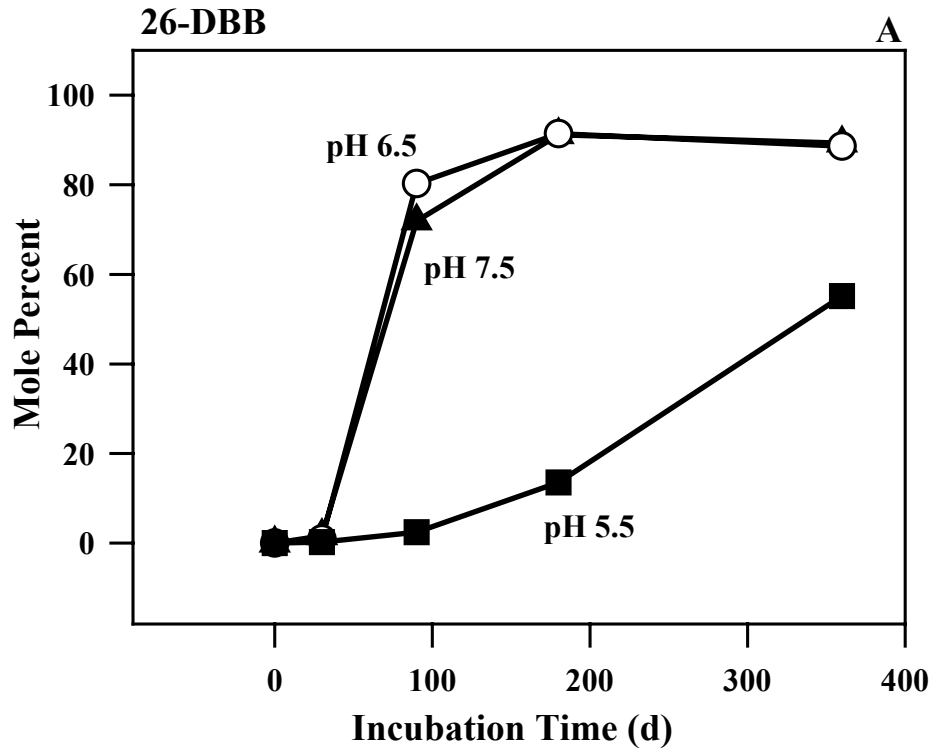

23456-CB
B
28°C
13°C
Mole Percent
100
80
60
40
20
0
0
100
200
300
400
Incubation Time (d)

Figure 2.3AB: Plot of the first halogen removed for (A) 2,6-DBB and (B) 23456-CB versus incubation time for three experimental pH conditions. ▲ pH 7.5, ○ pH 6.5, ■ pH 5.5.


26-DBB
A
100
80
60
40
20
0
Mole Percent
pH 6.5
pH 7.5
pH 5.5
0
100
200
300
400
Incubation Time (d)

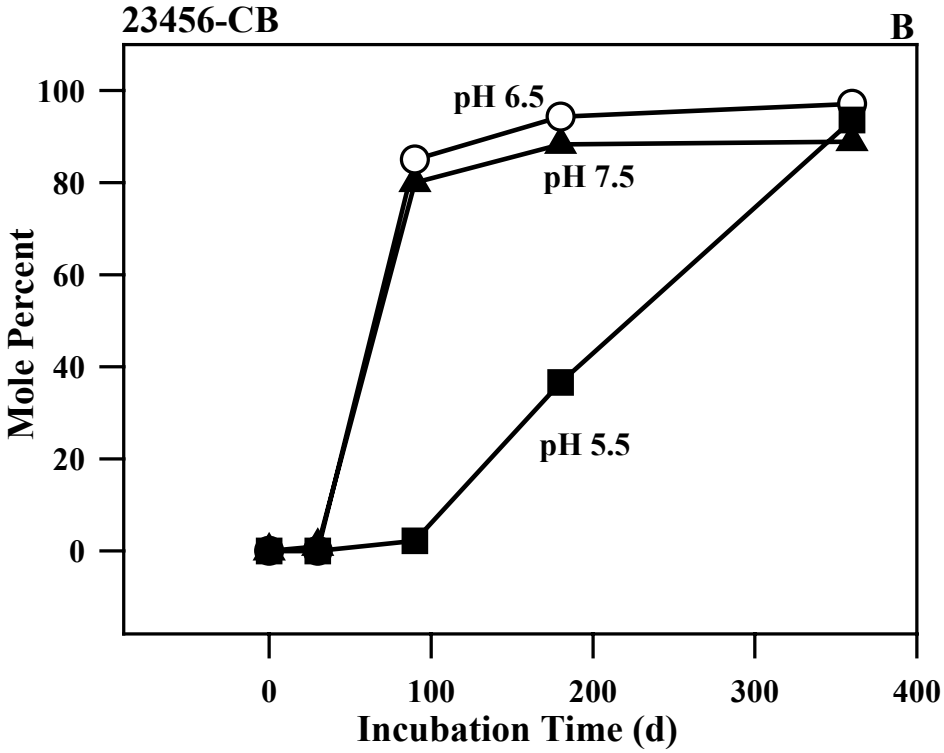
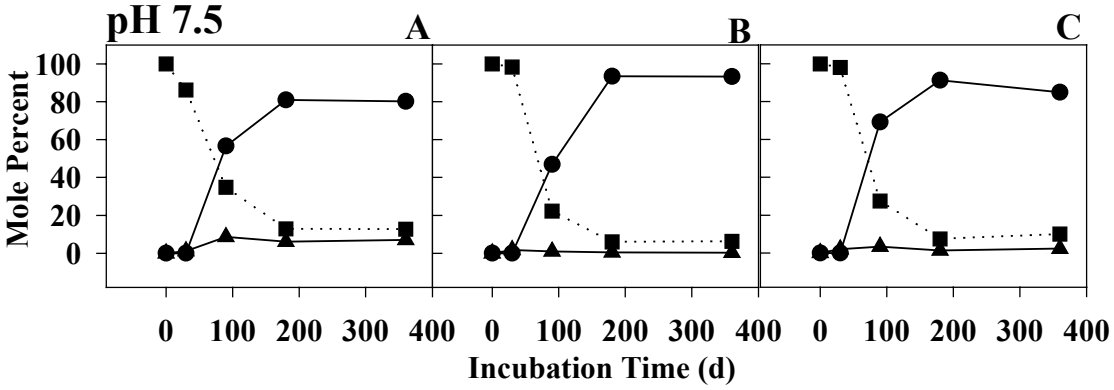
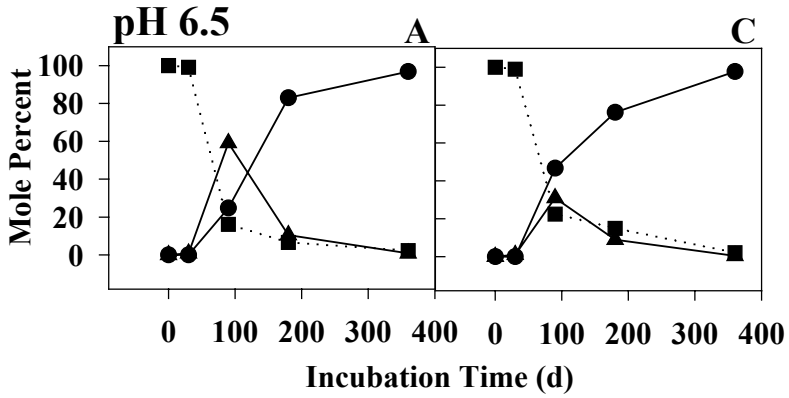

23456-CB
B
100
80
60
40
20
0
Mole Percent
pH 6.5
pH 7.5
pH 5.5
0
100
200
300
400
Incubation Time (d)

Figure 2.4: Molar abundances of dehalogenation products of 2,6-DBB versus incubation time for three experimental pH conditions in triplicate (A, B, C). Data for replicates B at pH 6.5 and pH 5.5 were discarded due to erroneous analysis.

■ 26-DBB, ▲ 2-BB, ● BP.


pH 7.5
A
B
C
Mole Percent
100
80
60
40
20
0
0 100 200 300 400 0 100 200 300 400 0 100 200 300 400
Incubation Time (d)


pH 6.5
A
C
Mole Percent
100
80
60
40
20
0
0 100 200 300 400 0 100 200 300 400
Incubation Time (d)

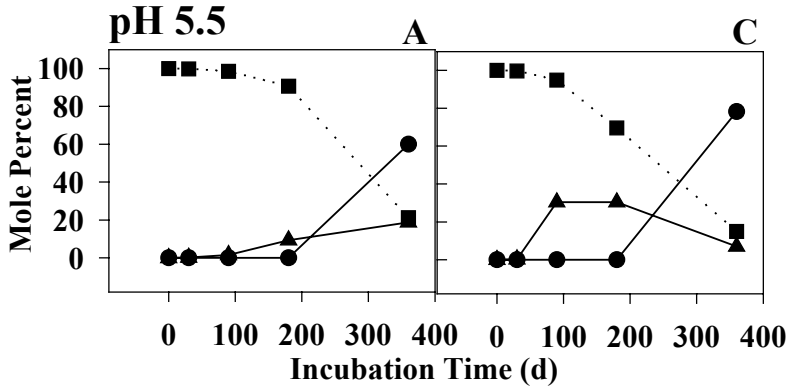

pH 5.5
A
C
Mole Percent
100
80
60
40
20
0
0 100 200 300 400 0 100 200 300 400
Incubation Time (d)

Figure 2.5: Molar distribution of 2,6-DBB and dehalogenation products in incubations (replicates A and B) with no pH maintenance (pH 6.7-7.6). The congeners are indicated as: ■ 26-DBB, ▲ 2-BB, ● BP.

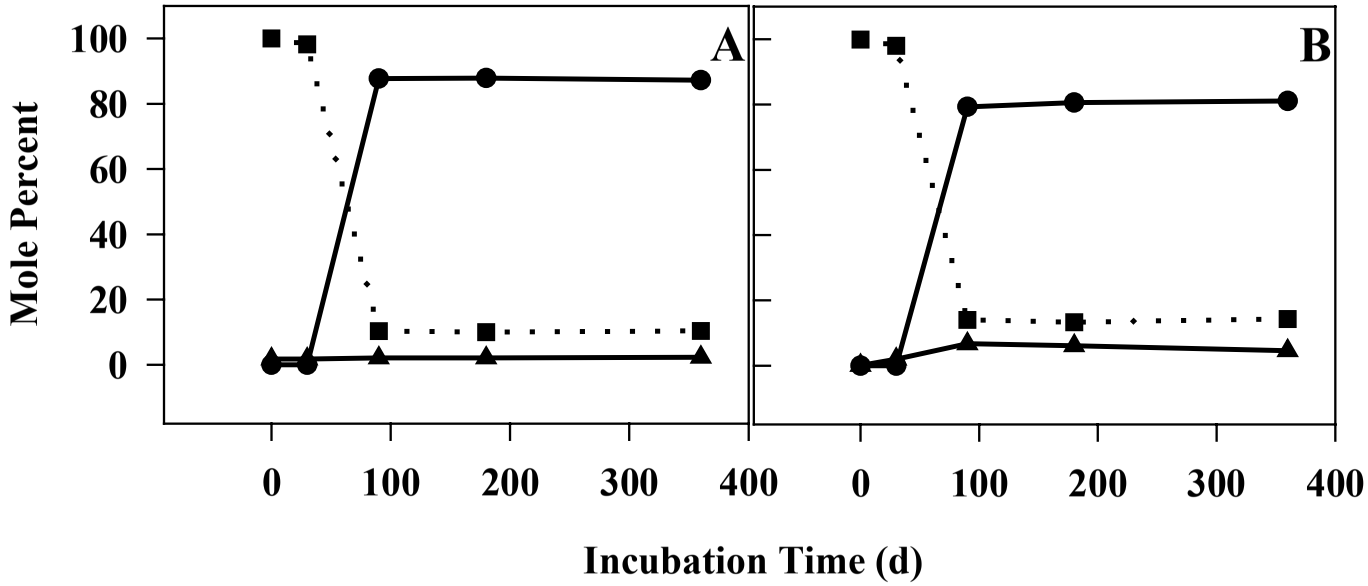

A
B
Mole Percent
100
80
60
40
20
0
0
100
200
300
400
Incubation Time (d)

Figure 2.6: The variability in the molar distribution of 23456-CB and dechlorination products among triplicate incubations (A, B, C) maintained at pH 7.5. The 10 congeners depicted are separated into high (top) and low (bottom) abundance. Red arrows indicate the time point sulfate amendments ceased. The congeners are designated as:

—◆— 23456-CB, —■— 2345/2356-CB, —▲— 235-CB, —△— 236-CB,

···△··· 246-CB, —●— 25-CB, —○— 26-CB, ···○··· 35-CB, ····•···· 3-CB,

—◇— Biphenyl (BP).

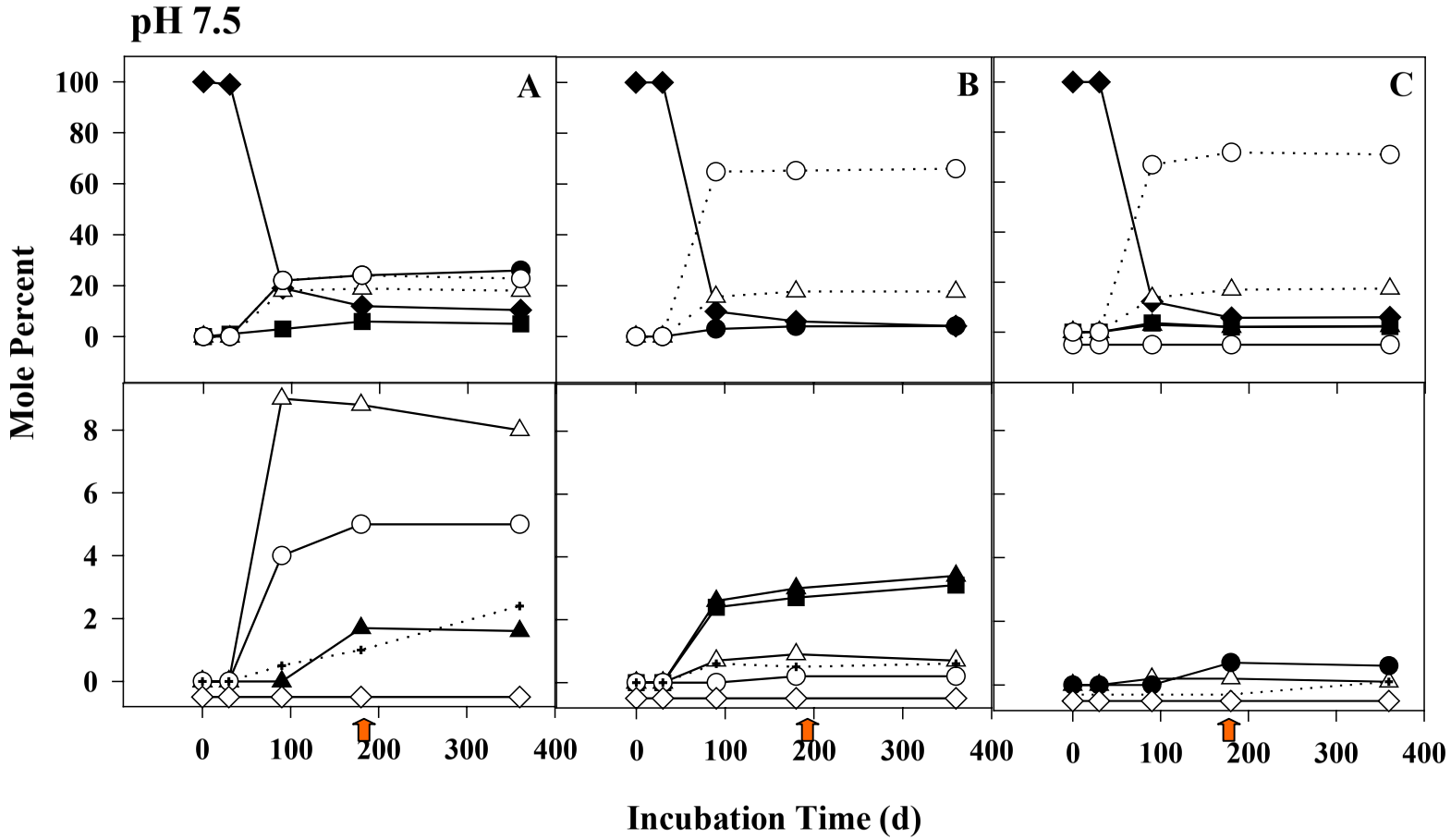

pH 7.5
A
B
C
Mole Percent
100
80
60
40
20
0
8
6
4
2
0
0
100
200
300
400
Incubation Time (d)

Figure 2.7: The variability in the molar distribution of 23456-CB and dechlorination products among triplicate incubations maintained at pH 6.5. The 10 congeners depicted are separated into high (top) and low (bottom) abundance. Red arrows indicate the time point sulfate amendments ceased. Data for replicate C was discarded due to erroneous analysis. The congeners are designated as: —◆— 23456-CB, —■— 2345/2356-CB, —▲— 235-CB, —△— 236-CB, ···△··· 246-CB, —●— 25-CB, —○— 26-CB, ···○··· 35-CB, ····•···· 3-CB, —◇— Biphenyl (BP).

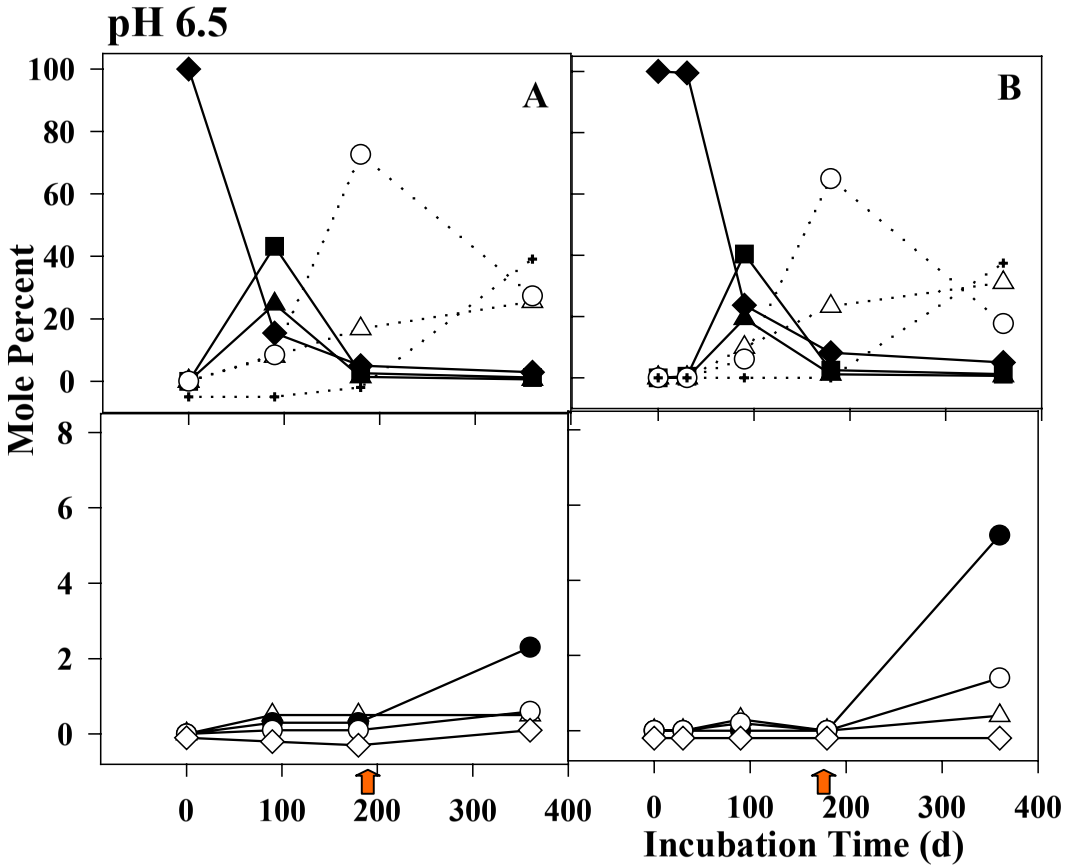

pH 6.5
A
B
Mole Percent
100
80
60
40
20
0
8
6
4
2
0
0
100
200
300
400
Incubation Time (d)

Figure 2.8: The variability in the molar distribution of 23456-CB and dechlorination products among triplicate incubations maintained at pH 5.5. The 10 congeners depicted are separated into high (top) and low (bottom) abundance. Red arrows indicate time point sulfate amendments ceased. The congeners are designated as:

—◆— 23456-CB, —■— 2345/2356-CB, —▲— 235-CB, —△— 236-CB,

···△··· 246-CB, —●— 25-CB, —○— 26-CB, ···○··· 35-CB, ····•···· 3-CB,

—◇— Biphenyl (BP).

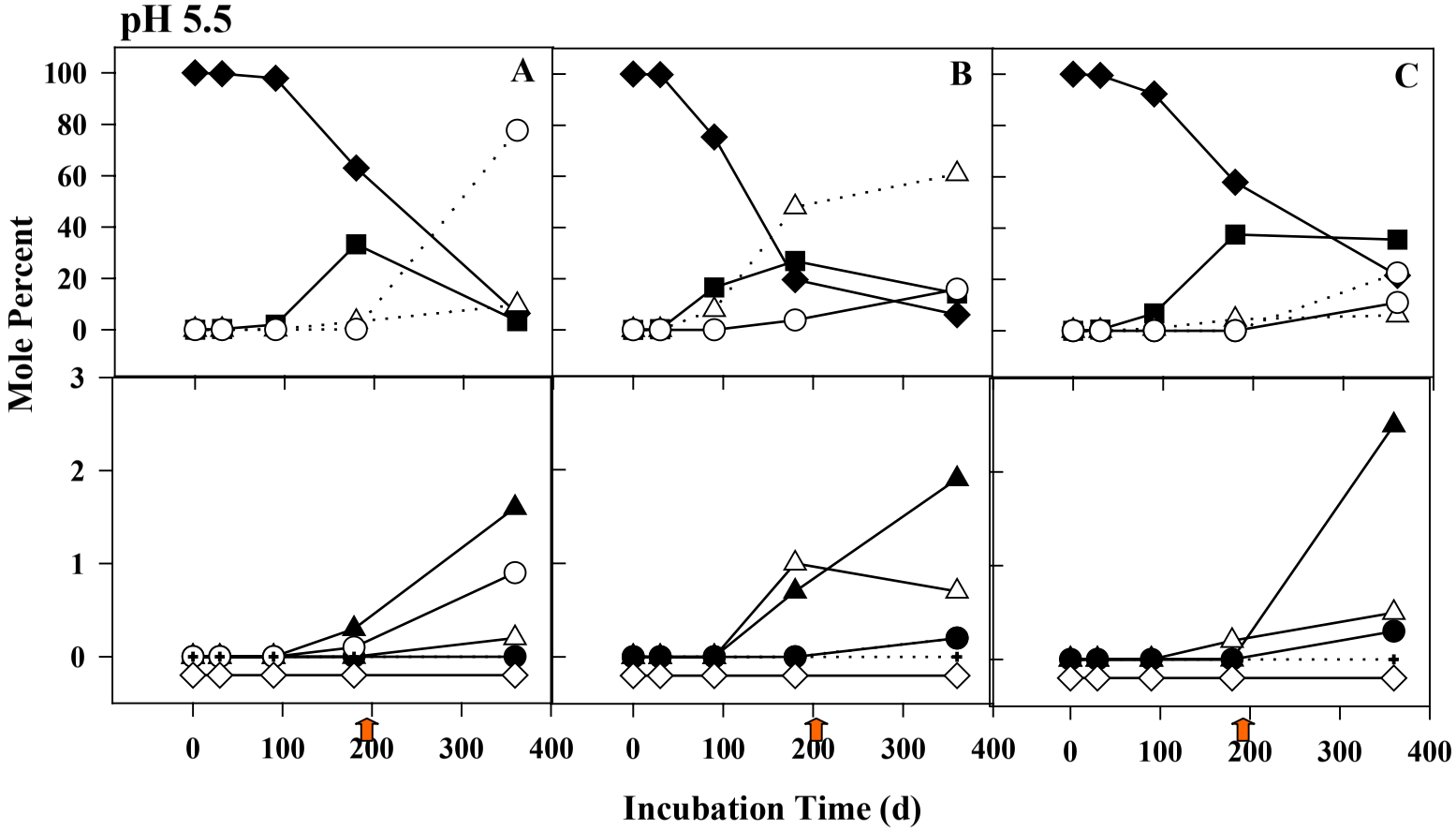

pH 5.5
A
B
C
Mole Percent
100
80
60
40
20
0
3
2
1
0
0
100
200
300
400
Incubation Time (d)

Figure 2.9: Molar distribution of 23456-CB and dechlorination products in incubation replicates (A and B) with no pH maintenance. The congeners depicted are separated into a top graph (0-100 mole percent y-scale) and resolved in the bottom graph (0-40 mole percent y-scale). Data for replicate B was discarded due to erroneous analysis. The congeners are indicated as: —◆— 23456-CB, —■— 2345/2356-CB, —▲— 235-CB, —△— 236-CB, ···△··· 246-CB, —●— 25-CB, —○— 26-CB, ···○··· 35-CB, ····•···· 3-CB, —◇— Biphenyl (BP).

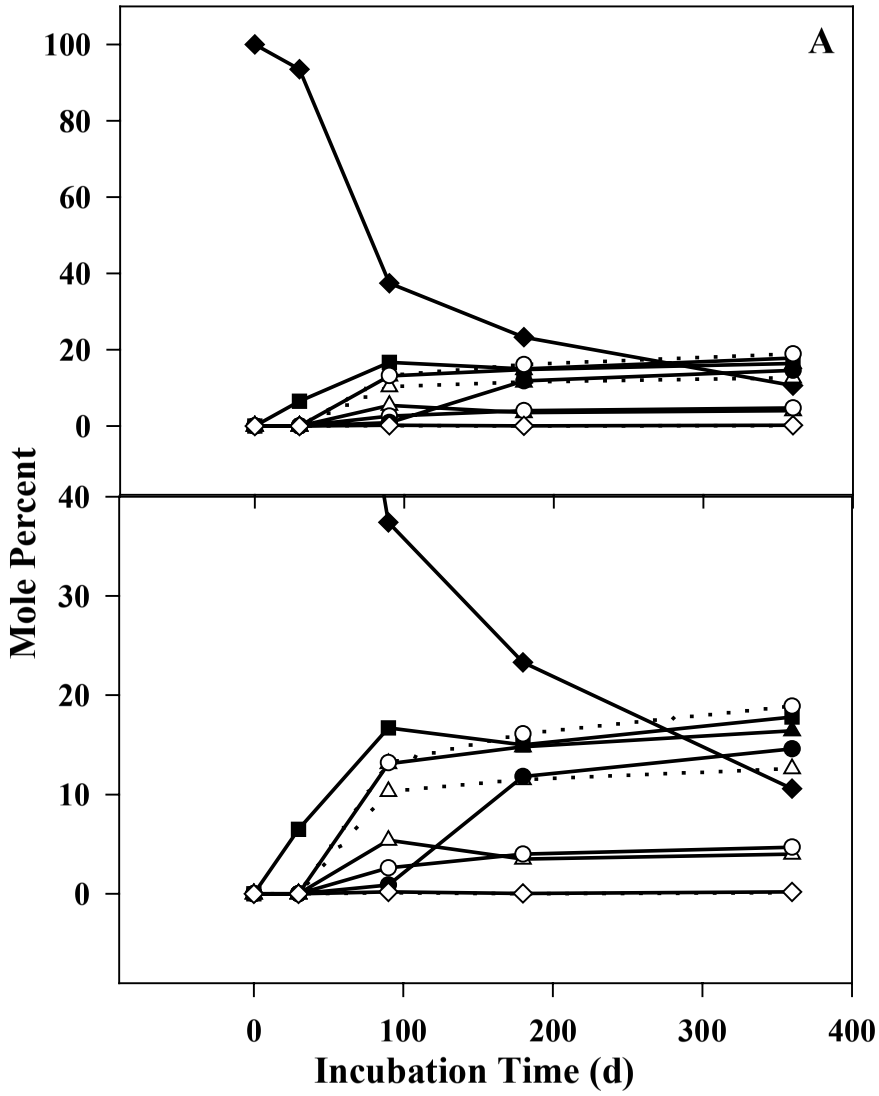

A
100
80
60
40
20
0
40
30
20
10
0
Mole Percent
0
100
200
300
400
Incubation Time (d)

Figure 2.10: Molar abundance of 2,6-DBB and dehalogenation products in control (no sulfate) incubations (replicates A and B). Data for replicate A was discarded due to erroneous analysis. The congeners are indicated as: ■ 26-DBB, ▲ 2-BB, ● BP.

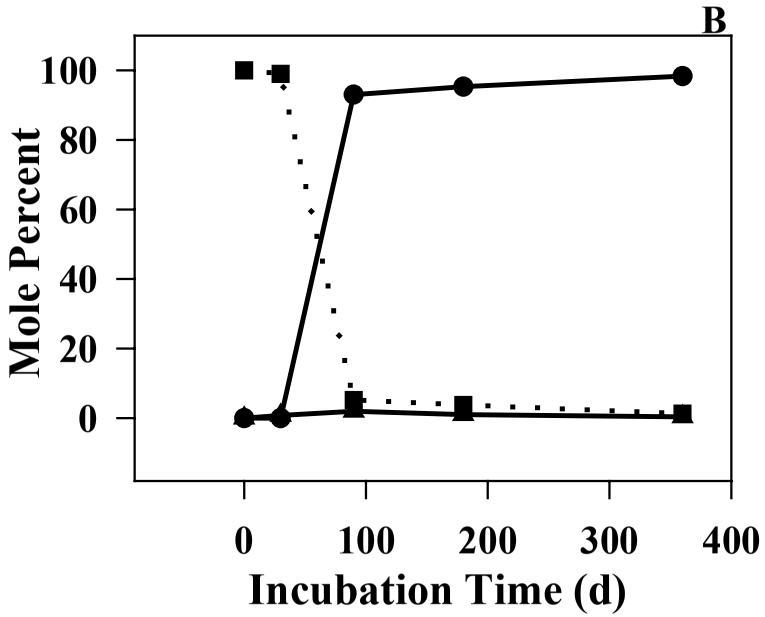

B
100
80
60
40
20
0
Mole Percent
0
100
200
300
400
Incubation Time (d)

Figure 2.11: Molar abundance and distribution of 23456-CB and dechlorination products in control (no sulfate) incubations (replicates A and B). The congeners are indicated as:

—◆— 23456-CB, ···△··· 246-CB, ···○··· 35-CB,

—◇— 2345/2346-CB, 235-CB, 236-CB, 25-CB, 26-CB, 3-CB, Biphenyl.

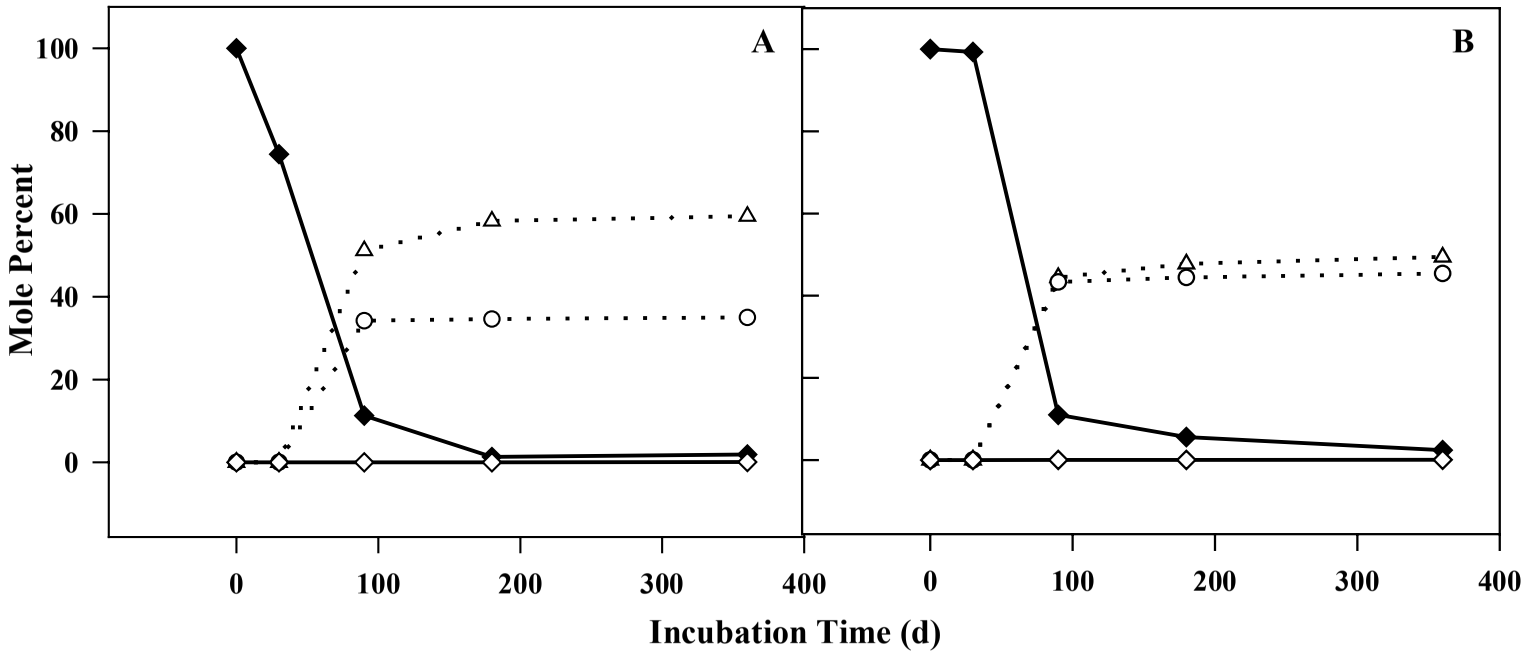

A
B
Mole Percent
100
80
60
40
20
0
0
100
200
300
400
Incubation Time (d)

Table 2.1: Comparison of PCB dechlorinating microbial consortia in sediment. The presence (+) or absence (-) of dechlorination is indicated for specific reactions.

Observed Reactions	Woods Pond Lenox, MA[a] freshwater	Sandy Creek Athens, GA[b] freshwater	Baltimore Harbor Baltimore, MD[c] estuarine	LCP Superfund Brunswick, GA estuarine
23456-CB → 2346-CB	+	-	-	+
→ 2356-CB	+	-	+	+
2356-CB → 235-CB	+	-	+	+
→ 236-CB	+	-	+	+
2346-CB → 236-CB	+	+	-	+
→ 246-CB	+	+	-	+
2345-CB → 235-CB	+	-	+	-
→ 245-CB	+	-	+	-
236-CB → 25-CB	+	-	+	+
→ 26-CB	+	+	+	+
235-CB → 25-CB	+	-	+	+
→ 35-CB	-	-	+	+
246-CB → 24-CB	+	-	-	-
→ 26-CB	+	+	-	+
24-CB → 4-CB	+	-	+	-
→ 2-CB	+	-	-	-
35-CB → 3-CB	-	-	+	+
25-CB → 3-CB	-	-	+	+

[a] References 3-7
[b] References 22, 23, 25, 26
[c] References 8, 24

CHAPTER 3

INVESTIGATION OF DECHLORINATION OF BIOAVAILABLE NONA- CHLORINATED PCBS IN ESTUARINE SEDIMENTS

Introduction:

The purpose of this experiment was to investigate whether (i) the sediment-associated microorganisms from an Aroclor 1268 contaminated estuary could dechlorinate freshly spiked nonachlorobiphenyls and, (ii) whether nonachlorobiphenyls could prime the dechlorination of aged Aroclor 1268. In previous experiments the microbial community was capable of dehalogenating the primers, 2,3,4,5,6-pentachlorobiphenyl (23456-CB) and 2,6-dibromobiphenyl (26-DBB), to biphenyl. However, no evidence of dechlorination of the weathered PCBs after a year of incubation in the microcosms was obtained. It was observed that the primers were easily biotransformed compared to the highly chlorinated congeners of Aroclor 1268 in the contaminated LCP marsh sediment. This may have been due a low bioavailability of the weathered PCBs, deficiencies of the microbial dehalogenases, or a relatively low concentration of Aroclor 1268. It was also observed that sulfidogenic conditions were conducive to PCB dehalogenation. In addition, a pH range of 6.5-7.5 created a successful dehalogenating environment for the bacteria. Another research group did not maintain a particular pH with their batch cultures of estuarine sediment containing Aroclor 1260

(with trace levels of nonachlorobiphenyls), but they observed that dechlorination occurred at pH 6.8 (L. Cutter, personal communication). In the previous experiment dechlorination occurred at 28^{o}C, but not at 13^{o}C. Buffering the batch cultures based on the controls of the last experiment was not necessary for dehalogenation, however, adjusting the pH periodically at sample points provided enough maintenance for a dehalogenating culture.

In this experiment, freshly spiked nonachlorobiphenyls (IUPAC #206, 207, 208) were added to sediment slurry incubations to see whether they can be dehalogenated, and if so, whether they would prime the dechlorination of Aroclor 1268 congeners. Although the concept of a primer is to use a PCB or halogenated compound that is less difficult to dehalogenate, the previous attempt did not demonstrate this because either the concentration of the primer was too low and not re-added upon complete biotransformation or the compounds were not the effective candidate when priming for the Aroclor 1268 dechlorination.

Materials and Methods:

Sediment:

PCB contaminated sediments were collected from the LCP Superfund Site, Brunswick, GA as described in Chapter 2. Uncontaminated sediments were collected from Skidaway Island, Savannah, GA near the rhizosphere of *Spartina* sp. The LCP sediment was stored for 2 years at 4^{o}C prior to use. The Skidaway sediment was stored for 1 year at 4^{o}C prior to use.

Microcosm Preparation:

Sediment was homogenized in a blender under a stream of nitrogen. Slurries were prepared by mixing 1 volume of wet sediment with 1 volume of autoclaved filtered pond water from the site of sampling. Each batch of contaminated or uncontaminated slurry was initially contained in 1L glass bottles, under anoxic conditions, in the dark at 27^{o}C for 2.5 months. Twenty milliliters of slurry was dispensed into 30 mL serum bottles maintained anoxic with Teflon-lined butyl rubber stoppers and aluminum crimp caps. The pH in batch slurries was adjusted with 30 mM sodium phosphate. In addition, each was fed 10 mM acetate and 10 mM lactate. PCB 206 (2,2',3,3',4,4',5,5',6-nonachlorobiphenyl), 207 (2,2',3,3',4,4',5,6,6'-nonachlorobiphenyl), or 208 (2,2',3,3',4,5,5',6,6'-nonachlorobiphenyl) was added at final concentrations of 308, 88, and 308 μM, respectively. Sediment microcosms were fed Na_2SO_4 or were not supplemented with an electron acceptor ("live control"). Malate was found to stimulate the onset of 2346-CB primed dechlorination of Aroclor 1260 in Woods Pond sediment samples (CITE WU). Therefore, malate was added to the methanogenic sediment cultures ("live control"). At each sampling time (0, 30, 90, 180 days), bottles were shaken by hand and vortexed for at most 10 seconds. Sterile controls were autoclaved three times for 1 hour at 121^{o}C on each of three consecutive days before the individual PCB congeners were added. Samples in triplicates and controls in duplicate were incubated in the dark at 27^{o}C without agitation.

Sample Extraction:

Slurry samples for PCB analysis were taken at 0, 30, 90, and 180 days. After vigorous shaking, 2 mL slurry was poured directly into a graduated 30 mL glass

scintillation vial and then sealed with a foil-lined screw cap in an anaerobic chamber. Approximately 100 μL of the subsample was poured into an Eppendorf microcentrifuge tube and set aside for sulfate analysis while the remaining slurry sample was extracted for PCBs. Dibromooctafluorobiphenyl was added to each slurry sample as an internal standard prior to extraction. Slurry subsamples were shaken vigorously on a shaker table at 200 strokes per minute for 24 hours with 5 mL ethyl acetate. After shaking, samples were allowed to settle for 30-60 minutes. The ethyl acetate extract was then eluted through a 5 cm column of a pre-washed mixture of Florisil and dried activated granular copper (4:1). PCBs were eluted with 5 mL ethyl acetate. The final eluant (~10 mL) was concentrated down to 1 mL under gently flowing nitrogen gas and then transferred into GC vials.

PCB Analysis:

A Varian 3400CX gas chromatograph equipped with a Saturn 3 ion trap mass spectrometer (GC-MS) and an 8200 autosampler was used for congener-specific identification of PCBs. The column oven temperature program started at 60^{o}C (2 min hold), increased to 150^{o}C at 20^{o}C/min (4.5 min hold), then ramped to 260^{o}C at 2^{o}C/min (13.5 min hold). The injector was programmed as follows: 60^{o}C (1 min hold), ramped to 280^{o}C at 200^{o}C/min (15 min hold). The ion trap and transfer line were maintained at 240^{o}C and 280^{o}C, respectively. Nine standard solutions containing all 209 PCB congeners (Accustandard, New Haven, CT) at 1 μg/ml were used to calibrate the GC-MS.

Results:

Uncontaminated (Skidaway) sediment slurries:

No treatments amended with PCB 206, 207, and 208 showed evidence of dechlorination under sulfate-reducing or methanogenic conditions. As expected, the sterile controls did not dechlorinate the nonachlorobiphenyls (Figure 3.1).

Contaminated (LCP) sediment slurries:

The anaerobic microorganisms indigenous to the LCP Superfund Site were unable to dechlorinate PCBs 206, 207, and 208 under sulfate-reducing or methanogenic conditions (Figures 3.2 and 3.3). Changes in the mole percentages of the nonachlorobiphenyls were attributed to recovery inconsistencies rather than dechlorination because no new PCB products were detected. There were neither detectable products of the Aroclor 1268 congeners nor a decrease of Aroclor 1268 congeners. Replicate C amended with 206 showed a sharp increase in 207, which appeared to be a result of contamination during the extraction clean-up (Figure 3.2). The sterile controls showed no evidence of dechlorination of PCBs, as expected.

Discussion:

The Persistence and Bioavailability of Nonachlorobiphenyls:

Freshly supplemented nonachlorobiphenyls (PCB 206, 207, and 208) at elevated concentrations were not dechlorinated by the microorganisms in either the PCB-contaminated or uncontaminated sediment. These results do not indicate that the lack of dehalogenation is attributed to decreased bioavailability. It was expected that the

contaminated sediment would probably dechlorinate the nonachlorobiphenyls because the microorganisms have been exposed to the Aroclor 1268 mixture weathered in the sediment. However, 2 year-old sediment was used and the equilibration time for the sediment slurries may have been too short (2.5 months) for the dechlorinating microorganisms to recover their activity. Also, dechlorination of highly chlroinated compounds by indegenous consortia (i.e. non-enriched) may require extended incubation periods (more than 1 year).

The bioavailability of sediment-associated contaminants depends on contaminant and sediment characteristics and microorganism behavior and physiology (3). Weathering, or the age of contamination, may affect bioavailability by physically trapping, hindering, and/or slowing desorption of contaminants from the soil. Desorption from field-contaminated sediments with extended contact times may not be accurately depicted in laboratory experiments of relatively short duration (i.e. weeks) (5).

Contaminants in aged sediments differ from spiked sediments in their chemical and biological availability. Biphasic desorption is often used as an explanation. Biodegradation occurs in (at least) a biphasic system comprised of immiscible components. The microbial utilization of solid, hydrophobic substrates requires solubilization, or emulsification, prior to uptake and metabolism (3). The challenge created by the hydrophobicity of PCBs is that microorganisms require an aqueous environment for optimal growth and activity. Solubilization of the substrate can be rate limiting to biodegradation. The low aqueous solubility of nonachlorobiphenyls may have resulted in an intrinsically low bioavailability via the aqueous phase.

Sediment organic carbon is a major variable that affects the bioavailability of non-polar, sediment –associated contaminants (4). PCBs may be limited in accessibility to bacteria or extracellular enzymes if sediment organic matter is relatively high (TOC ~5%) as is typical in fine-grained estuarine/saltmarsh sediments.

The bioavailability of aromatic hydrocarbons in marine or estuarine sediments can be difficult to determine due to extraction, analytical, and matrix barriers (1). Studies performed on highly variable systems such as soils can provide results that are mixed and often difficult to compare, owing to a difference in matrix (soil types) or contaminant.

The nonachlorobiphenyls in this experiment were also ineffective primers for the dechlorination of Aroclor 1268. The first report of dechlorination of non-native nonachlorobiphenyls after 16 weeks was in two PCB dechlorinating cultures derived from Esquimalt Harbor, Canada (2). However, the level of congener 207 dechlorination amounted to products that ranged between 1.2-3.1 mol %, while dechlorination of 206 and 208 ranged between 1.2-1.5 mol % and 1.8- 2.8 mol %, respectively. It is unclear what type of sediment the microorganisms were eluted from, what primers (if any) were used, and if the cultures could extensively dechlorinate congeners 206, 207, and 208 if incubated longer than 16 weeks. Bioavailability limitations due to sorption and/or aging were circumvented in this study by using a non-sorbing artificial solid phase (sand).

These same researchers also investigated the dechlorination of PCBs 195 (23456-234-CB), 199 (2345-2356-CB), 203 (23456-245-CB). For the octa- and nonachlorinated congeners with a 23456- group, the order of chlorine removal was similar to that of the 23456-CB primer used in the previous experiment (Chapter 2). That is, doubly flanked *meta* chlorines were removed first followed by the unflanked *para* chlorine of the same

ring. Also, octachlorinated congeners with a 2346- group (similar to the 2346-CB daughter congener in the previous experiment) were dechlorinated by the removal of doubly flanked *meta* chlorines to produce a 246- group. The 246- group is subsequently biotransformed by singly flanked *ortho* dechlorination or unflanked *para* dechlorination to produce a 24-group or 26-group, respectively. The 2346-CB intermediate from the previous experiment reacted similarly to produce 246-CB to 26-CB, but it was not *ortho*-dechlorinated.

The dechlorinating bacteria described by Kuipers et al. exhibited more diversity in their ability to biotransform octa- and nonachlorobiphenyls, relative to the microbial consortia from Georgian salt-marshes (this study). The mole conversions reported by were significant in the dechlorination of octachlorobiphenyls, but were extremely low for daughter congeners from nonachlorobiphenyls (2). This reflects the inherent difficulty in dechlorinating the nonachlorinated biphenyls 206, 207, and 208. The microorganisms in the Georgian salt marsh were unable to dechlorinate the spiked nonachlorobiphenyls.

The implications of this study reveals little of the influence bioavailability has on this potentially dechlorinating salt marsh system. However it reinforces the perception that nonachlorobiphenyls are difficult to dechlorinate, if at all. One of the explanations for this could be their solubility limitations.

References

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sediment for biodegradation experiments: chemical results. Org. Geochem. **30**(8B)**:**891-900.

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3. **Landrum, P. F., and W. R. Faust.** 1994. The role of sediment composition on the bioavailability of laboratory-dosed sediment-associated organic contaminants to the amphipod, Diporeia (spp). Chem. Speciat. Bioavailab. **6**(2-3)**:**85-92.

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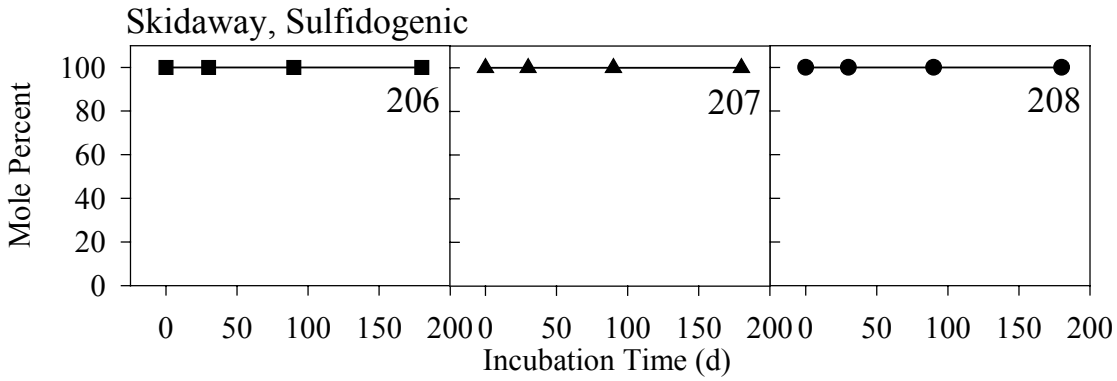
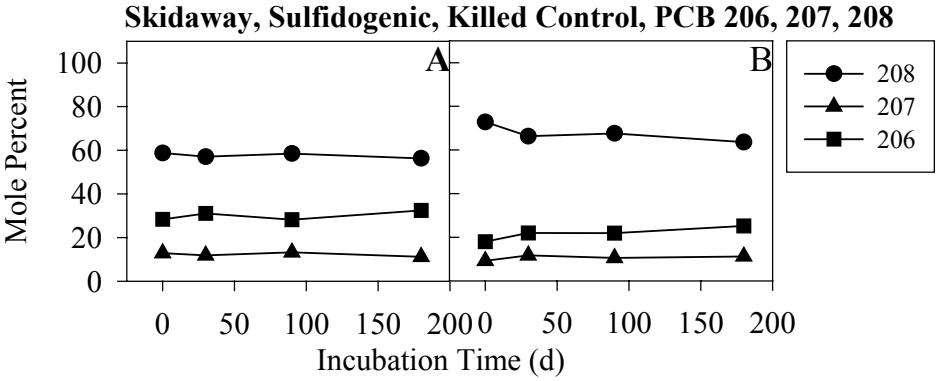
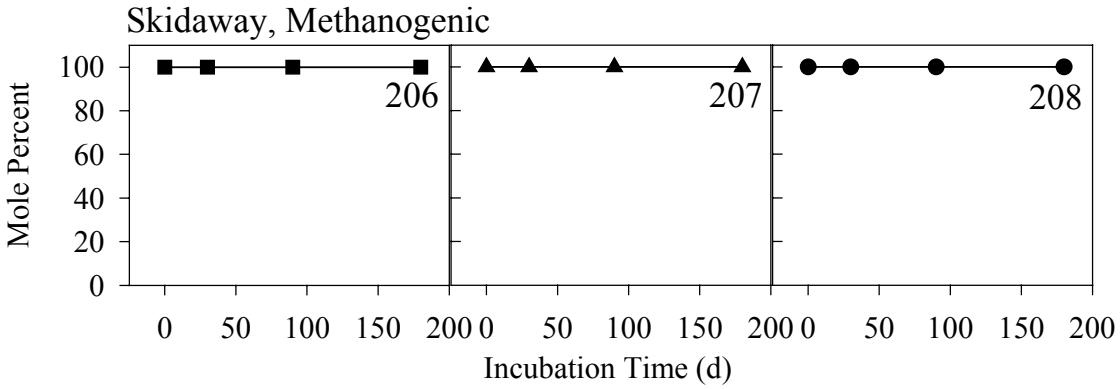
Figure 3.1: Mean molar abundance of PCB 206, 207, and 208 versus incubation time in uncontaminated sediment under sulfidogenic and methanogenic conditions. There was no dechlorination activity after 6 months of incubation at 27^{o}C. Duplicates of the sterile controls treated with sulfate or malate and amended with PCB 206, 207, and 208 did not exhibit dechlorination activity, as expected.


Skidaway, Sulfidogenic
Mole Percent
100
80
60
40
20
0
206
207
208
0
50
100
150
200
Incubation Time (d)


Skidaway, Sulfidogenic, Killed Control, PCB 206, 207, 208
Mole Percent
100
80
60
40
20
0
A
B
208
207
206
0
50
100
150
200
Incubation Time (d)


Skidaway, Methanogenic
Mole Percent
100
80
60
40
20
0
206
207
208
0
50
100
150
200
Incubation Time (d)

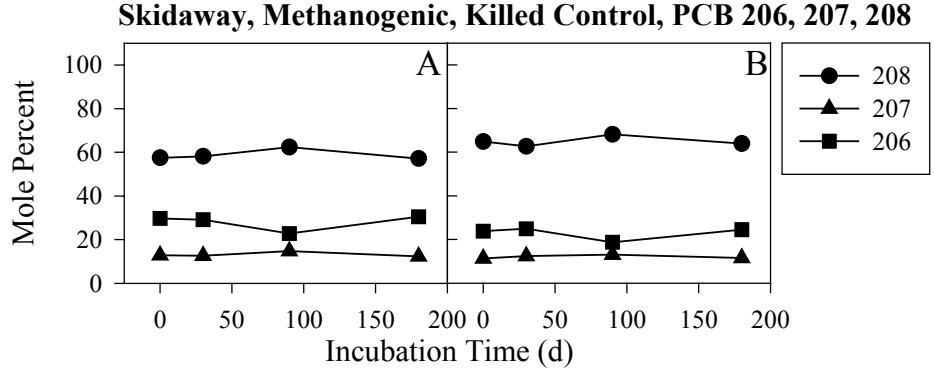

Skidaway, Methanogenic, Killed Control, PCB 206, 207, 208
Mole Percent
100
80
60
40
20
0
A
B
208
207
206
0
50
100
150
200
Incubation Time (d)

Figure 3.2: Mean molar abundance of selected PCB congeners versus incubation time in contaminated LCP Superfund Site sulfidogenic sediment slurries. There was no dechlorination activity after 6 months of incubation at 27^{o}C in experimental or sterile control batch cultures. Included are plots for nonachlorinated primers as well as the most abundant congeners in Aroclor 1268.

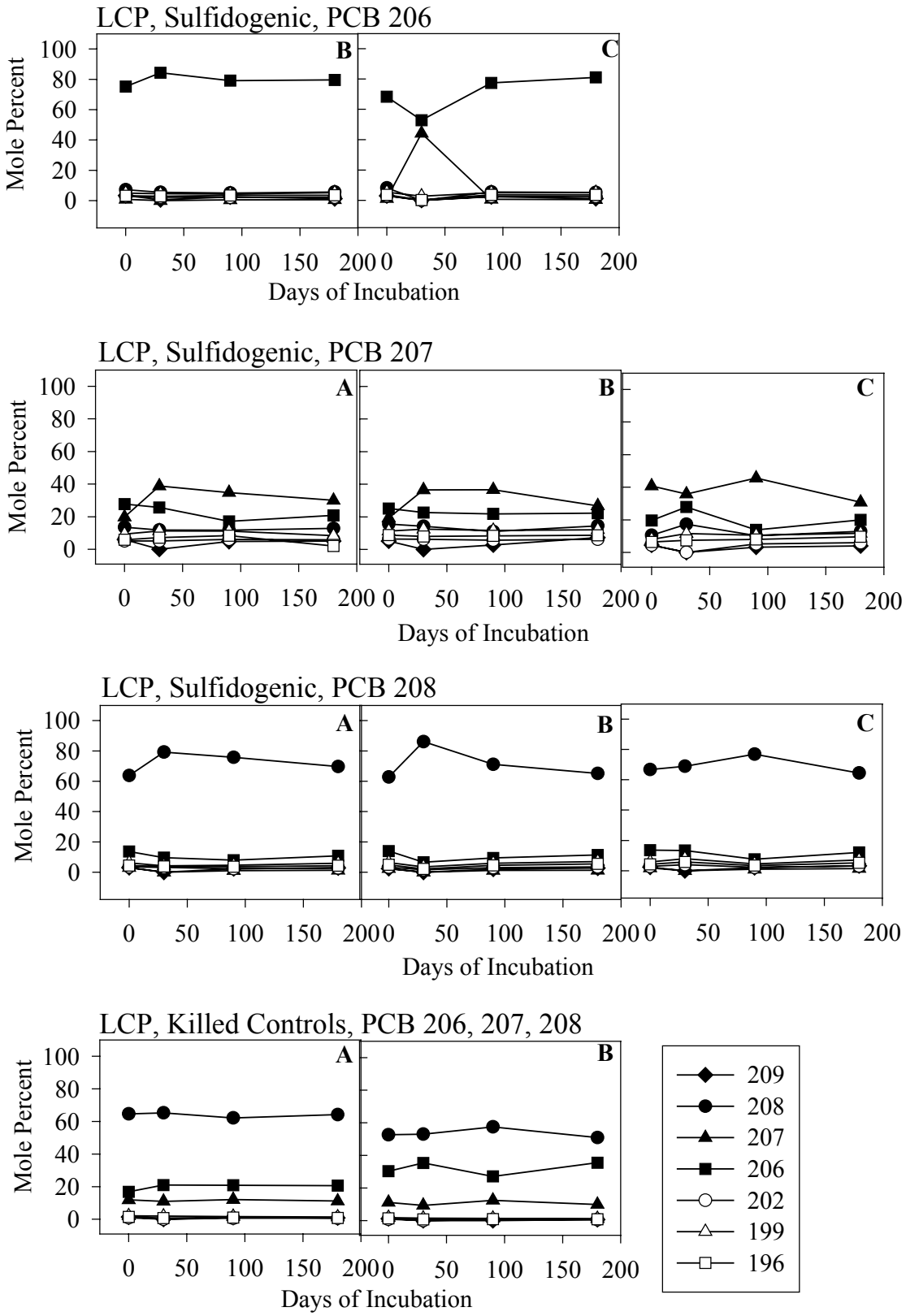
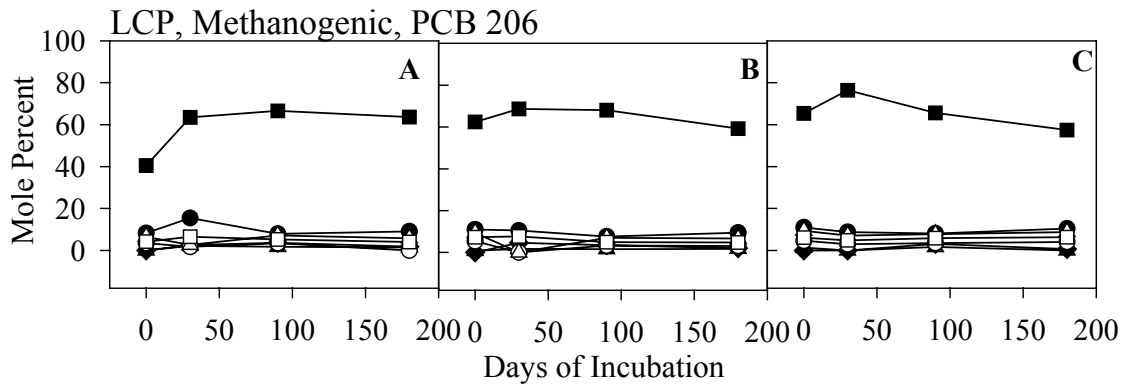
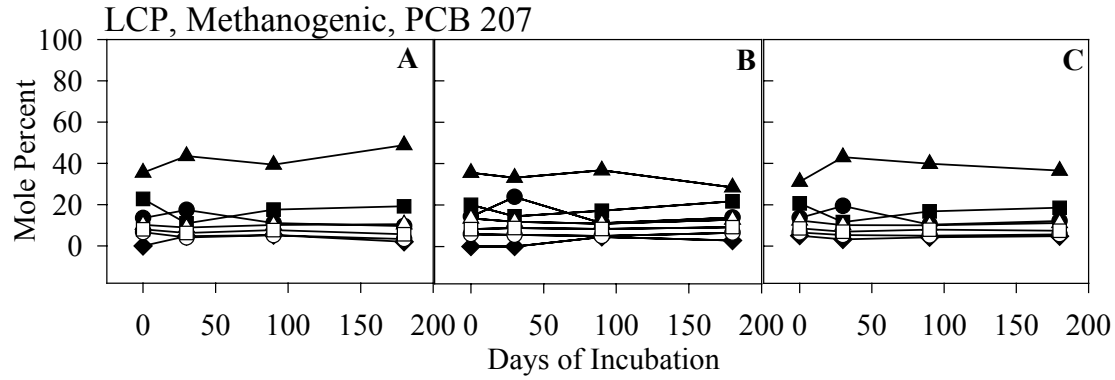
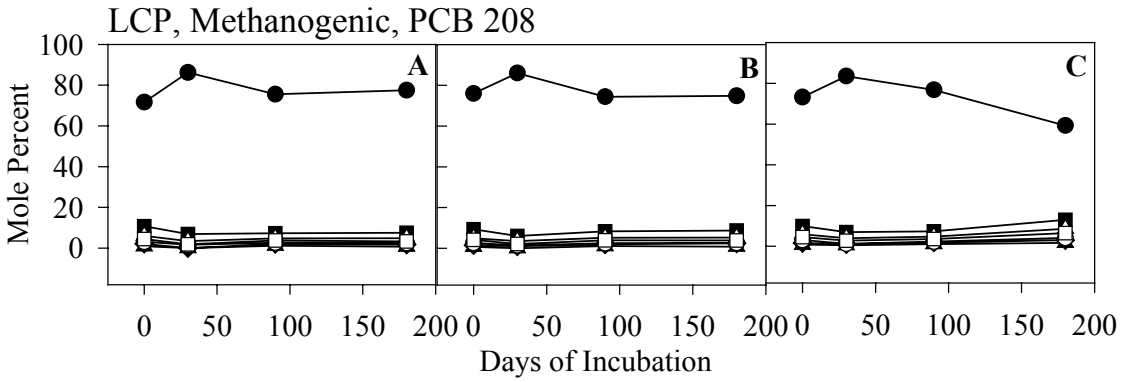
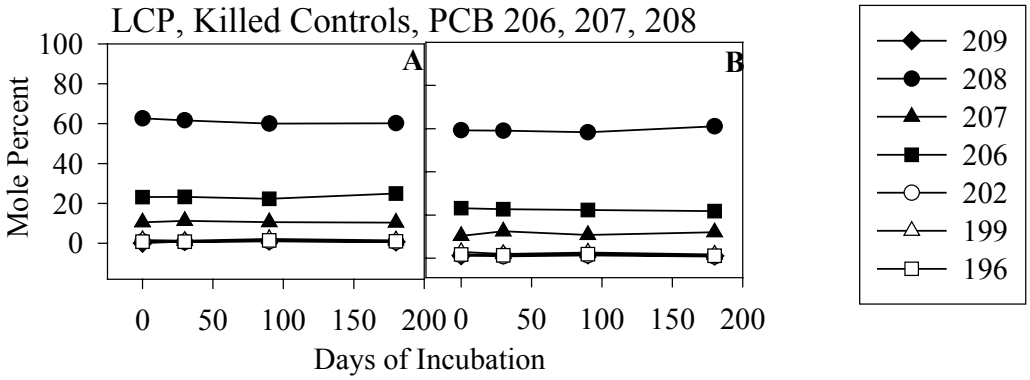

LCP, Sulfidogenic, PCB 206
LCP, Sulfidogenic, PCB 207
LCP, Sulfidogenic, PCB 208
LCP, Killed Controls, PCB 206, 207, 208
Mole Percent
Days of Incubation
A
B
C
209
208
207
206
202
199
196

Figure 3.3: Mean molar abundance of selected PCB congeners versus incubation time in contaminated LCP Superfund Site methanogenic "live control" sediment slurries. There was no dechlorination activity after 6 months of incubation at 27^{o}C in experimental or sterile control batch cultures. Included are plots for nonachlorinated primers as well as the most abundant congeners in Aroclor 1268.


LCP, Methanogenic, PCB 206
Mole Percent
A
B
C
Days of Incubation


LCP, Methanogenic, PCB 207
Mole Percent
A
B
C
Days of Incubation


LCP, Methanogenic, PCB 208
Mole Percent
A
B
C
Days of Incubation


LCP, Killed Controls, PCB 206, 207, 208
Mole Percent
A
B
Days of Incubation
209
208
207
206
202
199
196