ABSTRACT

Title of Document: THE ROLE OF DEHALORESPIRING BACTERIA IN THE REDUCTIVE DECHLORINATION OF POLYCHLORINATED BIPHENYLS IN BALTIMORE HARBOR SEDIMENT MICROCOSMS

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Baltimore Harbor sediment microcosms were incubated with the 12 most predominant congeners in Aroclor 1260 and their intermediate products to identify the major dechlorination pathways. Most congeners were dechlorinated in the *meta* position, although some dechlorination in the *para* and *ortho* positions was observed. The major dechlorination products were tetrachlorinated biphenyls with unflanked chlorines. Specific dechlorination rates of parent and intermediate PCB congeners were determined to identify the rate limiting reactions. To identify the microorganisms responsible for the dechlorination pathways, I developed PCR primers specific for the 16S rRNA genes of known PCB dehalogenating bacteria. These PCR primers were used in conjunction with DGGE to selectively identify the microorganisms that catalyzed each dechlorination reaction. Only three phylotypes were identified that catalyze the dechlorination of Aroclor 1260, and the selective activities of these phylotypes were determined. Phylotype DEH10 had high sequence

similarity to *Dehalococcoides* spp., while phylotype SF1 had high sequence similarity to the *o*-17/DF-1 group of PCB dechlorinating bacteria. The third phylotype had 100% sequence similarity to the *ortho*-dechlorinating bacterium *o*-17 described previously from Baltimore Harbor sediments. Most dechlorination reactions for all three phylotypes were growth-linked, indicating that PCB-impacted environments have the potential to sustain populations of PCB dechlorinating organisms. To investigate whether bioaugmentation would be feasible for bioremediation of PCB contaminated sites, Baltimore Harbor sediment microcosms were supplemented with known dechlorinators and their effects on PCBs dechlorination patterns determined. The addition of different dechlorinators resulted in different dechlorination patterns. Finally, novel putative reductive dehalogenases were identified from the PCB dechlorinating bacterium DF-1 using degenerate PCR primers. Comparative sequence analyses indicated that they had high sequence similarity to both confirmed and putative dehalogenases from several *Dehalococcoides* species. In conclusion, microorganisms that can dechlorinate Aroclor 1260 have been identified for the first time and dechlorination of congener mixtures was shown to occur by the growth-linked complementary activities of bacterial consortia within the *Chloroflexi*. Demonstration that bioaugmentation with these organisms can influence rates and pathways of dechlorination, combined with the development of molecular assay for monitoring their fate, provide potentially valuable tools for the development of bioremedial strategies for PCB contaminated sediments.

THE ROLE OF DEHALORESPIRING BACTERIA IN THE REDUCTIVE DECHLORINATION OF POLYCHLORINATED BIPHENYLS IN BALTIMORE HARBOR SEDIMENT MICROCOSMS

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2007

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Dedication

To my mom and dad who have always given me tremendous support.

They have always believed in me and been there for me.

I can get a real job now, Dad!

Acknowledgements

First of all I would like to thank my professor and mentor Dr. Kevin Sowers for giving me the chance to come to Baltimore and perform this work. Without him, this thesis would not exist! I have had some great years at the Center of Marine Biotechnology under his supervision. The next in line to thank is Dr. Joy Watts who taught me all I know about molecular methods while putting up with a lot of questions from someone without any prior molecular biology experience. I would also like to thank all the other members of the Sowers lab, Ethel Apolinario, for her support throughout the years, Kimberly Anderson, for help revising my papers and thesis, Dr. Birthe Kjellerup and Sheridan MacAuley for fruitful discussions.

I would like to acknowledge the work of the group at MUSC, Dr. Harold D. May and his group, especially Charlie Milliken and Greg Miller. They have been great collaborators. I would also like to acknowledge the Office of Naval Research Life Sciences Research Division, award no. N00014-03-1-0035/06-1-0090 for financial support.

Thanks also go to the COMB community and the many helpful lunch discussions, especially with Dr. Yossi Tal and Dr. Mohammed Alavi. Another COMB person I would like to mention is my good friend Naglaa Mohammed who has given me great personal support.

Last but not least I would like to thank my dear husband Marcelino T. Suzuki. He has been a tremendous support for me personally but has also been of great help for scientific discussions and for reviewing papers and this thesis.

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Chapter 1: Introduction

1.1. General background

Polychlorinated biphenyls (PCBs) are environmental contaminants found throughout the planet, in the air, water, sediments, fish and wildlife. PCBs were released into the environment through the production and use of commercial mixtures called Aroclors (Monsanto, USA and UK) and Clophen (Bayer, Germany), among other names. These mixtures were mainly used as a dielectric medium in transformers and capacitors hydraulic fluids, solvent extenders and flame-retardants (96). Due to their physical and chemical properties, PCBs are not readily biodegradable and have been shown to bioaccumulate in the food chain (60). As a result, PCBs are also found in adipose tissue, milk and serum of humans (108). Toxic effects of PCBs include developmental, reproductive and dermal toxicity, as well as endocrine effects, hepatotoxicity, carcinogenesis, and the induction of diverse phase I and phase II drug-metabolizing enzymes (108).

Polychlorinated biphenyls can be substituted with chlorine or hydrogen atoms in 10 different positions on the biphenyl skeleton (Figure 1.1). This results in 209 possible different isomers and homologs called congeners. The production of PCB involves batch chlorination of biphenyls with anhydrous chlorine in the presence of a catalyst, and the extent and pattern of chlorination is dependent on the reaction time and the amount of chlorine added. The names and chemical properties of PCB

mixtures depend on their chlorine content. One such mixture, Aroclor 1260, is a soft sticky, resin and was primarily used in transformers, as hydraulic fluids, in synthetic resins and as anti-dusting agents. Aroclor 1260 contains 60 weight % chlorine and represents one of the most prevalent mixtures that contaminate the environment. Since Aroclor 1260 is highly chlorinated, its congeners are more hydrophobic and generally less bioavailable than those in less chlorinated Aroclor mixtures. Thus, Aroclor 1260 is less susceptible to biodegradation in the environment than lower chlorinated Aroclors.

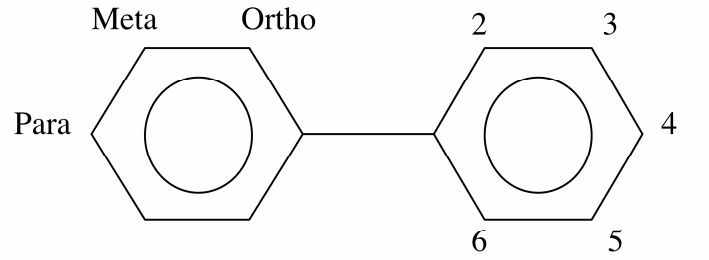


Figure 1.1. The basic structure of PCBs and the nomenclature according to the chlorine positions on the biphenyl rings

1.2. Reductive dechlorination of PCBs

1.2.1. Early studies on the reductive dechlorination of PCBs

The first reports of a biological process that changed the composition of Aroclor mixtures were published in the mid 1980's (17, 19, 20). Brown and colleagues examined chromatograms of PCBs extracted from sediments of different Hudson River sites and found that the congener compositions had changed compared to the composition of the original Aroclor 1242 mixture that had been released into the river from a single point source. The congener distribution showed that deeper

sediments contained a higher proportion of mono- and di-chlorinated congeners, as well as a higher proportion of *ortho* chlorines, while surface sediments were more similar to the original Aroclor 1242. Another site, Silver Lake (Pittsfield Massachusetts) was originally contaminated with Aroclor 1260 and, upon examination, samples from this site also supported the conclusion that reductive dechlorination had taken place (17). Although there was some initial resistance to the dechlorination hypothesis (21, 25), reductive dechlorination was widely accepted after Quensen *et al.* (99) showed that anaerobic laboratory microcosms containing Hudson River sediments dechlorinated Aroclor 1242.

It was hypothesized (20) that some microorganisms use PCBs as electron acceptors and may gain an environmental advantage by being able to perform this process in anaerobic environments where electron acceptors are scarce. Brown *et al.* (17, 20) began to classify the different activities observed in contaminated sediments into "patterns". This classification was mostly based on differences in the chromatograms generated by gas chromatographic analysis of the PCB congeners. This same approach was followed by Quensen *et al.* (98) who compared the patterns of dechlorination of four different Aroclors in sediment microcosms from Hudson River and Silver Lake. Brown and colleagues hypothesized that these various dechlorination patterns resulted from the action of different microbial populations with distinct dechlorinating activities (17, 20). Dechlorination in sediments has subsequently been observed in many locations around the world, for example, the New Bedford Harbor, MA (69), the Acushnet Estuary, MA (18), the St Lawrence River, NY (116), Lake Ketelmeer, Netherlands, and in soil microcosms in Italy (43).

1.2.2. “Patterns” of dechlorination of PCB mixtures

Bedard and Quensen (12) published a comprehensive review in which they classified dechlorination patterns observed at different sites into different processes that can occur separately or in combination. **Process M** is defined as flanked or unflanked *meta* dechlorination, mostly observed in lighter Aroclor mixtures and in Hudson River sediments (98). **Process Q** is defined by *para* dechlorination, unflanked or flanked, but also some *meta* dechlorination activity of Aroclor 1242 in Hudson River sediments. **Process C** is the combined result of processes M and Q. **Process H’** is the removal of both *meta* and *para* chlorines, but only if these chlorines are flanked, and **Process H** is similar to H’, but does not dechlorinate the *meta* chlorine in 23-groups. This process was observed when Aroclor 1260 was dechlorinated in Hudson River sediments, and the accumulation products were 25-25-CB (98). This process prefers *para* chlorines if doubly flanked *meta* chlorines are not present. **Process P**, defined as mostly flanked (single or double) *para* dechlorination, was observed in Woods Pond sediment with Aroclor 1260 and results in accumulation of 25-25-CB (9). **Process N** is defined by double or single flanked *meta* dechlorination and results in the accumulation of 24-24-CB. This process has been observed in Silver Lake sediments (4, 98) and Woods Pond sediments (10). **Process LP** can dechlorinate unflanked *para* chlorines and results in greater dechlorination of Aroclor 1260 when combined with process N (14). Finally, **Process T** is defined by very restricted *meta* dechlorinating activity observed in Woods Pond sediment microcosms with Aroclor 1260 incubated at 50-60 degrees (140).

Based on the patterns of dechlorination, some of the factors that may influence which chlorine on a biphenyl will be subjected to dechlorination include: 1) the position of the chlorine relative to the bond between the biphenyl rings (*ortho, meta or para)*, 2) the surrounding configuration of chlorines (unflanked, single-flanked or double-flanked), 3) the chlorine configuration of the opposite ring, 4) environmental conditions and 5) the microbial populations present (12).

1.2.3. Dechlorination of single congeners

There are several reports by investigators who have studied the dechlorination of single PCB congeners to infer clear daughter-parent congener relationships. It appears that relative chlorine positions on the biphenyl ring are a major factor influencing the dechlorination of single congeners, but different studies have also seen different dechlorination activities with the same PCB congener. For example, Nies and Vogel (93) showed that 23456-CB was *meta* dechlorinated to 2346-CB, while others (15, 114) observed either double-flanked *meta* or double-flanked *para* dechlorination of 23456-CB. In addition, Wu and Wiegel (138) observed flanked *para* dechlorination of 2346-CB, while Van Dort *et al.* (123) showed that 2356-CB was mostly dechlorinated in the *meta* position, also showing for the first time *ortho* dechlorination *in vitro*. Dechlorination in the *ortho* position was also observed in microcosms with Baltimore Harbor sediments (15).

Complete dechlorination to mono-chlorobiphenyl has been rarely observed, but Abramowicz *et al.* (2) observed *meta* dechlorination of 234-34-CB to 24-34-CB, followed by two subsequent *para* dechlorination steps to 2-3-CB and finally a *meta*

dechlorination to 2-CB in Hudson River microcosms. This might have occurred because 234-34-CB only had a single chlorine in the *ortho* position. On the other hand, Boyle *et al.* (16) observed *meta* dechlorination of 236-CB to 26-CB in enrichment cultures inoculated with Hudson River sediment, but no further dechlorination. 26-CB was also the final product of an unflanked *para* dechlorination of 246-CB (138). Finally, Williams (134) tested all possible combinations of trichlorobiphenyls chlorinated on one ring. He generally found that double-flanked chlorines were dechlorinated first, before *meta* and *para* chlorines. In most cases double flanked chlorine positions are preferentially dechlorinated, but other factors appear to have an effect of the dechlorination of single congeners.

1.2.4. Dechlorination of Aroclor 1260

Generally, the rate of dechlorination of Aroclor 1260 is much slower than the dechlorination rates of less chlorinated Aroclor mixtures (4, 98). This might be due to different factors such as i) availability, Aroclor 1260 is less bioavailable due to greater hydrophobicity, ii) toxicity, some congeners in Aroclor 1260 might be toxic to some dechlorinating microorganisms or iii) lack of inducing congeners, as some lesser chlorinated PCB congeners have been shown to stimulate PCB dechlorination (98).

Environmental dechlorination of Aroclor 1260 was reported in Silver Lake (20) and Woods Pond (10). In Silver Lake "process N", which is defined as a preference for dechlorination of single or double flanked *meta* chlorines, was the primary dechlorination activity observed (12, 98). In Woods Pond, both *meta*

dechlorination (process N) and flanked *para* dechlorination (process P) was observed (10). In addition, Bedard *et al.* (14) identified process LP, which can dechlorinate unflanked *para* chlorines and results in a more extensive dechlorination of Aroclor 1260 in conjunction with process N. The authors proposed that these two activities are mediated by different microbial populations with distinct PCB dechlorinating activities (14). Likewise, Baltimore Harbor sediment microcosms showed dechlorination activity of Aroclor 1260 primarily towards *meta* chlorines, process N, but some *ortho* dechlorination was also observed (141). Recently, a sediment-free mixed enrichment culture has been shown to dechlorinate Aroclor 1260, mostly by process N (8).

1.3. Factors influencing PCB dechlorination

1.3.1. Effect of electron acceptors

Since it was hypothesized that microorganisms use PCBs as electron acceptors (17, 20, 99), several laboratories have investigated whether alternative electron acceptors influence PCB dechlorination. The results of these studies vary in the literature. Rhee *et al.* (103) performed several experiments with the addition of different electron acceptors in Hudson River sediment microcosms and showed that methanogenic conditions were the most conducive to reductive dechlorination, while nitrate completely inhibited dechlorination of Aroclor 1242. However, others have found that nitrate supports dechlorination (84).

There are conflicting reports on the effect of sulfate addition on dechlorination. Some studies found that sulfate inhibits or partially inhibits PCB dechlorination (4, 23, 103) while Øfjord and coworkers (95) concluded that sulfate did not inhibit dechlorination of Aroclor 1254 in marine sediment microcosms. However, in the examples above, the sulfate concentration was not measured over time. Indeed, when the sulfate concentration was measured, several studies have showed that dechlorination did not start before sulfate was depleted (79, 146), thus some hypothesized that the sulfate reducers were stimulated by sulfate, and switched to use PCBs after the sulfate was depleted (146).

1.3.2. Effect of electron donors

Various electron donors have also been shown to affect PCB dechlorination. Nies and Vogel (92) showed that addition of glucose and acetone to microcosms supported dechlorination of Aroclor 1242 to a greater extent than microcosms with acetate or methanol. Morris *et al.* (84) found that pyruvate and hydrogen stimulated dechlorination of Aroclor 1242 in microcosms more effectively than formate, but the pyruvate had to be completely consumed before dechlorination started, with acetate accumulating as a transient product. Some studies investigated the effect of electron acceptors on the lag time prior to dechlorination. Alder *et al.* (4) found that adding a fatty acid mixture decreased the lag period before dechlorination was observed in microcosms dechlorinating Aroclor 1242. However, after 11 months of incubation the total dechlorination was similar to microcosms with no added fatty acid mix. Similarly, Abramowitz *et al.* (2) found that adding a complex carbon source

decreased the lag period before dechlorination was observed. Finally, Sokol *et al.* (114) found that the pathway of dechlorination changed and the rate increased when adding hydrogen instead of nitrogen. However, as pointed by Bedard *et al.* (12), these experiments can be complicated by the fact that these sediments contained other organic carbon sources and the actual electron donor was not known.

1.3.3. Effect of heavy metals

The effect of heavy metals, chemicals that are frequent co-contaminants in PCB contaminated sediments, was the subject of only a few studies. Alder *et al.* (4) observed less *in situ* dechlorination in sediments from Silver Lake and New Bedford Harbor than from Hudson River and hypothesized that this could be an effect of different heavy metal concentrations between these sites. Sokol *et al.* (116) also observed a similar inhibition of *in situ* PCB dechlorination at sites with extraordinary high concentrations of heavy metals and polyaromatic hydrocarbons (PAHs). These reports suggest that heavy metals might have a negative effect on PCB dechlorination.

1.3.4. Effect of PCB concentration

The concentration of PCBs has been shown to be a factor in the dechlorination process. Quensen *et al.* (99) found that higher concentrations of Aroclor 1242 resulted in higher dechlorination rates, possibly due to higher solute concentrations available for the microorganisms. Abramowitz *et al.* (2) observed that the highest

dechlorination rates of Aroclor 1242 occurred at concentrations over 750 ppm (μg/ml), and that dechlorination rates were concentration dependent below 250 ppm. Others have suggested that there are certain "threshold" concentrations below which no dechlorination occurs (102, 104). Experiments with lower concentrations of individual congeners showed that dechlorination was concentration dependent down to 4 ppm (μg/g dry sediment) for 234-CB, and the authors suggested that both the lag times and the dechlorination rates were congener specific (115). However, several of these studies give the concentration in relation to the amount of dry sediment in the microcosms, while others (2) give the concentrations in mL of cultures. It is therefore difficult to directly compare these values. Also, the amount of sediment in the microcosm studied will also have on effect on how much PCB is available for the microorganisms as some of the PCB will be absorbed to sediment particles.

1.3.5. Effect of the microbial community

There are many uncertainties as to what drives the different patterns of Aroclor 1260 dechlorination that were observed at different sites. For example, microcosm experiments showed that Silver Lake sediments, which were originally contaminated with Aroclor 1260, dechlorinated this same Aroclor at a higher rate and to a greater extent than microcosms containing Hudson River sediments that were originally contaminated with lower chlorinated compounds (4, 98). This higher rate of dechlorination might be due to natural attenuation by a dechlorinating population that was already dechlorinating Aroclor 1260. The dechlorination patterns were also different, which the authors attributed to the possibility that different microbial

communities were present. The results suggest that microbial populations have an effect on Aroclor 1260 dechlorination. However, since the microorganisms responsible for the dechlorination activities observed in several of these studies could not be identified, the specific effects of different PCB dechlorinators on the dechlorination of Aroclor 1260 is not known.

1.4. Identification of the microorganisms responsible for reductive dechlorination of PCBs

Several groups of organisms have been hypothesized to be involved in PCB dechlorination, most predominantly sulfate reducers and methanogens. The first attempt to identify the microorganisms responsible for the dechlorination of PCBs was a study by Ye *et al*. (143). Using Hudson River sediments, this study concluded that the microorganisms responsible for the *meta* dechlorination of Aroclor 1242 survived treatment with both heat and ethanol and based on this observation, were believed to be spore-forming sulfate reducing bacteria (12, 143). Furthermore, microorganisms that were killed by this treatment were believed to be responsible for dechlorination in the *para* position. Similarly, Zwiernik *et al.* (145) hypothesized that sulfate reducers were responsible for the *para* dechlorination observed in $FeSO_4$ amended Hudson River cultures. Ye and coworkers (143) also concluded that methanogenesis was not required for dechlorination (i.e. these organisms are not methanogens), as did Rhee *et al*. (103). Finally, Kim *et al.* (62) concluded that neither sulfate reducers nor methanogens were responsible for the PCB dechlorination in their cultures, and they showed that dechlorination was growth-linked for the first

time based on Most Probable Number (MPN) enumeration of the dechlorinating population.

The breakthrough on the identification of PCB dechlorinating bacteria came with the establishment of an *ortho* dechlorinating enrichment cultures grown without soil or sediment (32). The microbial community in these selective enrichment cultures was analyzed by 16S rRNA analysis that revealed several "candidates" for the microbial catalyst (97). This dechlorinator, designated *o*-17, was later identified to belong to the *Chloroflexi* group, related to *Dehalococcoides* (33). Subsequently, Wu *et al.* (136) established a second sediment-free microbial consortium that dechlorinated double-flanked chlorines. Later analysis of these enrichment cultures showed that they contained three dominant microorganisms and the dechlorinator was identified by 16S rRNA analysis as DF-1, most similar to *o*-17 and the *Dehalococcoides* group (137). Members of the *Dehalococcoides* group have also been shown to dechlorinate a number of chlorinated compounds, including strains VS (31), FL2 (51), BAV1 (50), CBDB1 (3, 22) and KB-1/VC-H_2 (37). More significantly, Fennel and co-workers (45) reported that *Dehalococcoides ethenogenes* 195 dechlorinated 23456-CB and other aromatic organochlorines when grown with tetrachloroethene, and this was the first isolated organism to dechlorinate PCBs. Despite these significant developments on the identification of dechlorinating microorganisms, the specific activities of these microorganisms in the dechlorination of Aroclor mixtures and their role in the environment remains unanswered.

1.5. Enhancement of PCB dechlorination

1.5.1. Addition of stimulating substrates (i.e. "priming")

Several studies have investigated a possible stimulation of the overall or specific dechlorination activities by adding halogenated substrates to sediment microcosms. Klasson *et al.* (63) reported that dechlorination of endogenous PCB occurred more quickly in soil microcosms with the addition of 236-CB, but that the total dechlorination was not necessarily increased. Furthermore, specific dechlorinating activities have been shown to respond to stimulation. Bedard *et al.* (9) showed stimulation of *para* dechlorination of endogenous Aroclor 1260 in sediments from Woods Pond by the addition of 2534-CB, while most of the dechlorination in unstimulated Woods Pond sediment was *meta* dechlorination (10). Other studies showed that "process N" could be stimulated by the addition of single congener PCBs with flanked *meta* chlorines (140). Other halogenated compounds have also been shown to stimulate dechlorination of PCBs (13, 35) presumably by increasing the number of dechlorinating bacteria (26).

1.5.2. Bioaugmentation

Bioaugmentation is defined as the addition of specific microorganisms to the local population to enhance degradation of contaminants. The use of bioaugmentation as a bioremediation tool is not new, and several studies have been preformed with varying success, [for reviews see El Fantroussi *et al*. (40) and Gentry *et al.* (49)]. Anaerobic bioaugmentation studies have been shown to be successful *in*

situ with the complete dechlorination of TCE to ethene in groundwater aquifers using additions of either strain KB-1 (77), a consortia of different *Dehalococcoides* strains (38), or enrichment cultures from the same site that were "enhanced" by growth in the presence of TCE (72). In all these cases increased amounts of *Dehalococcoides* species were observed after treatment. Also, Natarajan *et al.* (88) showed that the addition of anaerobic microbial consortia to sediment microcosms had a stimulating effect on the dechlorination of Aroclor 1258 and the single congener 2,3,4,5,6-pentachlorbiphenyl. However, to date, no successful bioaugmentation approach has been developed for bioremediation using PCB reductive dechlorinators.

1.6. Reductive dehalogenases

The term dehalorespiration is used to indicate the use of chlorinated compounds as electron acceptors in respiration. This process has been associated with members of several phylogenetic groups including the δ- and ε- *protebacteria,* low G+C Gram positive bacteria, and *Dehalococcoides* within a deep branch of the green non-sulfur bacteria. These organisms can generally use chlorinated ethenes and some chlorinated phenolic compounds as electron acceptors (113). Enzymes mediating this transfer of electrons are called reductive dehalogenases, and they are the key catalysts in the respiratory chain of halorespiring microorganisms (113).

Generally, these enzymes contain iron-sulfur clusters and use a corrinoid as a cofactor. Their catalytic units are about 60 kDa and are thought to be membrane associated. In addition, a protein that appears to be co-transcribed with dehalogenases has also been identified and is thought to act as a membrane anchor for

the catalytic subunit. Despite these physiological similarities, reductive dehalogenases have overall low sequence similarity. However, some regions of the reductive dehalogenase genes present some sequence conservation and degenerate PCR primers have been designed based on these regions (65, 101, 127). Using these degenerate primers, investigators have been able to identify several putative dehalogenases (57, 65, 129) and have shown that several contaminated environments contain putative reductive dehalogenase genes (66). However, no PCB reductive dehalogenases have been identified to date.

It is still unclear if the specificity of the dechlorination processes of PCB congeners is a result of different enzymes within the same microorganism, or conserved enzymes within different microbial species or a broad physiological group of organisms. Although dehalorespiration is catalyzed by microorganisms in different genera, PCB dechlorination has only been reported in organisms from the *Dehalococcoides*/*o*-17/DF-1 group within the green non-sulfur bacteria, which shows about 90% sequence similarity in their 16S rRNA genes. Since *o*-17 and DF-1 dechlorinate PCB congeners in specific patterns (80, 136), it is possible that specific microorganisms in this broad phylogenetic group confer the specificity of dechlorination pathways through species-specific dehalogenases in each of the organisms. These enzymes might be similar to other dehalogenases, but the nature of these dehalogenases is currently unknown. Only two reductive dehalogenases from microorganisms within the "*Dehalococcoides*" group has been identified through biochemical method (58, 74); TceA (74) and VcrA (86). Reductive dechlorination of chlorobenzenes has also been identified in cell extracts of CBDB1, but the

dehalogenase(s) have not yet been purified (58). Using whole genomic sequencing and PCR, followed by sequence similarity analysis, other *Dehalococcoides* species have been shown to have several putative dehalogenase homologues, including BAV 1 with 7 homologues (65), CBDB1 with 32 homologues (67) and FL2 with 14 homologues (57). Analysis of the genome of *Dehalococcoides ethenogenes* strain 195 (112) has shown that several of the putative dehalogenases are situated within "atypical regions" of the genome (100). This suggests that these putative reductive dehalogenases have been incorporated in the genome of *Dehalococcoides ethenogenes* through lateral gene transfers (100). Indeed, a recent study suggests that lateral gene transfer of the trichloroethene reductive dehalogenase gene (*TceA*) has occurred between different *Dehalococcoides* species (66).

1.7. Significance of studying reductive PCB dechlorination

1.7.1. Sequential anaerobic-aerobic treatment

PCBs can be biologically transformed both aerobically and anaerobically. In aerobic environments PCBs undergo microbial degradation with oxygen addition at the 2,3 positions by a dioxygenase and subsequent dehydration to catechol, followed by ring cleavage. Anaerobic reductive dechlorination of PCBs is fundamental for PCB degradation because most extensively chlorinated congeners (i.e. those with more than four chlorines) are not transformed under aerobic conditions (1). Several investigators have proposed that a sequential anaerobic dechlorination step followed by an aerobic step would be plausible as a bioremediation strategy (121). Indeed Master *et al.* (78) saw a decrease of total PCB concentration when applying a

sequential anaerobic-aerobic treatment of soil microcosms with Aroclor 1260. A 4-month anaerobic incubation resulted in dechlorination pattern N and several of the major dechlorination products were subsequently degraded aerobically.

1.7.2. Role of microorganisms in remediation of PCB contaminated sites

Although some dechlorinators have been identified and their specificities PCB dechlorination capabilities determined, it is still unknown what drives these specificities. By identifying the PCB dechlorinators that are responsible for the dechlorination pathways of Aroclor 1260, it will be possible finally to determine the biological factors behind the different dechlorination patterns. With these data it will also be possible to develop models that predict the rates and major dechlorination products at a given site. Finally, by identifying microorganisms involved in PCB dechlorination, assays could be developed (i.e. by using specific PCR primers) to detect and monitor specific PCB dechlorinators at PCB contaminated sites. This information could be used to confirm whether natural attenuation is occurring, or if bioaugmentation or biostimulation would be a preferential approach. If the addition of specific dechlorinating microorganisms could change the dechlorination pathways to produce products that are more susceptible to complete degradation, this could be a valuable tool for the purpose of bioremediation.

1.8. Hypothesis and objectives

1.8.1. Hypothesis

The main objective of my studies is to elucidate the driving factors behind the different patterns of dechlorination that have been observed. My hypothesis is that different microorganisms confer the specificity of the dechlorination reactions and that several different microorganisms are required to dechlorinate Aroclor 1260.

1.8.2. Objective 1: Identification of the specific reductive dechlorination pathways of Aroclor 1260

To investigate what drives the specific patterns of reductive PCB dechlorination in Aroclor 1260, I initially aimed to identify the specific pathways of dechlorination for this PCB mixture. My approach was to incubate the 12 most predominant congeners in Aroclor 1260 with Baltimore Harbor sediment microcosms**.** Individual pathways were elucidated, and dechlorination rates were determined. Intermediate PCB congeners were also incubated individually to verify pathways of dechlorination and to determine the specific rates of dechlorination of these intermediate PCB congeners.

In addition, I investigated whether any clear relationships existed between PCB congeners' specific dechlorination rates and their chemical parameters such as water solubility, the number of chlorines on the biphenyl ring, the number of *meta*, *ortho* or *para* chlorines and differences in Gibbs free energy between the parent and

the daughter congeners (i.e. how much energy is available for the microorganisms by performing these reactions). Results of this work are presented in Chapter 3.

1.8.3. Objective 2: Identification of the microorganisms responsible for the reductive dechlorination of the 12 most predominant Aroclor 1260 congeners in Baltimore Harbor microcosms

In order to further investigate whether different microorganisms might influence specific dechlorination pathways in Aroclor 1260, I wanted to identify the microorganisms responsible for each of these pathways. Because PCB dechlorinating microorganisms are a minor part of sediment microcosms in terms of numbers, it was necessary to develop a specific and rapid screening technique. Denaturing Gradient Gel Electrophoresis (DGGE) PCR primers targeting the 16S rRNA genes for a monophyletic group within the *Chloroflexi* were designed to rapidly detect and identify putative dechlorinating microorganisms in dechlorinating microcosms (Chapter 2). I was able to identify microorganisms responsible for the major dechlorination pathways in Aroclor 1260 by comparing the microbial communities of dechlorinating cultures with those present in no-PCB controls. I also investigated whether these specific dechlorination activities were growth-linked, using a technique called competitive PCR (cPCR) (Kjellerup *et al.*, 2007, in preparation). Results of this research are presented in Chapter 3.

1.8.4. Objective 3: Changing the patterns of PCB dechlorination with the addition of dechlorinating enrichment cultures

I aimed to test the feasibility of anaerobic bioaugmentation of PCB contaminated sediments. The approach was to add enrichment cultures containing PCB dechlorinating bacteria with defined selective dechlorination activities to Baltimore Harbor sediments. Since I had previously observed that PCB 151 (2346-25) could be dechlorinated via different pathways (Chapter 3), I chose to follow PCB 151 dechlorination over time, as well as that of Aroclor 1260. I used a rapid screening technique, PCR-DGGE with specific primers (Chapter 2), to investigate whether the microorganisms in enrichments could compete with the natural population in Baltimore Harbor sediment microcosms. Finally, competitive PCR was also performed to investigate whether these microorganisms could grow throughout incubation. Results of this work are presented in Chapter 4.

1.8.5. Objective 4: Identification of a PCB reductive dehalogenase

Since microorganism DF-1 dechlorinates different PCB congeners as well as chlorinated benzenes and ethenes (82, 135), I attempted to identify possible dehalogenases in this organism. I constructed a clone library using degenerate primers targeting putative reductive dehalogenases (65) and retrieved several putative dehalogenase. Specific PCR primers were developed with the goal of measuring the expression of each of these putative dehalogenase genes. I then used specific primers to compare the expression of putative dehalogenases in DF-1 grown with PCE,

pentachlorobenzene and PCB. In addition, clone libraries of putative dehalogenases generated from mRNA of DF1 grown with PCB, pentachlorobenzene and PCE were constructed and Amplified Ribosomal DNA Restriction Analysis (ARDRA) was performed to evaluate the differential expression of these enzymes. This work is presented in Chapter 5.

Chapter 2: Sequential reductive dechlorination of *meta*-chlorinated PCB congeners in sediment microcosms by two different phylotypes of *Chloroflexi**.

** Fagervold, S. K., J. E. M. Watts, H. D. May, and K. R. Sowers. 2005. Appl. Environ. Microbiol. 71:8085-8090.*

2.1. Abstract

Three species within a deeply branching cluster of the *Chloroflexi* are the only microorganisms currently known to anaerobically transform polychlorinated biphenyls (PCBs) by the mechanism of reductive dechlorination. A selective PCR primer set was designed that amplifies the 16S rRNA genes of a monophyletic group within the *Chloroflexi* including *Dehalococcoides* spp. and the *o*-17/DF-1 group. Assays for both qualitative and quantitative analyses by denaturing gradient gel electrophoresis (DGGE) and Most Probable Number (MPN) -PCR, respectively, were developed to assess sediment microcosm enrichments that reductively dechlorinated PCBs 101 (2,2',4,5,5'-CB) and 132 (2,2',3,3',4,6'-CB). PCB 101 was reductively dechlorinated at the *para*-flanked *meta* position to PCB 49 (2,2',5,5'-CB) by phylotype DEH10, which belongs to the *Dehalococcoides* group. This same species reductively dechlorinated the *para* and *ortho*-flanked *meta* chlorine of PCB 132 to PCB 91 (2,2',3',4,6'-CB). However, another phylotype designated SF1, which is more closely related to the *o*-17/DF-1 group, was responsible for the subsequent dechlorination of PCB 91 to PCB 51 (2,2',4,6'-CB). Using the selective primer set, an increase in 16S rRNA gene copies was observed only in actively dechlorinating

cultures indicating that PCB dechlorinating activities by both phylotype DEH10 and SF1 were linked to growth. The results suggest that individual species within the *Chloroflexi* exhibit a limited range of congener specificities and that a relatively diverse community of species within a deeply branching group of *Chloroflexi* with complementary congener specificities is likely required for the reductive dechlorination different PCBs congeners in the environment.

2.2. Introduction

Polychlorinated biphenyls (PCBs) have been an environmental concern for several decades due to their widespread use, chemical stability and biological toxicity (107, 117). Historically, harbor regions have been heavily impacted by the accumulation of PCBs released during commercial activities. Commercial production of PCBs was banned in the United States in 1978, but reports of the distribution of PCBs in marine coastal harbor regions demonstrate the tenacity of PCB contamination (7, 48, 59, 118).

Although PCBs persist in the environment, some microbial processes are able to transform these chemically stable molecules. Aerobic degradation involves biphenyl ring cleavage. However, PCBs are hydrophobic and tend to adsorb to particles that settle and accumulate in the anaerobic zone of sediments, where microbial reductive dehalogenation results in the sequential removal of chlorine atoms from the biphenyl backbone (12, 20). Two anaerobic PCB dechlorinating microorganisms, strains DF-1 and *o*-17, within the green non-sulfur *Chloroflexi* phylum, have been shown to link their growth to the reductive dechlorination of

PCBs (33, 97, 133, 137). Fennel and co-workers (45) reported that another species within the *Chloroflexi, Dehalococcoides ethenogenes* 195, dechlorinated the PCB 2,3,4,5,6-pentachlorobiphenyl and other aromatic organochlorines when grown with perchloroethene. This microorganism was the first species to be isolated and described in the *Dehalococcoides* group (81). Although other *Dehalococcoides* spp. including strains VS (31), FL2(51), BAV1 (50), CBDB1 (3, 22) and KB-1/VC-H_2 (37) use chlorinated ethenes and other chlorinated compounds as electron acceptors, no other species have been reported to reductively dechlorinate PCBs.

PCB dechlorinating microorganisms are difficult to isolate and are generally a small portion of the total microbial community in natural sediments (97, 133). Little is therefore known about the catalytic diversity of PCB dechlorinating bacteria and their distribution in nature. A recently developed PCR based assay using primers specific for the 16S rRNA genes of PCB dechlorinating microorganisms similar to *o*-17 and DF-1 revealed a diverse group of organisms within a deep branch of the *Chloroflexi* that are distinct from *Dehalococcoides* spp. (132). Sequence similarity among *Dehalococcoides* strains is very high (> 98 %), while the similarity between the *o*-17/DF-1 group and the *Dehalococcoides* strains are less than 90 %. Nevertheless, all these microorganisms form a monophyletic clade within the *Chloroflexi*. Using PCR primers designed to detect both *Dehalococcoides* spp. and *o*-17/DF-1-like microorganisms with denaturing gradient gel electrophoresis (DGGE) we report that two phylotypes, one closely related to phylotype m-1 (132) within the *o*-17/DF-1 group and the second a *Dehalococcoides* sp., sequentially dechlorinate the double flanked and single flanked *meta* chlorines of PCB 132 in Baltimore Harbor

sediment microcosms. Enumeration by most probable number PCR with the specific primers shows that individual PCB congeners can be sequentially dechlorinated by a succession of two phylotypes that link their growth to reductive dehalogenation.

2.3. Experimental procedures

2.3.1. Sediment samples

Sediments were sampled from the Northwest Branch of Baltimore Harbor with a petite Ponar grab sampler at 39°16.8'N, 76°36.1'W as described by Berkaw et al. (15) and stored anaerobically under nitrogen prior to use.

2.3.2. Anaerobic enrichment cultures

A defined low-sulfate (<0.3mM) estuarine salts medium (E-Cl) was prepared as described by Berkaw et al. (15) with the exclusion of $Na_2S•9H_20$, dispensed in 10 ml aliquots into 20 ml Balch anaerobe tubes and sealed under N_2-CO_2 (80:20). The medium was autoclaved for 20 minutes having a final pH at 6.8. All subsequent additions were performed aseptically in an anaerobic glove box under N_2-CO_2-H_2 (75:20:5) atmosphere. Prior to inoculation, a fatty acid mixture of sodium salts (acetate, propionate and butyrate) was added to a final concentration of 2.5 mM each. Microcosms were initiated by the addition of 2 g of Baltimore Harbor (BH) sediment slurry into 8 ml of medium. PCB congeners 91, 101 and 132 (AccuStandard, Inc., New Haven, CT) were solubilized in 10 µl acetone, and separately added to triplicate microcosms to a final concentration of 50 ppm (mg/L). Sterile controls were

prepared by twice autoclaving sediment-inoculated tubes containing medium and the fatty mix, with a 48 hour interval between treatments followed by addition of PCB. Active cultures were maintained by transferring 1 ml of homogenized slurry into freshly prepared medium containing 0.5 g dried, sterile BH sediment approximately every 8 months. One control microcosm containing 10 µl acetone without PCB was also transferred for each PCB congener set. Dried BH sediment was prepared by baking BH sediment at 115°C for 72 hours, followed by five times autoclaving in a sealed container for 60 min. All cultures were incubated at 30°C in the dark.

2.3.3. Analytical techniques

PCBs were analyzed by extracting 0.5 ml of culture with 3 ml of hexane for 12 hours on a wrist shaker. The organic phase was passed through a copper/Florisil® (1:4) column and analyzed using a Hewlett Packard 5890 series II gas chromatograph (GC) with a DB-1 capillary column (30 m by 0.25 mm by 0.25 µm; JW Scientific, Folsom, CA) and a Ni^{63} electron capture detector (ECD) as described by Berkaw et al. (15). Nine mixes containing in total 209 congeners (AccuStandard, Catalog name: C-CSQ-SET) were used to identify the PCB congeners by matching their retention times. Individual PCB congeners were quantified with a 10-point calibration curve using PCB 65 and PCB 204 as external and internal standards.

2.3.4. Bacterial community 16S rRNA gene analysis

DNA from pooled samples (0.5 ml from each culture replicate) was extracted according to Holoman et al. (97) with minor modifications. Briefly, samples were subjected to bead beating with a Fastprep Cell Disruptor (Qbiogene, Carlsbad, CA) and phenol chloroform extraction was followed by electrophoresis in a 1.3 % (wt/vol) low-melt agarose gel containing 2% (wt/vol) polyvinylpyrrolidine (PVP). DNA was excised from the gel and recovered using the Promega Wizard PCR Prep Kit (Promega, Madison, WI.). Total DNA was probed for dechlorinating microorganisms within the *o*-17/DF-1 group with universal primer 14F (39) and specific primer Dehal1265R (131, 132). The same DNA samples were screened for the presence of *Dehalococcoides* spp. with forward primer DHC 1 and the reverse primer DHC 1377 (52). Amplified rDNA restriction analyses (ARDRA) were conducted as described by Pulliam Holoman et al. (97). The 16S rRNA gene clone library was generated with *Dehalococcoides*-specific primers DHC 1 and DHC 1377 (52) and fragments were ligated into pCR2.1 using the TA Cloning® Kit (Invitrogen, Carlsbad, CA). The library was screened using the primers DHC 1 and DHC 1377 followed by restriction fragment polymorphism analysis with restriction endonucleases *Hae*III and *Hha*I. Digestion products were discriminated by gel electrophoresis on a 3% (wt/vol) Trevigel at 25V for 3 hours on ice. Five plasmids containing the 16S rRNA gene from strains DEH10, *D. ethenogenes 195*, strain *o*-17, DF-1 and *C. aurantiacus* were constructed using the TA Cloning® Kit to use as controls.

New group-specific primer set was developed by using Probe Design in the ARB software package (128). Forward primer Chl348F (5'-

GAGGCAGCAGCAAGGAA-3') is specific for *Chloroflexi* and reverse primer Dehal884R (5'-GGCGGGACACTTAAAGCG-3') is specific for putative dechlorinating microorganisms. The product size is approximately 470 base pairs. The primers were checked for compatibility and possible self-annealing using Primer Express (Applied Biosystems, Foster City, CA).

For ARDRA, clone libraries were generated by PCR with Chl348F and Dehal884R as described above, except that restriction enzymes *Rsa*I and *Hin*fI were used for restriction fragment analysis. For DGGE, a GC clamp (87) was added to primer Chl348F (5'-CGC CCG CCG CGC GCG GGA GGC AGC AGC AAG GAA-3') (Genosys Biotechnologies), and designated Chl348FGC. PCR reactions (50 μl) with 10 ng DNA were performed using the GeneAmp PCR kit (PE Applied Biosystems, Foster City, CA) containing 1X PCR buffer, a mixture of dNTPs (200nM each), 1.5 mM $MgCl_2$, 160 nM of each primer, 192 mM dimethylsulfoxide (DMSO) and 1 unit Ampli*Taq* DNA polymerase. Amplification was performed in a PTC200 thermal cycler (MJ Research, Watertown, MA.) with the following cycle parameters: Initial denaturing (1 min at 95°C), 26 cycles of denaturation (45 s at 95°C), annealing (45 s at 60°C), and elongation (45 s at 72°C), followed by a final extension (30 min at 72°C) (61). The sensitivity of the DGGE assay with the PCR conditions described above was determined by dilution of plasmids containing the 16S rRNA gene of *o*-17 (33). PCR products were checked for correct size and yield on a 0.8% (wt/vol) TAE agarose gel (Fisher Biotech, NJ.). DGGE was performed as described by Watts et al. (133) using the D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA.). The 6% (wt/vol) polyacrylamide gels (Sigma, St. Louis, MO.) contained a 39-

48% denaturing gradient and fragments were separated by electrophoresis for 18 hours at 75 V. The gels were stained with SYBR-Green I DNA stain (Molecular Probes, Eugene, OR) and visualized using a Storm PhosphorImager (GE Healthcare, Piscataway, NJ). DGGE bands of interest were excised and eluted by incubation in 30 µl TE overnight at 4°C. PCR and DGGE were repeated twice to assure purity of each eluted band and the last PCR reaction used primers without the GC clamp before DNA sequencing as described below.

2.3.5. Sequencing and analysis

Plasmids from the two clone libraries were purified using the Qiagen Plasmid Mini Kit (Qiagen, Chattsworth, CA) according to the manufacturer's protocol. Plasmids and PCR products were used as templates for dye terminator cycle sequencing using Big Dye 3.1 kit (Applied Biosystems) and an ABI 3100 (Applied Biosystems). Sequences were examined for errors and assembled using the software Pregap4 and Gap4 of the Staden software package (http://sourceforge.net/projects/staden). Chimera formation was examined using Chimera Check (28). The sequences were aligned using the ARB software package (128) and a phylogenetic tree was generated based on published *Chloroflexi* sequences over 1200 base pairs. A manual filter was developed to exclude hypervariable regions sequences (*E. coli* positions 71-98, 452-483, 838-849, 1004-1037, 1126-1148, 1163-1174). A second filter was created using the "filter by base frequency" tool in ARB that excluded positions in the alignment where gaps were more frequent than characters and positions with ambiguous characters. DNA

distance matrices were generated with the ARB software package using the Kimura 2-parameter evolutionary distance correction and phylogenetic trees were generated using the neighbor joining (110) algorithm. Bootstrap analyses (100 replicates) were performed using the PHYLIP package (44).

2.3.6. Quantitative assessment of PCB dechlorination populations

Putative dehalogenating *Chloroflexi* were enumerated by MPN-PCR using primers Chl348F and Dehal884R. DNA samples (10 μg/mL) were serially diluted 10-fold and amplified as described above with 40 PCR cycles. 16S rRNA gene copies per μl of DNA sample were determined using a standard Most Probable Numbers table (27). Dilutions of a plasmid with the 16S rRNA gene of the PCB dechlorinating strains *o*-17 (33), DF-1 (137) and phylotype DEH10 were used as controls and to determine the sensitivity of the assay. In order to test whether non-homologous DNA would interfere with the MPN assay, 10 ng DNA from a *Chloroflexus aurantiacus* isolate were added to dilution series and MPN numbers calculated as described above.

2.3.7. Nucleotide sequence accession numbers

The 16S rRNA gene sequences for phylotype DEH10 and phylotype SF1 have been submitted into GenBank under accession numbers DQ21869 and DQ21870, respectively.

2.4. Results

2.4.1. PCB dechlorination in initial enrichment cultures

Cultures containing BH sediment were amended with PCBs 101 (2,2',4,5,5'-CB) and 132 (2,2',3,3',4,6'-CB), which are predominant congeners (about 4 and 3 mol %, respectively) in Aroclor 1260 (46). Complete reductive dehalogenation of congeners in only the *meta* positions was detected within 3-6 months by the pathways shown in Figure 2.1.

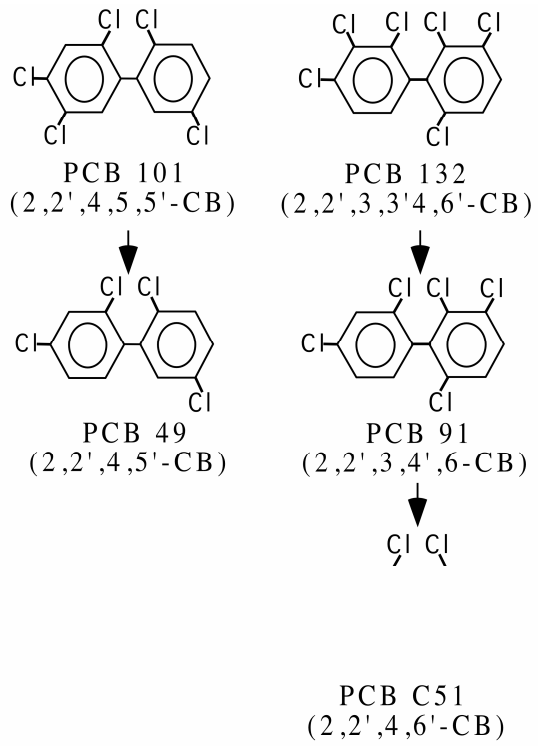


Figure 2.1. Dechlorination patterns and products observed from Baltimore Harbor sediment microcosms containing PCBs 91, 101 and 132.

These cultures were transferred with their respective PCB congener, then screened for dechlorinating organisms with primers specific for the 16S rRNA genes of strains DF-1 and *o*-17 (131, 132) and *Dehalococcoides* spp. (52). Unexpectedly, cultures dechlorinating PCB 101 were positive only for *Dehalococcoides* spp., while the cultures dechlorinating PCB 132 were positive for both groups.

To investigate if one specific *Dehalococcoides* phylotype is enriched in the PCB101 microcosms, we performed an ARDRA with PCR products generated with primers targeting *Dehalococcoides* spp. (52) from cultures dechlorinating PCB 101 and from the no-PCB control after 6 months of incubation. There was a clear enrichment of a single phylotype (17 out of 18 clones), which we designated DEH10, with no apparent enrichment of an individual ARDRA pattern in the no-PCB control (8 different patterns out of 9 clones). The RFLP pattern representing DEH10 was not found in the clone library from the no-PCB control.

2.4.2. Development of PCR primers for detection of PCB dechlorinating species

In order to detect both *Dehalococcoides* spp. and *o*-17/DF-1-like PCB dechlorinating species, a group-specific primer set was developed to target the 16S rRNA genes of this group of *Chloroflexi* (Figure 2.2). Chl348F and Dehal884R amplify the 16S rRNA genes from *o*-17, DF-1, phylotype DEH10 and *Dehalococcoides ethenogenes* 195, but not *Chloroflexus aurantiacus* (Figure 2.3).

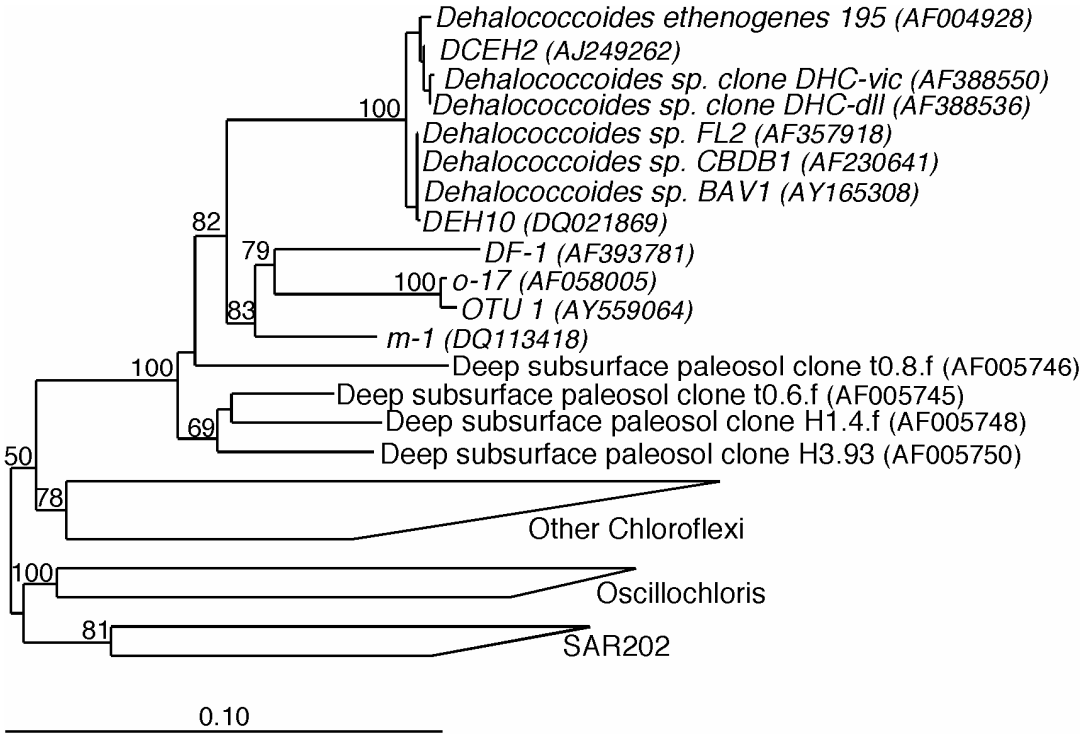


Figure 2.2. Phylogenetic analysis (neighbor joining) of *Chloroflexi* 16S rRNA genes. Tree reconstruction was based on 998 positions between *E. coli* positions 44 and 1232 from published sequences. The tree is rooted with *Bacillus subtilis* (AB016721). Bootstrap analysis was performed using the PHYLIP software package (44) and values over 50 are indicated at the branch points. The scale bar indicates 10 substitutions per 100 nucleotide positions. Microorganisms that are confirmed dechlorinators are italicized.

PCR product was not detected with species outside of the green non-sulfur bacteria including those from several Bacteria and Archaeal phyla (data not shown). Furthermore, sequences retrieved from a clone library generated using Chl348F and Dehal884R with Baltimore Harbor sediments included only sequences within the *Dehalococcoides*/*o*-17/DF-1 *Chloroflexi* group. The detection limit of these primers was $\geq 10^5$ copies per 50 µl PCR reaction mixture with 26 PCR cycles and 8 µl loaded in agarose gel. The detection limit in 8 µl with 40 PCR cycles ranged between 10 and

65 gene copies per 50 µl PCR reaction mixture for strains *o*-17, and DF-1 and phylotype DEH10. The addition of up to 10 µg *Chloroflexus aurantiacus* DNA had no effect.

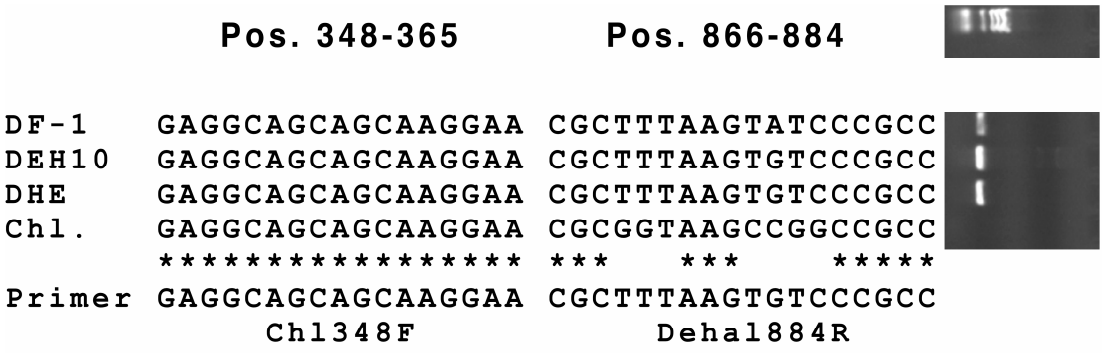


Figure 2.3. Primer specificity. Alignment of 16S rRNA genes of *o*-17, DF-1, DEH10, *Dehalococcoides ethenogenes* 195 (DHE) and *Chloroflexus aurantiacus* (Chl.). Numbering is based on the *E. coli* 16S rDNA positions. Panel on the right shows an agarose gel of PCR products using primers Chl348F and Dehal884R and plasmids with cloned 16S rRNA gene templates from the organisms indicated. The top lane is the DNA size marker (fXDNA-Hae III digest).

2.4.3. Analysis of dechlorinating activity and microbial community profiles

Group-specific primer set Chl348F and Dehal884R were used to identify putative PCB dechlorinating bacteria in sediment microcosms actively dechlorinating PCB 101 and 132 and PCB 91. The progressive dechlorination of the congeners 132, 101 and 91 at intervals of 0, 100, 150, and 200 days is shown in Figure 2.4. PCB101 was dechlorinated in a flanked *meta* position to PCB 49 (2,2',4,5'-CB) and no further dechlorination was observed after 250 days. PCB 132 was dechlorinated sequentially in two *meta* positions to PCB 91, then to PCB 51 (2,2',4,6'-CB), which was the terminal product after incubation for 300 days. Inoculum from PCB 132 microcosms

was also used to initiate PCB 91 microcosms. Negligible dechlorinating activity was detected in sterile controls (less than mol 6% over 200 days).

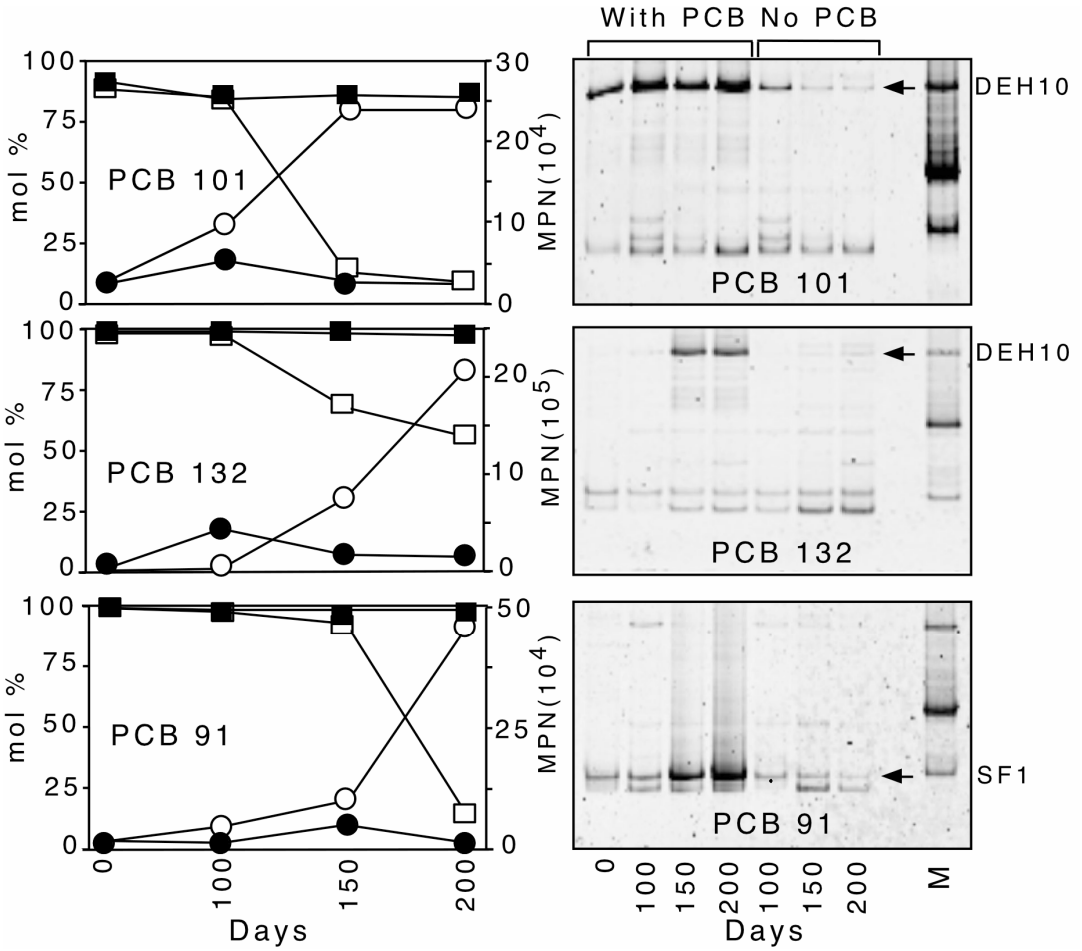


Figure 2.4. Quantitative and qualitative analyses of 16S rRNA genes during active dechlorination of PCBs 132, 101 and 91. Left column: Dechlorination of PCB congener 101, 91 132 showing mol % of parent compound in active culture (□) and sterile control (■); MPN-PCR analyses of 16S rDNA copies per µl of DNA in active culture (○) and sterile control (●). Right column: DGGE results from dechlorinating cultures and the no PCB controls. Cultures were sequentially transferred four times on the respective PCB congener prior to analysis. All bands were excised and sequenced. Bands in far right lane are products from (from the top) DEH10, DF-1 and *o*-17.

DGGE profiles in Figure 2.4 show enrichment of a single phylotype in each of the dechlorinating cultures over the course of 200 days. The DGGE band representing phylotype DEH10 was enriched in cultures dechlorinating PCB 101 and PCB 132. Another phylotype designated SF1, was enriched in PCB 91 dechlorinating cultures. Although other PCR amplified 16S rRNA genes appeared in both the actively dechlorinating cultures and the no PCB controls, there is no apparent enrichment of these bands during the incubation period. The lowermost bands in the DGGE gels (Figure 2.4) were chimeras.

2.4.4. Quantitative assessment of PCB dechlorinating populations

A MPN-PCR based assay with primers Chl348F and Dehal884R was used to determine whether the apparent enrichment of selected 16S rRNA genes showed by DGGE analyses of actively dechlorinating microcosms was the result of growth by specific phylotypes. PCB 101 microcosms exhibited a 10-fold increase in 16S rRNA gene copies during active dechlorination (Figure 2.4). The controls initiated without added PCB showed a steady decrease of dehalogenating *Chloroflexi* 16S rRNA gene copies during the same incubation period. During active dechlorination of PCB 132 and PCB 91 the cultures exhibited a 20-fold and 50-fold increase in dehalogenating *Chloroflexi* 16S rRNA gene copies, respectively.

2.5. Discussion

2.5.1. Patterns of dechlorination in sediment microcosms

All microcosms incubated with PCB 132, 101 and 91 exhibited reductive dechlorination in the *meta* position. Dechlorination of *ortho, para* and unflanked *meta* chlorines was not detected, indicating that these enrichment microcosms selectively dechlorinated double- and single-flanked *meta* chlorines. Dechlorination of double flanked chlorines on a biphenyl backbone has been previously reported for bacterium DF-1 (136, 137) and *D. ethenogenes* 195 (45). This study provided compelling evidence that phylotype DEH10 was responsible for both double-flanked *meta* dechlorination of PCB132 and single-flanked *meta* dechlorination of PCB 101. As *D. ethenogenes* 195 was only tested with PCB 116 (2,3,4,5,6-CB) (45), which contains two double-flanked *meta* chlorines, the ability to reductive dechlorinate a PCB congener such as PCB 101 with a single-flanked *meta* chlorine cannot be discounted.

These results are consistent with the reductive dechlorination of Aroclor 1260 in microcosms with Baltimore Harbor sediments (141), which showed significant decreases in PCB 101 and PCB 132 and significant accumulation of PCB 51 and PCB 49 after 181 days of incubation. This pattern of dechlorination is most similar to "Process N" described in enrichment microcosms from Silver Lake and Woods Ponds (12), which exhibit extensive dechlorination of flanked *meta* chlorines. Other studies have also reported this to be a frequent dechlorination pattern (4, 20, 69, 84, 98).

2.5.2. Identification of dechlorinating microorganisms

One of the goals of this study was to develop a rapid and comprehensive assay for monitoring the microorganisms responsible for the different dechlorination patterns observed in sediment microcosms. Although primers for both the *Dehalococcoides* group (52) and the *o*-17/DF-1 group (131, 132) are available, the group-specific primer set developed in this study selectively amplifies both groups of putative PCB dechlorinating bacteria within this *Chloroflexi* clade in a single PCR reaction. Figure 2.2 shows that the bootstrap values separating this clade from the rest of the *Chloroflexi* is high, suggesting that this group is monophyletic.

Enrichment of phylotype DEH10 in the PCB 101 and PCB 132 cultures compared to the no PCB control is apparent in the DGGE gel (Figure 2.4). Phylotype DEH10 16S rRNA gene sequence has the "Pinellas group" signature of *Dehalococcoides* spp. in variable region 2 and 6 (52), and a single base pair difference over 1378 base pairs compared to *Dehalococcoides* sp. strain FL2 (51). However, due to the small size of the PCR products, the DGGE assay described here will not distinguish between different members of the Pinellas group.

Phylotype SF1 was clearly enriched in the microcosm dechlorinating PCB 91 compared to the no-PCB control. Phylotype SF1 is most similar to phylotype m-1 (only 1 bp difference of the 16S rRNA gene sequence over 466 bp), which was detected in cultures dechlorinating 3,5-dichlorobiphenyl (132). Previously DGGE using universal primers (87) was performed on these same cultures and no changes were observed in the microbial community. Although the DNA concentrations were normalized among samples and PCR cycles were kept at a minimum to minimize

PCR biases, DGGE is only a semi-quantitative method. The use of MPN-PCR confirmed the DGGE assay results (Figure 2.4). MPN-PCR was used instead of real time quantitative PCR because fluorescence quenching and auto-fluorescence associated with sediment samples can adversely affect enumeration accuracy using the latter assay (119).

Isolation of PCB dechlorinating microorganisms has proven difficult (15, 32, 33, 97, 133, 136, 137). Several isolates in the *Dehalococcoides* group have been reported (3, 31, 37, 50, 51) but a direct link between growth and PCB dechlorination activity has not been shown for any of these isolates. The development of primers targeting a broader range of putative dehalogenating phylotypes within *Chloroflexi*, including uncultured microorganisms, is an important advance in the study of this diverse group of bacteria in laboratory microcosms as well as *in situ*.

Increases in 16S rRNA gene copies were observed only in cultures actively dechlorinating PCBs. This is the strongest evidence reported thus far to indicate that PCB dechlorinating activity is linked to growth of dehalogenating bacteria, in this case DEH10 and bacterium SF1, possibly by the proposed mechanism of dehalorespiration. The predominance of these *meta* dechlorinating pathways in the reductive dechlorination of Aroclor 1260 in BH sediments further suggests that phylotypes DEH10 and SF1 may have a significant and complementary role in this process.

Chapter 3: Microbial reductive dechlorination of Aroclor 1260 in Baltimore Harbor sediment microcosms is catalyzed by three phylotypes within the *Chloroflexi**

**Fagervold, S. K., H. D. May, K. R. Sowers. Accepted. Appl. Environ. Microbiol.*

3.1. Abstract

We have identified three microbial phylotypes that reductively dechlorinate Aroclor 1260 in Baltimore Harbor sediment microcosms. The specific dechlorination pathways for Aroclor 1260 were determined in microcosms developed with the 11 most predominant congeners from this commercial mixture and their resulting dechlorination intermediates. Most of the PCB congeners were dechlorinated in the *meta* position, and the major products were tetrachlorobiphenyls with unflanked chlorines. Using PCR primers specific for the 16S rRNA genes of known PCB dehalogenating bacteria, we detected three phylotypes that had the capability to dechlorinate PCB congeners present in Aroclor 1260 and identified their selective activities. Phylotype DEH10, which belongs to *Dehalococcoides* spp., generally removed the double-flanked chlorine in 234-substituted congeners and exhibited a preference for *para*-flanked *meta* chlorines when no double-flanked chlorines were available. Phylotypes SF1 had similarity to the *o*-17/DF-1 group of PCB dechlorinating bacteria. Phylotype SF1 dechlorinated all the 2345-substituted congeners mostly in the double-flanked *meta* position and 2356-, 236- and 235-substituted congeners in the *ortho*-flanked *meta* position, with a few exceptions. Phylotype SF2 was responsible for one *ortho* and one *ortho*-flanked *meta*

dechlorination activity. Most of the dechlorination pathways for all three phylotypes were growth-linked, which indicates that PCB-impacted environments have the potential to sustain populations of PCB dechlorinating organisms. The results demonstrate that the variation in dechlorination patterns of congener mixtures typically observed at different PCB impacted sites can potentially be mediated by the co-metabolic and synergistic activities of relatively few dehalogenating species.

3.2. Introduction

Polychlorinated biphenyls (PCBs) were widely used between 1929 and the late 1970's for industrial applications requiring chemical stability, low flammability and high vaporization temperature. The stable properties of these compounds led to their widespread accumulation in the environment, first documented in the 1960's, and to growing concerns about the effects of these environmental contaminants on the health of humans and wildlife (109). Although the manufacture of PCBs stopped in most countries by the late 1970's, they remain ubiquitous contaminants transported globally in the air, water and in suspended sediment (64, 71). As a result of these concerns, PCBs are listed as Priority Organic Pollutants by the US EPA (http://nlquery.epa.gov).

Historically, their use as dielectric fluid of liquid-filled transformers represented the second largest usage of PCBs (approximately 30 %), of which the predominant commercial form between 1930 and 1971 was Aroclor 1260 (125). Aroclor 1260 is a mixture of highly chlorinated PCB congeners and is less susceptible to transformation by partitioning and microbial activity than less chlorinated Aroclor

mixtures (4, 98). This might be due to a combination of factors including: 1) lower bioavailability caused by greater hydrophobicity, 2) greater toxicity of individual higher chlorinated congeners, and 3) the lack of lesser-chlorinated PCB congeners associated with the stimulation of microbial transformation (98). However, despite this lower susceptibility to biotransformation, microbial transformation of Aroclor 1260 by anaerobic reductive dechlorination was reported as early as 1987 (17, 20), and several investigators since then have shown reductive dechlorination of Aroclor 1260 in sediment, as well as laboratory microcosms (4, 8, 10, 14, 68, 98, 99, 121, 123, 139, 141). Brown and coworkers (20) proposed that microorganisms could use PCBs as electron acceptors for respiration thus occupying a unique niche in anaerobic environments where other electron acceptors are limiting.

Aroclor 1260 is reductively dechlorinated through diverse patterns of congener transformations, depending on the contaminated sediment source and, presumably, the community of PCB dechlorinating bacteria present (4, 10, 12, 17, 20, 98). Several investigators have attempted to isolate or identify microorganisms responsible for the reductive dechlorination of PCBs (79, 84, 103, 143), and although earlier studies suggested that dechlorination was growth-linked (26, 62, 139), identification of the microbial catalysts by enrichment and isolation remained elusive. The first PCB reducing bacteria were identified when the microbial communities in two sediment-free cultures with different dechlorination specificities were characterized by comparative sequence analysis of PCR-amplified 16S rRNA genes (32, 97, 133, 136). The dehalogenating microorganism *o*-17 (33) and DF-1 (137) were shown to belong to a deep branch of the *Chloroflexi* phylum with their 16S

rRNA gene sequences approximately 90 % identical to the chloroethene dechlorinating microorganism *Dehalococcoides ethenogenes* (81). Species within the *Dehalococcoides* group have been shown to reductively dechlorinate a number of chlorinated compounds (3, 22, 31, 50, 51). Indeed, *D. ethenogenes* 195 has since been shown to dechlorinate 2,3,4,5,6-pentachlorobiphenyl and other aromatic organochlorines when grown with tetrachloroethene (45), however the authors did not investigate whether *D. ethenogenes 195* could gain energy for growth on PCB alone. Also, Bedard and coworkers (8) identified phylotypes similar to *Dehalococcoides* spp. in a sediment-free culture dechlorinating Aroclor 1260, further suggesting that *Dehalococcoides* spp. and related microorganisms within the *Chloroflexi* are the likely catalysts for the reductive dehalogenation of PCBs in the environment. However, there are currently no reports on how many different microorganisms are required to reductively dechlorinate a commercial PCB mixture such as Aroclor 1260 into congeners that have the potential to be aerobically degraded (i.e. four chlorines or less (1)).

Recently, we showed (42) that two phylotypes, DEH10 and SF1, with high sequence similarity to *Dehalococcoides* spp. and the PCB-dehalogenating strain *o*-17 sequentially dechlorinated 2,2'3,3',4,6'-hexachlorobiphenyl (abbreviated as 234-236-CB or PCB 132), a predominant congener in Aroclor 1260, to PCB 91 (236-24-CB) and to PCB 51 (24-26-CB). Here, we report that these two phylotypes, in addition to a third phylotype, SF2, are capable of reductive dechlorination of Aroclor 1260, as well as the 11 most predominant individual PCB congeners of this Aroclor in Baltimore Harbor sediment microcosms, and show that most steps in these processes

are linked to growth. Individual PCB dechlorination pathways, terminal end products and the microorganisms responsible for each step in the pathways are reported. Interestingly, only three phylotypes were responsible for the dechlorination of Aroclor 1260 to congeners containing unflanked chlorines as the terminal products. These findings, combined with the ability to monitor the fate of both indigenous and augmented dechlorinating microorganisms in soils and sediments, are essential developments for designing effective *in situ* treatment strategies of PCB impacted sites.

3.3. Materials and methods

3.3.1. Anaerobic enrichment cultures

Sediment from Baltimore Harbor (BH), an estuarine tributary in the Chesapeake Bay, was used to prepare microcosms as previously described (42) with a defined, low-sulfate (<0.3mM), estuarine salts medium (E-Cl) and a fatty acid mixture (acetate, propionate and butyrate, 2.5 mM each) as electron donor and carbon source (15). Aroclor 1260 and individual PCB congeners were each added to a final concentration of 50 ppm (mg/L) (42). Sterile controls were also prepared as described previously (42). Aroclor 1260 and 12 individual PCB microcosms (PCB 194, 187, 183, 180, 174, 170, 153, 151, 149, 138, 132 and 101) (table 3.1) were transferred four times after dechlorination activity had been detected (except microcosms with PCB 194, which never showed dechlorination activity). To confirm the PCB dechlorination pathways, individual microcosms were then sub-cultured with intermediate PCB congeners (PCB 147, 154, 146, 135, 90, 130, 137, 102, 99, 95, 92

and 91) detected after incubation with the parent PCB congener. All cultures were inoculated (1 ml) in triplicate and incubated at 30^{o} C in the dark. The results reported here, including the Aroclor 1260 cultures, represent microcosms assayed after 4 sequential transfers, with the exception of microcosms dechlorinating PCB 174, 153, 151 (*meta* activity), 135, and 95, which were all assayed after 3 sequential transfers.

3.3.2. Analytical techniques

Microcosms were sampled in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI) and analyzed for PCB dechlorination every 50 days as described previously (42). PCBs were analyzed by extracting 0.5 ml of culture with 3 ml of hexane for 12 hours on a wrist shaker. The organic phase was passed through a copper/Florisil® (1:4) column and analyzed using a Hewlett Packard 5890 series II gas chromatograph (GC) equipped with a DB-1 capillary column (30 m by 0.25 mm by 0.25 μm; JW Scientific, Folsom, CA) and a Ni^{63} electron capture detector (ECD) as described by Berkaw *et al.* (15). Nine mixes containing a total of 209 congeners (AccuStandard, Catalog name: C-CSQ-SET) were used to identify the PCB congeners based on retention times. Individual PCB congeners were quantified with a 10-point calibration curve using PCB 204 as an internal standard. Dechlorination curves for all the PCB congeners were based upon mol % as described previously (42). The total amount of PCBs was determined in each replicate, and the mol % was calculated for each congener in the sample. The average mol % and the standard deviation for each congener were determined from triplicate cultures.

3.3.3. Calculation of dechlorination rates

Dechlorination curves were made for all PCB congeners in 50-day intervals over the course of incubation. Dechlorination rates for each congener were determined by calculating the number of moles dechlorinated over time within the linear slope of the dechlorination curve, Appendix 1. The linear range was determined by eye. In instances where a congener was dechlorinated in several positions, the dechlorination rate was calculated from the total increase in concentration of each of the daughter products. The dechlorination rate was calculated by dividing the mole amount dechlorinated by the total number of moles present in the culture, and the time elapsed in days. The average rate and the standard deviation were calculated from triplicate cultures (Table 3.1).

3.3.4. Bacterial community 16S rRNA gene analyses

DNA from pooled samples (0.5 ml from each culture replicate) was extracted every 50 days using Fast DNA® SPIN For Soil kit (MP biochemicals, Solon, OH) or UltraClean™ Soil DNA Kit (Mo Bio, Carlsbad, CA) according to manufacturer's protocols. The concentration was determined using a DU 650 spectrophotometer (Beckman, Fullerton, CA), and DNA extracts were diluted with TE buffer to 10 μg/ml. Diluted DNA (1 μl) was used in all subsequent PCR reactions, which means that each PCR reaction contained 1 ng of DNA.

Microbial community DNA from Aroclor 1260 and individual PCB congener microcosms was amplified by PCR with either universal 16S rRNA gene primers

(87), or primers specific for 16S rRNA genes of a monophyletic group within the *Chloroflexi*, Chl348FGC and Dehal884R, as described previously (42). Denaturing gradient gel electrophoresis (DGGE) was performed as described by Watts *et al.* (133) using the D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA). Briefly, 6% (wt/vol) polyacrylamide gels (Sigma, St. Louis, MO) containing a 30-70% denaturing gradient and fragments were separated by electrophoresis for 18 hours at 75 V. The gels were stained with SYBR-Green I DNA stain (Molecular Probes, Eugene, OR) and visualized using a Storm PhosphorImager (GE Healthcare, Piscataway, NJ). DGGE bands of interest were excised and DNA eluted by incubation in 30 µl TE overnight at 4°C. PCR and DGGE were repeated until purity was confirmed for DNA fragments in each eluted band.

3.3.5. DNA sequencing and analyses

PCR products from excised bands were used as templates for dye terminator cycle sequencing using the Big Dye 3.1 kit (Applied Biosystems, Foster city, CA) and an AB3100 Genetic Analyzer (Applied Biosystems). Sequences were examined for errors and assembled using the software Pregap4 and Gap4 of the Staden software package (http://sourceforge.net/projects/staden). Chimera formation was examined using Chimera Check (28). Sequences similarities were analyzed using the Basic Local Alignment Search Tool (BLAST) (5). In order to taxonomically classify sequences, we used the "classify" program by the Joint Genomic Institute (JGI) Greengenes server (http://greengenes.lbl.gov) after sequences were aligned using the align tool from Greengenes NAST server (34).

3.3.6. Enumeration of PCB dechlorinating phylotypes

Putative dehalogenating *Chloroflexi* were enumerated by competitive PCR using primers Chl348F and Dehal884R as described by Kjellerup *et al.* (Kjellerup, 2007, in preparation). Briefly, 16S rRNA gene copies per μl of normalized DNA sample (1 ng DNA per μl) were determined according to the manufacturer's instructions (Takara Bio, Shiga, Japan). DNA samples (10 μg/mL) were amplified as described above for 35 PCR cycles with 1/10 dilutions of a competitor template with known concentration. The ratio of the target PCR product to the competitor PCR product (T/C) measured by densitometry was determined using the image analysis software Quantity One (Biorad, Hercules, CA) and log (T/C) was plotted against the log of copies of the competitor. The regression equation was solved for log (C/T)= 0 (i.e. equal amounts of target and competitor).

3.3.7. Nucleotide sequence accession numbers

The 16S rRNA gene sequences for DGGE fragments have been submitted into GenBank under accession numbers EF150839-EF150845.

3.4. Results

3.4.1. Dechlorination of Aroclor 1260

Sediment from Baltimore Harbor (BH) was used to enrich for Aroclor 1260 dehalogenating microorganisms because it contains historically high concentrations of this PCB mixture. A previous report showed that these sediments contain dehalogenating activities for chlorines in the *ortho, meta* and *para* positions of PCB aromatic rings (6, 141). Baltimore Harbor microcosms incubated with Aroclor 1260 showed significant dechlorination activity within 100 days that continued through 400 days. Generally, the lag-time decreased with each transfer (data not shown). Figure 3.1 shows the mol % distribution of each of the 12 most predominant PCB congeners, the intermediate PCB congeners and products at day 0, 100 and 400 in the 5^{th} sequential transfer.

All of the 12 most predominant PCB congeners in the Aroclor 1260 mixture were dechlorinated, but the extent of dechlorination varied among congeners. The highly chlorinated congener PCB 194 (2345-2345) decreased by 1 mol % from day 0 to day 400, which is equivalent to 40% total dechlorination of this congener. In contrast, some of the less chlorinated congeners were dechlorinated to a greater extent. For example, the combined decrease of PCB 153 (245-245) and PCB 132 (234-236), which co-elute, was from 10 ± 0.5 mol % to 1.4 ± 1.3 mol %, constituting 86 % total dechlorination. The predominant dechlorination products of Aroclor 1260 after 400 days of incubation were PCB 47 (24-24) at 12.4± 2.1 mol %, PCB 49 (24-25) at 7.5 ± 1.0 mol % and PCB 51 (24-26) at 8.74± 1.68 mol %. Sterile controls did not show any dechlorination.

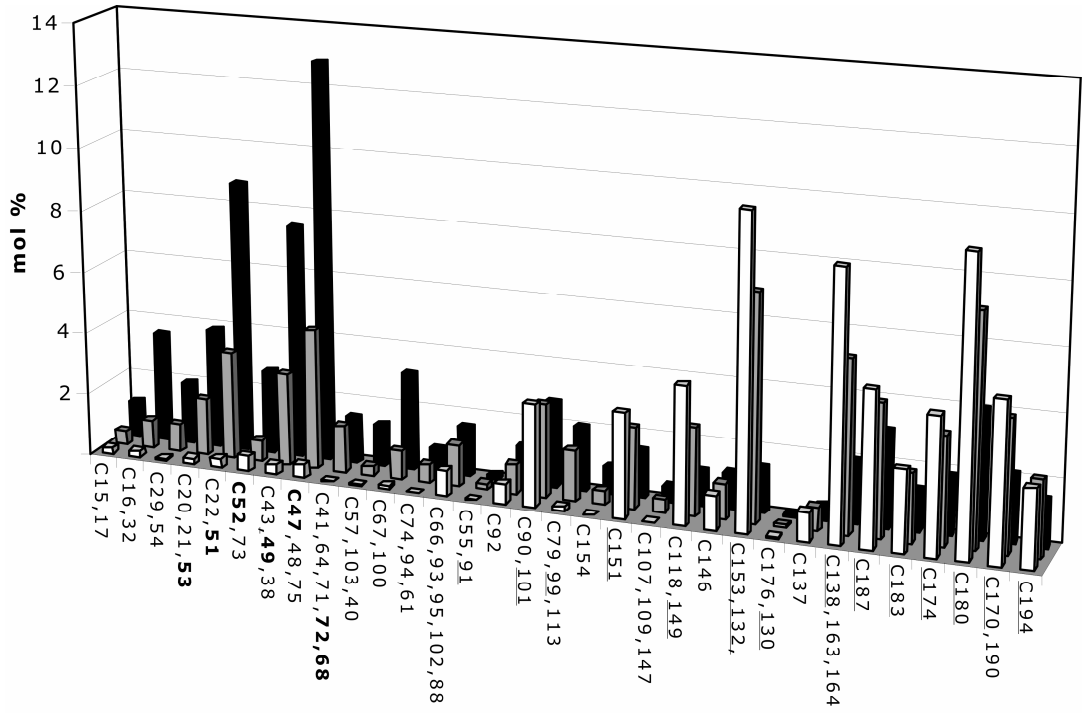


Figure 3.1. PCB congener distribution of the 12 most predominant congeners in Aroclor 1260 microcosms. Bars represent congener distributions at days 0 (□), 100 (▒) and 400 (■). Congeners that represent less than 0.05 wt % in Aroclor 1260 are not included (47). The 12 most dominant congeners are underlined and the major congener end products are shown in bold text.

3.4.2. Dechlorination of individual Aroclor 1260 congeners

BH sediment microcosms were incubated with each of the 12 most predominant PCB congeners in Aroclor 1260, which included octachlorobiphenyl 194, heptachlorobiphenyls 187, 183, 180, 174, 170, hexachlorobiphenyls 153, 151, 149, 138, 132, and pentachlorobiphenyl 101. Dechlorination lagged between 3 and 6 months in the initial microcosms, but the lag time generally decreased to less than 50 days by the fourth transfer (data not shown).

The PCB congeners used in this experiment are listed in Table 3.1, except PCB 194, which was not dechlorinated 18 months after the initial enrichment, and PCB 94, which was not dechlorinated after 300 days.

Table 3.1. Pathways and rates of reductive dechlorination for individual PCB congeners.

Parent	Product	Activity	Rate[a] (10^{-3}) (St. dev)	End mol%[b] (St. dev)
187 (2356-245)	149 (236-245)	*Ortho* fl *meta*	1.3 (0.3)	51 (1.5)
183 (2346-245)	154 (245-246)	Double fl *meta*	3.1 (0.3)	21 (3.8)
180 (2345-245)	153 (245-245)	Double fl *meta*	2.5 (0.2)	29 (4.8)
180 (2345-245)	146 (235-245)	Double fl *para*	2.4 (0.2)	29 (4.8)
174 (2345-236)	149 (236-245)	Double fl. *meta*	3.2 (1.9)	44 (3.3)
170 (2345-234)[c]	138 (234-245)	Double fl *meta*	1.5 (0.1)	47 (2.6)
	137 (2345-24)	Double fl *meta*		
	130 (234-235)	Double fl *para*		
154 (245-246)	100 (246-24)	*Para* fl *meta*	0.7 (0.2)	66 (4.0)
153 (245-245)	99 (245-24)	*Para* fl *meta*	3.3 (0.5)	12 (4.0)
151 (2356-25)	95 (236-25)	*Ortho* fl *meta*	5.5 (2.0)	24 (17)
151 (2356-25)	92 (235-25)	Flanked *ortho*	2.2	45
149 (236-245)	102 (245-26)	*Ortho* fl *meta*	6.7 (0.7)	30 (3.0)
147 (2356-24)	91 (236-24)	*Ortho* fl *meta*	0.8 (0.2)	70 (3.5)
146 (235-245)	90 (235-24)	*Para* fl *meta*	2.9 (0.5)	36 (0.9)
138 (234-245)	99 (245-24)	Double fl. *meta*	6.1 (0.3)	60 (6.6)
137 (2345-24)	99 (245-24)	Double fl *meta*	7.3 (1.2)	4 (0.7)
137 (2345-24)	90 (235-24)	Double fl *para*	4.5 (1.7)	4 (0.7)
135 (235-236)	94 (235-26)	*Ortho* fl *meta*	4.5 (0.5)	30 (8.9)
132 (234-236)	91 (236-24)	Double fl *meta*	6.2 (1.3)	61 (5.7)
130 (234-235)	90 (235-24)	Double fl *meta*	2.0 (0.6)	41 (5.7)
102 (245-26)	51 (24-26)	*Para* fl. *meta*	10 (0.6)	14 (2.3)
101 (245-25)	49 (24-25)	*Para* fl *meta*	15 (2.4)	13 (9.3)
99 (245-24)	47 (24-24)	*Para* fl *meta*	9.5 (1.3)	19 (8.8)
95 (236-25)	53 (25-26)	*Ortho* fl *meta*	8.2 (1.8)	32 (9.1)
92 (235-25)	52 (25-25)	*Ortho* fl *meta*	3.0 (0.4)	13 (5.7)
92 (235-25)	72 (25-35)	Flanked *ortho*	0.9 (0.1)	13 (5.7)
91 (236-24)	51 (24-26)	*Ortho* fl *meta*	17 (1.5)	12 (7.7)
90 (235-24)	49 (24-25)	*Ortho* fl *meta*	1.6 (0.7)	19 (2.4)
90 (235-24)	68 (24-35)	Fl *ortho*	1.7 (0.5)	19 (2.4)

[a] Chlorines removed per biphenyl per day
[b] After 150-400 days incubation.
[c] Multiple intermediates detected

Figure 3.2 shows the dechlorination pathways from each of the starting congeners to the final products. Table 3.1 describes the positions of the target chlorines, the rate of each reaction, and the end mol % for each of the starting congeners. The dechlorination rates of the parent compounds were lower than the dechlorination rates of the daughter compounds, with one exception; PCB 183 (2346-245) was dechlorinated more rapidly than PCB 154 (245-246). Also, the products in the single congener experiments were in agreement with the products observed in the Aroclor 1260 mixture (Figures 3.1 and 3.2).

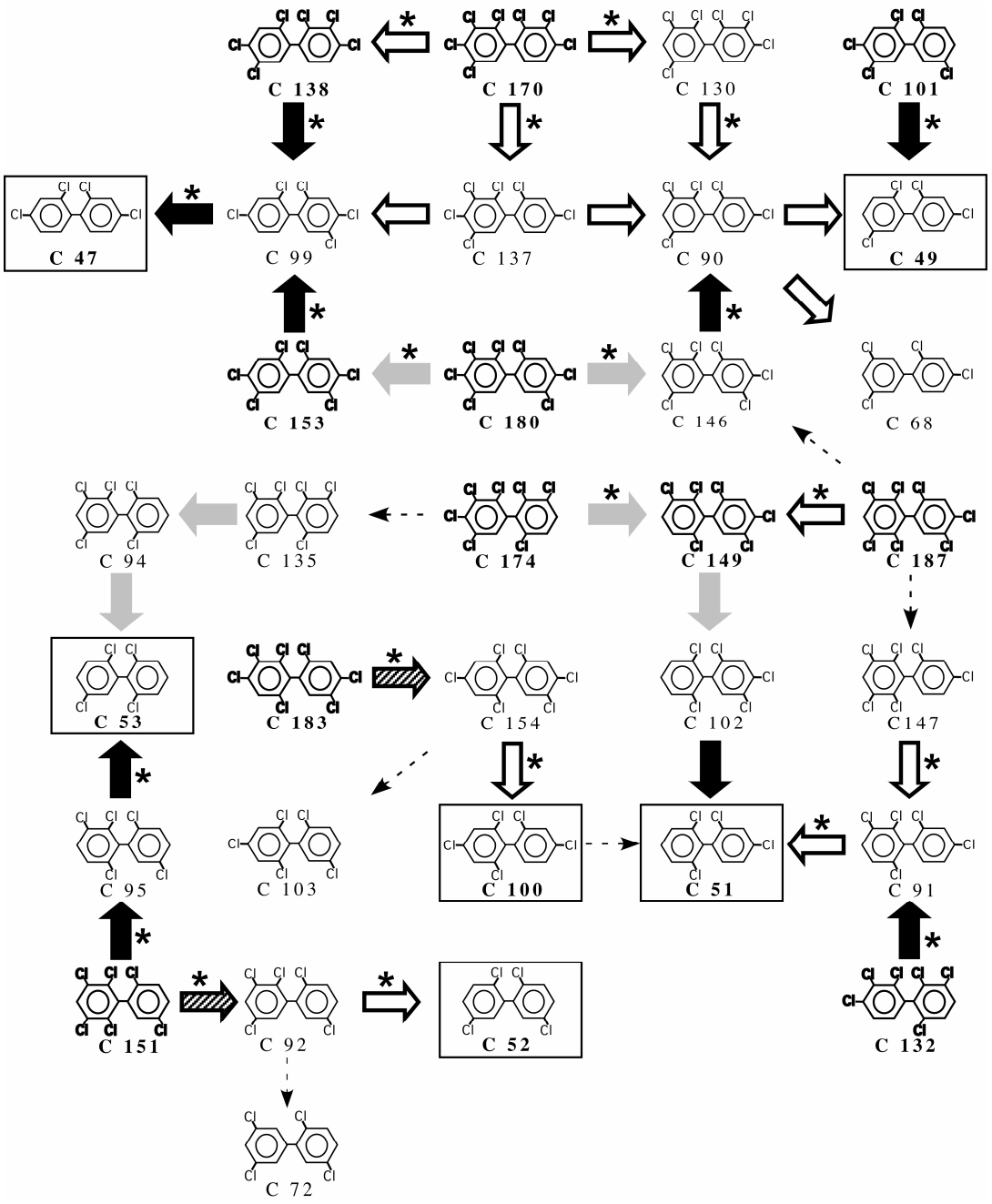


Figure 3.2. PCB dechlorination pathways of the predominant PCB congeners in Aroclor 1260. Parent congeners are shown in bold. The pathways are shown with large arrows that indicate different phylotypes: black solid, DEH 10; open arrows, SF1; hatched arrow, SF2; grey solid, both DEH10 and SF1. Small arrows indicate minor pathways. The predominant end products are boxed. Reactions in which there was at least a 2-fold increase in the number of dechlorinating phylotypes relative to the no-PCB control are indicated by asterisks.

3.4.3. Identification of dechlorinating phylotypes in Aroclor 1260 microcosms

DGGE analysis of 16S rRNA genes amplified with universal primers from the dechlorinating microcosms as well as the no-PCB control at day 0, 100 and 400, are shown in Figure 3.3 (Panel A).

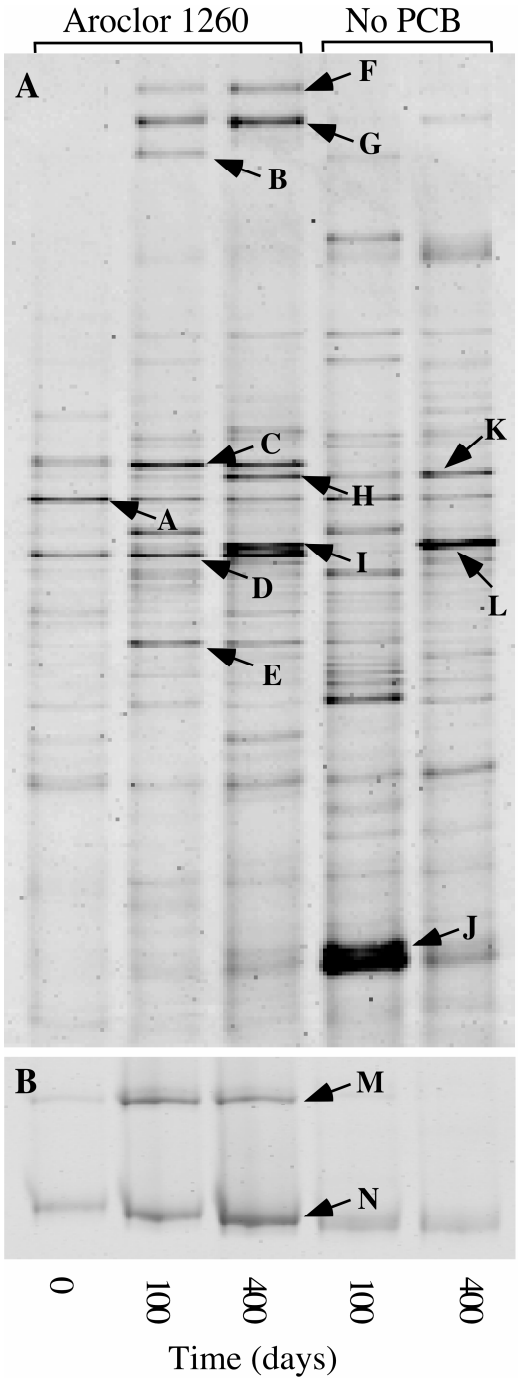


Figure 3.3. DGGE of Aroclor 1260 dechlorinating microcosms and no-PCB controls. Panel A is total DNA PCR amplified with universal primers (87) and panel B is total DNA PCR amplified with primers specific for PCB dechlorinating phylotypes (42). DNA fragments labeled A-N were sequenced and comparative sequence analyses are shown in Table 3.2.

DNA fragments identified by DGGE of samples from the Aroclor 1260 microcosm (Figure 3.3) were excised, purified and sequenced (Table 3.2). The sequence from fragment A was 97% identical to several clones identified in PCB- and dioxin-dechlorinating microcosms (142, 144). However, since the DGGE fragment corresponding to this sequence was present with PCB and in the no-PCB controls, this phylotype is likely not growing by reductive dechlorination of the added PCB. Sequence data suggest that fragments B and J are chimeras and that DNA fragments F and G represent the same sequence.

Table 3.2. Phylogenetic assignment of microorganisms in Aroclor 1260 microcosms.

Band [a]	Closest relative (accession no.)	% Identity	Phylogenetic group [b]
A	Dechlorination associated phylotypes [c]	97	Thermotogae (Firmicutes)
B [e]	Uncultured bacterium clone (EF031090)	86	Spirochaetes (Proteobacteria)
C	Dehalococcoides spp. (DEH10)	100	Chloroflexi
D	SF1 (DQ021870)	100	Chloroflexi
E	Uncultured Bacteriodetes (DQ167087)	96	Bacteriodetes
F	Uncultured bacterium (AJ853575)	96	Spirochaetes [d]
G	Uncultured bacterium (AJ853575)	96	Spirochaetes [d]
H	Uncultured Thermotogales (AM184116)	100	Thermotogae
I	Uncultured bacterium (AB177206)	89	SAR406 marine group A (Proteobacteria)
J [e]	Paper mill wastewater (AY426469.1)	82	Spirochaetes [d]
K	Uncultured Thermotogales (AM184116)	100	Thermotogae
L	Uranium mining waste pile clone AJ532716.1)	86	Chloroflexi (Proteobacteria)
M	Dehalococcoides spp (DEH10)	100	Chloroflexi
N	SF1 (DQ021870)	100	Chloroflexi

[a] Corresponds to DNA fragments in Figure 3.
[b] Classified according to Hugenholtz with the NCBI classification in parenthesis using Greengenes(34). When these two classifications agree, there is no parenthesis
[c] The sequence from fragment A was 97% identical to several clones identified in PCB- and dioxin-dechlorinating microcosms (142, 144)
[d] NCBI could not classify
[e] Possible chimera

We could detect phylotypes for two putative dehalogenators: fragment D was 100% identical to SF1, and fragment C was 100% identical to DEH10. Neither of these phylotypes was detected in the no-PCB control. Fragment H was 100% identical to a sequence found in a fosmid library constructed from Baltimore Harbor sediments (90).

In contrast, DGGE analysis of the Aroclor 1260 microcosm with PCR primers with higher specificity for a monophylogenetic group within the *Chloroflexi* only revealed two phylotypes, previously described from Baltimore Harbor sediment (Figure 3.3, panel B) (42). The DNA sequence of fragment M was 100% identical to *Dehalococcoides* spp. DEH10 and fragment N was 100% identical to phylotype SF1. Both phylotypes were reported previously to have PCB dechlorinating activity (42).

3.4.4. Enumeration of Aroclor 1260 dechlorinating phylotypes

To determine whether dechlorination of Aroclor 1260 was growth-linked, microorganisms were enumerated in microcosms by competitive PCR (cPCR) using a primer specific set for PCB dechlorinating phylotypes. Since this approach employs an internal standard that is nearly identical to the target sequence, the assay is not adversely affected by inherent differences in sediment composition between cultures that could bias PCR reactions. The number of 16S rRNA gene copies of putative dechlorinators per µl of normalized DNA from microcosms dechlorinating Aroclor 1260 shows putative dechlorinators increase in numbers as Aroclor 1260 is dechlorinated (Figure 3.4). In contrast, the control culture incubated without added Aroclor 1260 showed only a slight increase over 400 days (2.46 ± 0.18 E+04 to 4.47

± 0.27 E+04), which could be accounted for by PCB that was carried over in the transfer from the Aroclor 1260 microcosm. After 400 days of incubation, the number of PCB dechlorinating phylotypes increased 25-fold in microcosms with Aroclor 1260 added compared to the no-PCB control.

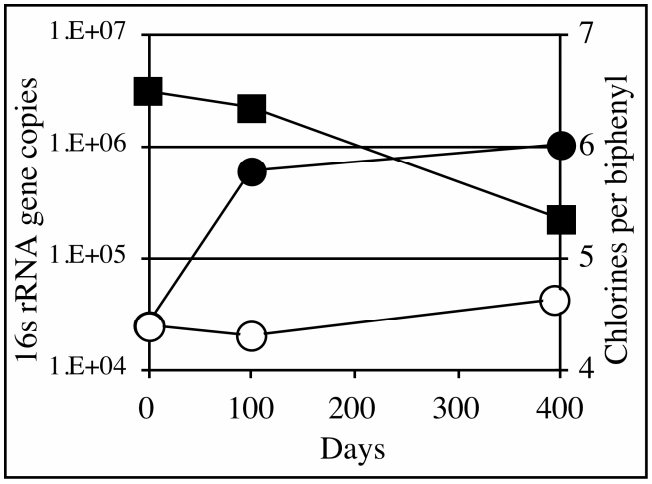


Figure 3.4. Enumeration of PCB dechlorinating phylotypes in Aroclor 1260 microcosms. 16S rRNA gene copies per µl of normalized DNA of putative dechlorinators are shown from Aroclor 1260 microcosms (●) and no-PCB controls (○). Dechlorination activity is shown as chlorines per biphenyl in Aroclor 1260 microcosms (■). Error bars (not shown) were smaller than the symbols.

3.4.5. Specific dechlorination pathways catalyzed by individual phylotypes

The phylotype responsible for each dechlorination reaction was identified in microcosms containing 11 of the 12 predominant Aroclor 1260 congeners, as well as in sub-enrichment microcosms with intermediate PCB congeners. Total community DNA from pooled replicates was amplified with specific primers Chl348FGC and

Dehal884R (42), and dechlorinating phylotypes were identified by DGGE gels as described above. Figure 3.5 shows the results from each PCB congener microcosm in a composite DGGE gel. Sequencing of all DNA fragments revealed three phylotypes. All of the uppermost DNA fragments were 100% identical to phylotype DEH10, which has previously been identified as a PCB dechlorinator (42). All the lower DNA fragments were 100% identical to phylotype SF1 identified previously from Baltimore Harbor sediments (42), except for two. The two exceptions, which were retrieved in microcosms that dechlorinated PCB 151 in the *ortho* position (marked by "o" in figure 3.5) and PCB 183, were 100% identical to *o*-17 (33) and are now called SF2. Figure 3.2 shows the pathways associated with each phylotype. Additional DNA fragments observed in some lanes (e.g., PCB 174, 135, 132 and 101, lower band) appeared to be PCR artifacts, as repeated attempts to sequence them were unsuccessful.

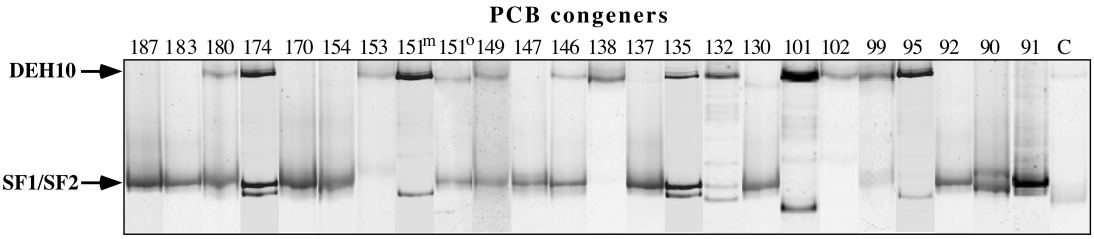


Figure 3.5. Composite DGGE of cultures dechlorinating individual PCB congeners. Primers used are specific for a monophyletic group within the *Chloroflexi* that includes known PCB dehalogenating microorganisms (42). Numbers indicate PCB congeners. 151^{m}, *meta* dechlorination of PCB 151; 151^{o}, *meta* and *ortho* dechlorination of PCB 151. Control microcosm (C) was incubated without PCB congener.

We performed cPCR on the same microcosms to investigate whether the specific reactions were growth-linked (Table 3.3). Reactions that supported at least a two-fold increase in the number of putative dechlorinators, compared to the no-PCB control, are indicated with asterisks in Figure 3.2.

Table 3.3. Enumeration of dechlorinating phylotypes by competitive PCR.

	16S rRNA gene copies per μl ($x10^3$) of normalized DNA				
Congener	Day 0 (st. dev)	PCB (st. dev)	No PCB (st. dev)	Fold	+/-PCB
187	74 (15)	410 (46)	65 (4.2)		6.2
183	0.3 (0.1)	370 (16)	33 (6.3)		11
180	9.8 (5.7)	880 (52)	58 (8.8)		15
174	19 (1.8)	150 (9.2)	25 (5.1)		5.9
170	14 (1.1)	350 (42)	35 (6.3)		9.9
154	45 (6.6)	360 (12)	94 (3.8)		3.8
153	3.1 (1.0)	26 (4.9)	2.3 (0.3)		11
151[a]	nd[c]	320 (20)	19 (1.4)		17
151	nd	110 (4.6)	12 (2.1)		8.8
149	nd	57 (12)	52 (3.7)		1.1
147	52 (4.2)	580 (21)	43 (6.7)		13
146	18 (3.0)	32 (6.3)	3.1 (0.4)		10
138	1.9 (0.1)	490 (44)	37 (5.5)		13
137	15 (2.9)	59 (6.6)	24 (3.8)		2.4
135	87 (26)	140 (17)	41 (1.2)		3.3
132[b]	7.5	2100	150		14
130	12 (1.7)	1300 (140)	34 (1.9)		38
102	nd	2.6 (0.1)	16 (1.3)		0.2
101[b]	23	240	23		10
99	1.6 (0.2)	460 (45)	53 (6.6)		8.5
95	nd	160 (12)	9.9 (3.0)		16
92	0.5 (0.3)	110 (11)	6.3 (1.4)		17
91[b]	9.3	460	4.3		110
90	8.8 (1.4)	530 (70)	430 (170)		1.2

[a] Only *meta* dechlorination of PCB 151 was observed
[b] Calculated from Fagervold *et al.* (42)
[c] Not detected

The extent of growth contributed by individual dechlorination reactions could not be distinguished in multi-step pathways. For example, PCB congener 138 (234-245) was dechlorinated in two steps, with PCB 99 (245-24) as an intermediate. While it is clear that there was an 8.5 fold increase of putative dechlorinators (Table 3.3) in the microcosms dechlorinating PCB 99, we cannot determine the specific increase for the reaction from PCB 138 to PCB 99, since dechlorination of PCB 99 occurs simultaneously in this microcosm. Generally, the extent of growth varied with different congeners. The highest increase was a 107-fold increase during *ortho*-flanked *meta* dechlorination of PCB 91 (236-24) while the dechlorination of PCB 102 (245-26) yielded no detectable growth. However there were no apparent differences between the overall growth yields when the reactions were catalyzed by SF1 compared to DEH10.

3.4.6. Congener specificity of the PCB dechlorinating phylotypes

Microbial dechlorination in Baltimore Harbor microcosms preferentially dechlorinated double flanked chlorines, and most of the double flanked dechlorination was catalyzed by SF1. SF1 dechlorinated all 2345-substituted chlorophenyl rings preferentially in the *meta* position, although some *para* dechlorination was observed, such as the dechlorination of PCB 137 (2345-24) to PCB 90 (235-24) (Figure 3.2). In addition, SF1 dechlorinated the 234-substituted chlorophenyl ring in PCB 130 (234-235) in the double-flanked *meta* position. SF1 also dechlorinated 2356-, 236- and 235-substituted chlorophenyl rings in the *ortho*-

flanked *meta* position when the other ring contained 245- or 24-substitutions (i.e. PCB 187, 147, 91, 90). SF1 dechlorinated PCB 92 (235-25), which contained a 25-substitution on the other ring, in the *ortho*-flanked *meta* position. Phylotype SF2 dechlorinated the only 2346-substituted chlorophenyl ring tested (PCB 183) in the double-flanked *meta* position and the 2356-substituted chlorophenyl ring of PCB 151 in the *ortho* position.

DEH10 dechlorinated the double-flanked chlorine in 234-substituted chlorophenyl ring (except in PCB 130). DEH10 showed a preference for *para*-flanked *meta* chlorines when no double-flanked chlorines were available and thus dechlorinated 245-substituted chlorophenyl rings in the *meta* position with one exception: PCB 154 (245-246), which contains a 246-substitution on the other ring. DEH10 also dechlorinated in the *ortho*-flanked *meta* position (PCB 151 and 95), when the other ring contained 25-substitutions.

3.5. Discussion

3.5.1. Patterns of dechlorination of Aroclor 1260 congeners by Baltimore Harbor microcosms

The pattern of Aroclor 1260 dechlorination in Baltimore Harbor sediment microcosms resembles "process N', first identified in Aroclor 1260 microcosms from Silver Lake sediments (98). Process N was described as exclusive dechlorination in the *meta* position, with a characteristically high accumulation of PCB 47 (24-24). This pattern has been subsequently observed in sediment microcosms from several PCB impacted freshwater sources, including Woods Pond (10), Hudson River (102), and from sediment-free microcosms developed from the Housatonic River (8). However, the patterns observed in sediments from Baltimore Harbor differ from the exclusive *meta* dechlorination pattern reported in freshwater sources by also showing dechlorination of Aroclor 1260 and individual PCB congeners in the *ortho* position (15, 32, 141). In the current study the major dechlorination products in the Aroclor 1260 and single congener experiments were PCB 100 (246-24), PCB 53 (25-26), PCB 52 (25-25), PCB 49 (24-25) and PCB 47 (24-24), all containing unflanked chlorines. Aroclor 1260 microcosms also yielded trace amounts of dichlorobiphenyls and trichlorobiphenyls with unflanked chlorines after 400 days of incubation.

All 12 major parent congeners, which account for over 50 wt % of Aroclor 1260, were dechlorinated in Aroclor 1260 microcosms including PCB 194, which was not dechlorinated when incubated with Baltimore Harbor sediment alone. The dechlorination of PCB 194, as well as the accumulation of tri- and dichlorobiphenyls in Aroclor 1260 microcosms, may be due to the presence of multiple congeners, that

have been shown to have both stimulatory and inhibitory effects on congener specificity by PCB-dehalogenating microorganisms (80, 98). Likewise, brominated biphenyls have been shown to have a stimulatory effect on both the rate, and the dechlorination patterns in Aroclor microcosms (13). In the current study, the stimulating effect of specific or multiple congeners in the Aroclor 1260 dechlorinating microcosm likely promoted the reductive dechlorination of PCB 194.

Several investigators have used single PCB congeners to infer PCB dechlorination pathways (2, 15, 16, 42, 93, 102, 114, 123, 134, 138). However, this is the first report on the dechlorination of all major PCB congeners present in Aroclor 1260 by single congener experiments. Several of the pathways in Figure 3.2 have been proposed previously from inference of Aroclor 1260 dechlorination products. For example, a previous report with Baltimore Harbor Aroclor 1260 microcosms (141) predicted the *ortho* dechlorination pathway of PCB 151 (2356-25) to PCB 72 (25-35). However, the previously predicted dechlorination pathway from PCB 170 (2345-234) to PCB 68 (24-35) was only partly consistent with our results (Figure 3.2), as most of PCB 170 was dechlorinated to either 26 mol % PCB 47 (24-24) or 13 mol % PCB 49 (24-25) at day 400. Several observations were consistent with the dechlorination pathways for PCB 101, 132, 138, 153, 170, and 180 in "Process N", proposed in a comprehensive review by Bedard and Quensen (12). We determined the dechlorination pathways for PCB 183, which is, to our knowledge, the first report of this pathway. In addition, we defined the pathways of PCB 174 and PCB 151, which were ambiguous in previous reports (8, 9).

In a previous report on dechlorination of Aroclor 1260 in Housatonic River microcosms (8) several proposed dechlorination pathways for 2345-substituted congeners were based upon the assumption that 60 % of these congeners were dechlorinated in the double-flanked *para* position and 40 % in the double-flanked *meta* position. Woods Pond sediments have also been shown to have *para* dechlorination activity (9, 10). Although we observed some examples of double-flanked *para* dechlorination, our results showed that dechlorination in Baltimore Harbor microcosms more often occurred in the double-flanked *meta* position. This was especially true for PCB 174 (2345-236), where all the dechlorination occurred in the double-flanked *meta* position and for PCB 180 (2345-245), where 67 % occurred in the double-flanked *meta* position and 33 % of the dechlorination occurred in the double-flanked *para* position. On the other hand, PCB 137 (2345-24) was dechlorinated equally in the double-flanked *meta* and *para* position.

Congeners with 2356-substitutions (i.e., PCBs 149, 151, 187) were dechlorinated in the *ortho*-flanked *meta* position. However, we also observed some minor *ortho* dechlorination of PCB 151 (2356-25), PCB 90 (235-24) and PCB 92 (235-25), which is consistent with prior reports of *ortho* dechlorination activity of both Aroclor 1260 and single congeners in Baltimore Harbor microcosms (15, 32, 33, 80, 141). Based on observations with Aroclor 1260 and the individual congeners, the general sequence of dechlorination in Baltimore Harbor microcosms is: double-flanked *meta* or *para* of 2345-substituted chlorophenyl rings; double-flanked *meta* of 234- or 2346-substituted chlorophenyl rings; *ortho*-flanked *meta* of 2356-substituted chlorophenyl rings; *para*-flanked *meta* of 245-substituted chlorophenyl rings; *ortho*-

flanked *meta* of 236-substituted chlorophenyl rings; *ortho*-flanked *meta* of 235-substituted chlorophenyl rings and flanked *ortho* of 2356-substituted chlorophenyl rings.

3.5.2. Effect of congener characteristics on dehalogenation

An analysis of 1) the differences in the estimated Gibbs free energy of formation between parent and daughter congeners (55), 2) the differences in relative retention time (24), 3) the aqueous solubilities (55), and 4) the number of total chlorines and the number of *ortho*, *meta* and *para* chlorines on the ring subjected to dechlorination, as well as on the opposite ring, showed that there were no significant relationships ($p < 0.05$) between these parameters, and the dechlorination rates, end mol % or the number of putative dechlorinators in Baltimore Harbor microcosms. However, we observed weak relationships (with low R^2 values) between the dechlorination rate and the aqueous solubility of the PCB congener (r^2=0.25), the total amount of chlorines (r^2=0.22), as well as the number of *meta* chlorines (r^2=0.28). Dechlorination rates increased in microcosms with more soluble PCB congeners and decreased with both the number of total chlorines and the number of *meta* chlorines.

Several possible factors could mediate the dechlorination patterns we observed in our cultures. Double-flanked chlorines are generally dechlorinated first despite the fact that these reactions yield the least amount of energy. This apparent preference for double and then single flanked chlorines may be explained based on the chemistry of chlorinated biphenyls. It has been proposed that microbial reductive dechlorination is a two-step process, the first of which is the transfer of an electron to

the chlorinated biphenyl and the formation of a carbanion intermediate (93). The negative charge is stabilized by resonance throughout the biphenyl molecule and the surrounding chlorine atoms. The ability of the molecule to stabilize through resonance also influences the overall reactivity, or standard potential (E°), of different PCB congeners. Generally, higher chlorinated congeners have higher E° values and are more reactive in environments with low redox potential. Furthermore, PCB molecules with *ortho* chlorines are less planar, have lower E° values, and are chemically less reactive (29, 106). However, this did not appear to determine the rate or the extent of PCB dechlorination in our microcosms. Brown *et al.* (20) observed similar results, where one dechlorination pattern would follow the reactivity of the chlorophenyl groups while another observed dechlorination pattern did not. Another explanation for the observed differences in reaction rates is the steric properties of the individual congeners, first proposed by Brown *et al.* (20), which could affect the specific activities of individual reductive dehalogenases. These results are consistent with an earlier suggestion by Williams (134) that the reactivity of a specific PCB chlorine is dependent upon both the chemical properties of the congener and catalytic properties of the microbes.

3.5.3. Diversity of Aroclor 1260 dehalogenating phylotypes

PCB dehalogenating microorganisms have been previously identified as belonging to either *Dehalococcoides* spp. or the *o*-17/DF-1 clade within a deep branch of the *Chloroflexi* (8, 33, 42, 45, 97, 132, 136, 137, 142) and these phylotypes have been detected in microcosms dechlorinating Aroclor 1260 (8). Although the microbial community in the Aroclor 1260 microcosms was diverse (Figure 3.3), several lines of evidence indicate that only two phylotypes, DEH10 and SF1, were the predominant biocatalysts of Aroclor 1260 dechlorination: 1) they were detected only in Aroclor 1260 and individual congener microcosms and not detected using universal PCR primers on the no-PCB controls; 2) they increased in numbers only during active dechlorination of Aroclor 1260 and individual congeners; 3) the phylotypes have high sequence similarity to phylotypes and isolates previously shown to reductively dechlorinate PCBs (42, 45, 132). Phylotypes belonging to the *Bacteriodetes* and the *Spirochaetes* (Figure 3.3, DNA fragments B and E) were also present only in the dechlorinating cultures, and although similar phylotypes have been previously detected in Baltimore Harbor microcosms (97), to date, there is no evidence they reductively dechlorinate PCBs.

There are some uncertainties using 16S rRNA primers due to the fact that microorganisms have been shown to have more than one 16S rRNA gene copy (130) and the inherent biases with PCR (120). In our experiments we compare the diversity and the growth of putative dehalogenators between cultures with and without PCB, and since this is the only difference, we assume that the difference in the microbial populations is due to dechlorination of PCBs. Also, since we directly compare the

presence of the same phylotypes between dechlorinating microcosms and no-PCB controls, any bias due to multiple 16S rRNA copies or PCR would be the same in both. We can detect putative dehalogenators in the no-PCB controls (Figure 3.3 and table 3.3), but this is likely due to enrichment resulting from traces of PCB co-transferred into no-PCB controls during inoculation from active cultures. *Dehalococcoides* species have been shown to have very similar or even identical 16S rRNA sequences, but still have different dechlorination activities (56). Although the 16S rRNA gene sequence of phylotypes DEH10, SF1 and *SF2* detected in this study are 100 % identical to the phylotypes detected previously in Baltimore Harbor sediment microcosms, we cannot confirm that they are each the same species or strain since they came from different microcosms. However, the high identity combined with the fact they each came from the same source and each have the same selective dechlorination activities as previously described phylotypes, indicates a high likelihood that they are the same microorganisms.

SF1 and DEH 10 exhibited specific activities towards the PCB congeners we tested. The combined activities of SF1 were different than those previously reported for either *o*-17 (flanked *ortho* and *ortho*-flanked *meta* chlorines) or DF-1 (double-flanked chlorines). SF2 was unequivocally only associated with these two pathways and we do not believe this represents an adequately comprehensive overview of its specific dechlorinating activities.

When grown with the single congeners or Aroclor 1260, the populations of PCB dechlorinating microorganisms increased only 1-2 orders of magnitude during the course of reductive dehalogenation. These results are consistent with prior reports

that showed a similar range of increase for congener mixtures by other methods, including Most Probable Number (MPN) enumeration and MPN PCR of 16S rRNA gene copies (26, 42, 62). However, we show unequivocally that phylotypes within *Dehalococcoides* spp. and the DF-1/*o*-17-group of the dehalogenating *Chloroflexi* are directly responsible for the reductive dechlorination of an Aroclor. The relative growth of the individual dechlorinating phylotypes varied among different PCB congeners, increasing up to 2 orders of magnitude with an average 13.5 fold increase for the 24 congeners tested. Although this supports a conclusion by Kim *et al.* (62) that the size of the dechlorinating population might be an indicator for PCB dechlorination potential in a site, the results also suggest that other factors, including the types of congeners and the indigenous dechlorinating phylotypes, will have an impact on the size of the population. By combining the relative dechlorination rates for individual congeners in Aroclor 1260 with growth rates of dehalogenating phylotypes on individual congeners, it might be possible to generate models to predict the dechlorination potential based on analyses of the congener distribution and enumeration of the total PCB dehalogenating population in specific PCB impacted sites

3.5.4. Conclusions

The results of this study showed that only three *Chloroflexi* phylotypes (SF1, SF2 and DEH10) were required to reductively dechlorinate the 11 major PCB congeners in Aroclor 1260 to unflanked tetra- and tri-chlorobiphenyls in our microcosms. Although these phylotypes were detected in estuarine microcosms, the similarity of dechlorinating patterns suggests that similar phylotypes might also be responsible for the Aroclor 1260 dechlorination previously reported in other sites such as the Hudson and Husatonic River (8, 102). Demonstration that dechlorination of Aroclor 1260, as well as most of the individual congeners is linked to growth, suggests that PCB-impacted environments can sustain populations of PCB dechlorinating organisms. This is particularly relevant for the development of biostimulation or biaugmentation strategies for the bioremediation of PCBs. The final products of Aroclor 1260 dechlorination by these three phylotypes (unflanked tetra- and tri-chlorobiphenyls), could potentially be further transformed by bioaugmentation with microcosms that have been shown to dechlorinate unflanked congeners (11), or could serve directly as substrates for aerobic mineralization by PCB degrading bacteria (78). Using molecular approaches for the specific detection of dehalogenating microbial communities in soils and sediments, it is now possible to identify the predominant dechlorinators at contaminated sites containing Aroclor mixtures and monitor the fate of both indigenous and augmented microorganisms, which is essential for the development of *in situ* treatment strategies.

Chapter 4: Assessing the potential of PCB dechlorinating microorganisms for bioaugmentation

4.1. Abstract

PCB dechlorinating populations with different PCB congener specificities were added to fresh Baltimore Harbor sediments microcosms in an attempt to evaluate the feasibility of using PCB dechlorinating microorganisms for bioaugmentation purposes. The dechlorinating microorganisms belong to a monophyletic group within the *Chloroflexi* and included phylotypes *o*-17, DF-1, DEH10 and SF1, previously shown to be perform different dechlorinating activities. Dechlorination of PCB 2,2',3,5,5',6-hexachlorobiphenyl was observed in all microcosms but the pattern of dechlorination was different depending on the dechlorinating populations added. Dechlorination of Aroclor 1260 was most extensive when all four dechlorinators were added to fresh Baltimore Harbor sediment microcosms. We also observed increased *ortho* dechlorination of Aroclor 1260 when *o*-17 and DF-1 were used to bioaugment these sediment microcosms. We used 16S rRNA gene primers specific for known dechlorinators to monitor the presence of putative dechlorinators. Generally, putative dechlorinators increased 1000-fold and phylotype DEH 10 was most successful at competing with the indigenous microbial population in Baltimore Harbor sediment microcosms. The ability to mediate both the type and rate of PCB dechlorinating activities in microcosms by adding selected PCB dechlorinating microorganisms indicates that

bioaugmentation might be a tractable approach for *in situ* treatment of PCB impacted sites.

4.2. Introduction

Bioaugmentation, or the addition of specific microorganisms to the local microbial population to enhance the degradation of contaminants, mostly at the experimental stage (40), although there are several reports of pilot scale studies using bioaugmentation as a strategy for bioremediation with various degrees of success (for reviews, see (40) and (49)).

Anaerobic bioaugmentation studies have been shown to be successful *in situ* with the complete dechlorination of TCE to ethene using KB-1 (77), a consortium of different *Dehalococcoides* strains (38), or a by using an enrichment inoculated with organisms from the same site (72). In both these cases, increased numbers of *Dehalococcoides* species were detected after treatment. Although, Natarajan *et al.* (88) showed that the addition of anaerobic microbial consortia to sediment microcosms had a stimulating effect on the dechlorination of Aroclor 1258 and the single congener 2,3,4,5,6-pentochlorbiphenyl, there are no reports of a successful bioaugmentation strategy for the reductive dechlorination of polychlorinated biphenyls.

Microbial reductive dechlorination is a process where microorganisms use PCBs as terminal electron acceptors in respiration (20). This process occurs mainly in anaerobic sediments and has been widely studied (12). It has long been proposed that the variety of patterns of reductive dechlorination that are observed at different

sites, was caused by differences in the microbial population with distinct dehalogenating enzymes (20). Thus, the idea of adding different strains with different dechlorination capabilities was first proposed by Quensen *et al.* (98) as early as 1990.

Several studies have been performed with "priming", or the addition of specific PCB congeners or other halogenated compounds to sediment microcosms, in order to either stimulate certain activities and/or to increase the number of dechlorinators (9, 13, 35, 63, 124). However, adding "primed" enrichment cultures alone did not appear to stimulate specific dechlorination activity in residual Aroclor 1260 in Woods Pond sediments (138), and similar results were reported from primed Housatonic River sediments (14).

We now have an arsenal of several different dechlorinating microorganisms with different dechlorinating specificities (33, 41, 137). In addition, the molecular tools to track the survival of these microorganisms in sediment microcosms (42) are now available. In order to investigate whether bioaugmentation could be a feasible strategy for treatment of PCB contaminated sites, we performed microcosm experiments with Baltimore Harbor sediments and tested whether adding known dehalogenators to fresh Baltimore Harbor sediment would change the pathways of dechlorination of an individual congener or Aroclor 1260. 2,2',3,5,5',6-hexachlorobiphenyl (2356-25 or PCB 151) was chosen for these studies because we had previously observed that this PCB congener could be dechlorinated via several alternative pathways (41)(Chapter 3). The presence of the added microorganisms was monitored by DGGE, with PCR primers specific for a monophyletic group within the *Chloroflexi* that contains all known PCB dechlorinators (42)(Chapter 2). We also

monitored the growth of putative dechlorinating microorganisms using competitive PCR (Kjellerup *et al.*, 2007, in preparation) (41). The addition of different microbial populations changed the dechlorination pathway of PCB 151 and also had a detectable, but less obvious effect on the nature of Aroclor 1260 dechlorination pathways. The microorganisms generally increased about three orders of magnitude during incubation, and phylotype DEH10 was especially successful in competing with the indigenous microbial community in Baltimore Harbor sediment microcosms.

4.3. Materials and methods

4.3.1. Source of microorganisms for bioaugmentation

Microorganisms from actively PCB-dechlorinating microcosms were used to bioaugment fresh or dry Baltimore Harbor sediment. A sediment microcosm enriched by sequential transfers with 236-25-CB (PCB 95) was used as an inoculum source of phylotype DEH 10, and a sediment microcosm enriched by sequential transfers with 236-24-CB (PCB 91) was used as an inoculum source of phylotype SF1. Both of these microcosms were initially enriched from Baltimore Harbor sediment (41). Bacterium DF-1, originally enriched from Charleston Harbor sediments, was maintained and grown in the lab in co-culture with a *Desulfovibrio* spp. with 10 mM sodium formate dechlorinating 2345-CB (PCB 61) as described previously (136). A co-culture containing the uncultured Bacterium *o*-17 originally enriched from Baltimore Harbor sediment was maintained and grown in the lab in co-culture with a *Desulfovibrio* spp. with 20 mM sodium acetate and 2356-CB (PCB 65) as described previously (33). Enumeration of cell numbers was performed by most

probable number estimation of 16S rRNA gene copies from total DNA in cultures containing DEH10, SF1, *o*-17 and DF1. Diluted DNA from these cultures was subjected to PCR (40 cycles) using primers Chl348F and Dehal884R, as described previously (42), and one copy of the gene per genome was assumed based on the genomes of *Dehalococcoides ethenogenes* and *Dehaloccoides* strain CBDB1 (67, 112).

4.3.2. Anaerobic enrichment cultures

Microcosms were prepared using methods similar to those described previously (42) with a defined, low-sulfate (<0.3mM), estuarine salts medium (E-Cl) and a fatty acid mixture (acetate, propionate and butyrate, 2.5 mM each) as electron donors (15). However, several different amendments and modifications were used. Each treatment was incubated with 50 ppm 2356-25-CB (PCB 151) and 100 ppm Aroclor 1260. Treatment 1 contained 1.5 g (wet) fresh Baltimore Harbor (BH) sediment as the only source of microorganisms. Treatment 2 contained 1.5 g BH sediment and 0.4 ml each of sediment microcosms enriched for phylotypes SF1 and DEH 10, which is equivalent to approximately 2.8 x 10^5 cells of each phylotype per 10 ml. Treatment 3 contained 1.5 g BH sediment and 1 ml each co-cultures containing *o*-17 and DF1, which is equivalent to 1 x10^5 and 2 x10^5 cells, respectively, of these phylotypes per 10 ml. Treatment 4 contained 1.5 g BH sediment and all the microorganisms at the same concentrations as in treatments 2 and 3. Treatment 5 contained 0.5 g dry BH sediment, as described earlier (42), with the addition of all the microorganisms combined at the same concentrations as treatments 2 and 3.

Treatment 6 contained 1.5 g BH sediment with the addition of an autoclaved (20 minutes, 121°C) mixture of all the microorganisms combined at the same concentrations as treatments 2 and 3. This treatment served as a control for the effect of small amounts of PCB carried over from the original enrichments on the activity of the indigenous microbial community. All cultures were prepared in triplicate and incubated at 30^{o} C in the dark.

4.3.3. Analytical techniques

Microcosms were sampled in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI) and analyzed for PCB dechlorination every 50 days as previously described (42). PCBs were analyzed by extracting 0.5 ml of culture with 3 ml of hexane for 12 hours on a wrist shaker. The organic phase was passed through a copper/Florisil® (1:4) column and analyzed using a Hewlett Packard 5890 series II gas chromatograph (GC) equipped with a DB-1 capillary column (30 m by 0.25 mm by 0.25 μm; JW Scientific, Folsom, CA) and a Ni^{63} electron capture detector (ECD) as described by Berkaw *et al.* (15). Nine mixes containing a total of 209 congeners (AccuStandard, Catalog name: C-CSQ-SET) were used to identify the PCB congeners based on retention times. Individual PCB congeners were quantified with a 10-point calibration curve using PCB 204 as an internal standard. Dechlorination curves for all the PCB congeners were based upon mol % as previously described (42). The total amount of PCBs was determined in each replicate, and the mol % was calculated for each congener in the sample. The average mol % and the standard deviation for each congener were determined from triplicate cultures.

4.3.4. Bacterial community 16S rRNA gene analysis

DNA from pooled samples (0.5 ml from each of replicates for each treatment) was extracted every 50 days using the Fast DNA® SPIN For Soil kit (MP biochemicals, Solon, OH) or UltraClean™ Soil DNA Kit (Mo Bio, Carlsbad, CA) according to the manufacturer's protocols. DNA concentration was determined using a DU 650 spectrophotometer (Beckman, Fullerton, CA), and DNA extracts were diluted with TE buffer to 10 μg/ml. Diluted DNA (1 μl) was used in all subsequent PCR reactions. Due to poor template quality (PCR inhibition), Day 0 samples extracted with the DNA® SPIN For Soil kit were subjected to an extra cleanup step with the Promega Wizard PCR Prep Kit (Promega, Madison, WI.).

Analysis of the microbial community within microcosms was evaluated by Denaturing gradient gel electrophoresis (DGGE) of total DNA PCR amplified with primers specific for 16S rRNA genes of a monophyletic group within the *Chloroflexi*, Chl348FGC and Dehal884R, as previously described (Chapter 2 and (42)). DGGE was performed as described by Watts *et al.* (133) and Fagervold *et al*. (42) using the D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA.). DGGE bands of interest were excised and the DNA eluted by incubation in 30 μl TE overnight at 4°C.

4.3.5. DNA sequencing and analysis

PCR products from excised bands were used as templates for dye terminator cycle sequencing using the Big Dye 3.1 kit (Applied Biosystems, Foster city, CA) and an AB3100 Genetic Analyzer (Applied Biosystems). Sequences were examined for errors and assembled using the software Pregap4 and Gap4 of the Staden software package (http://sourceforge.net/projects/staden). Sequences were identified using the Basic Local Alignment Search Tool (BLAST) (5).

4.3.6. Quantitative assessment of PCB dechlorinating population

Putative dehalogenating *Chloroflexi* were enumerated by competitive PCR (Kjellerup, 2007, in preparation) using primers Chl348F and Dehal884R as described by Fagervold *et al.* (41).

4.4. Results and discussion

4.4.1. Expected dechlorination activities

PCB 151 (2356-25) can be dechlorinated through different pathways (Figure 4.1). The first step involves either a *meta* dechlorination to PCB 95 (236-25), which is further dechlorinated in the *meta* position to PCB 53 (25-26), or dechlorination in the *ortho* position to PCB 92 (235-25). PCB 92 can either be dechlorinated in the *meta* position to PCB 52 (25-25) or in the *ortho* position to PCB 72 (25-35).

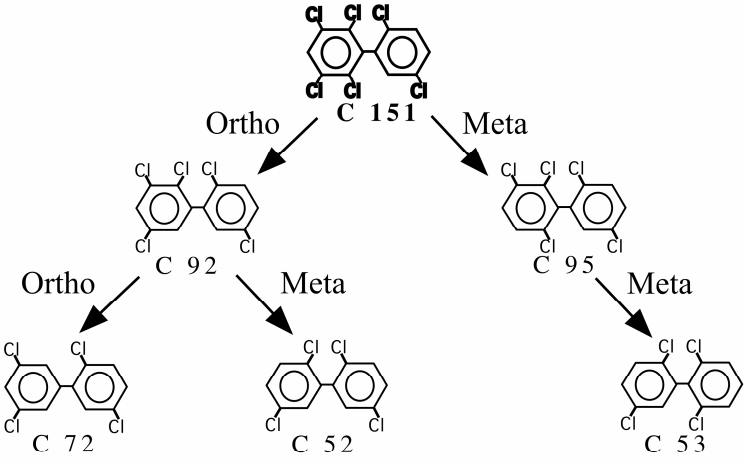


Figure 4.1. Possible dechlorination pathways of PCB 151 (2356-25). PCB 151 can be dechlorinated in *meta* or *para* positions.

The specific activities of the microorganisms we used to bioaugment the sediments have been characterized in Chapter 3. Bacterium *o*-17 has been reported previously to *ortho* dechlorinate 2356-CB and 2356-35-CB but does not dechlorinate 2356-2356-CB or 2356-26-CB, which contains four *ortho* chlorines (80). Also, *o*-17 has been shown to be responsible for the *ortho* dechlorination of PCB 151 to PCB 92 (235-25) (41). However, in a recent study, high concentrations of Aroclor 1260 was

shown to be inhibitory to *o*-17 (80). DF-1 is capable of double-flanked *para* or *meta* dechlorination, but does not dechlorinate single flanked chlorines (136). PCB 151 (2356-25) does not contain any double-flanked chlorines and therefore DF-1 was not expected to dechlorinate this congener. However, Aroclor 1260 should be a substrate for DF-1 as several PCB congeners in Aroclor 1260 contain double flanked chlorines.

Cultures with phylotypes SF1 and DEH10 were enriched from Baltimore Harbor sediments and because they were cultured with sediment they included a more complex microbial community than the *o*-17 and DF-1 cultures. Phylotype DEH10 has been shown to dechlorinate PCB 151 (2356-25) and PCB 95 (236-25) in the *ortho*-flanked *meta* position (41). Generally, DEH10 dechlorinates the double-flanked *meta* chlorines in 234-substituted chlorophenyl ring and *para*-flanked *meta* chlorines when no double-flanked chlorines are available. Phylotype SF1 dechlorinates all 2345-substituted chlorophenyl rings preferentially in the *meta* position, although some *para* dechlorination has been observed (41). SF1 is thought to be involved in the *ortho*-flanked *meta* dechlorination of PCB 92 (235-25) to PCB 52 (25-25).

To assure we added approximately the same number of dechlorinating microorganisms to the microcosms, we estimated the numbers of dechlorinators in the cultures that were used to bioaugment. The DEH 10 and SF-1 cultures contained approximately 7×10^5 copies of 16S rRNA genes of each phylotype per ml of culture. The DF-1 co-culture contained approximately 1×10^5 16S rRNA gene copies per ml of culture and the *o*-17 culture contained approximately 2×10^5 16S rRNA gene

copies per ml of culture. The assay likely underestimated how many microorganisms are present because the detection limit is probably higher than 10 copies.

4.4.2. Effect of bioaugmentation on the dechlorination patterns of PCB 151 (2356-25)

Fresh Baltimore Harbor (BH) sediment and dried, sterile BH sediment were amended with different populations of dechlorinating microorganisms, and the patterns of dechlorination were monitored over time. The results showed the dechlorination pathways were modified by the treatments (Figure 4.2). Treatment 1, with fresh BH sediment alone did not start to dechlorinate before day 100, and both *meta* and *para* dechlorination were observed. After 300 days of incubation, the major products were PCB 72 (25-35) at 49.2 ± 21.6 mol% and PCB 95 at 23.4 ± 24.3 mol%. However, standard deviations were very high because the triplicate microcosms behaved very differently. One microcosm dechlorinated PCB 151 to PCB 95 at 81.2 mol % at day 300, while the two other replicates dechlorinated PCB 151 to PCB 72 (69.1 mol % and 67.3 mol % at day 300). Furthermore, the latter two replicates did not show any dechlorination until day 200, while the microcosm dechlorinating PCB 151 to PCB 95 showed dechlorination at day 150.

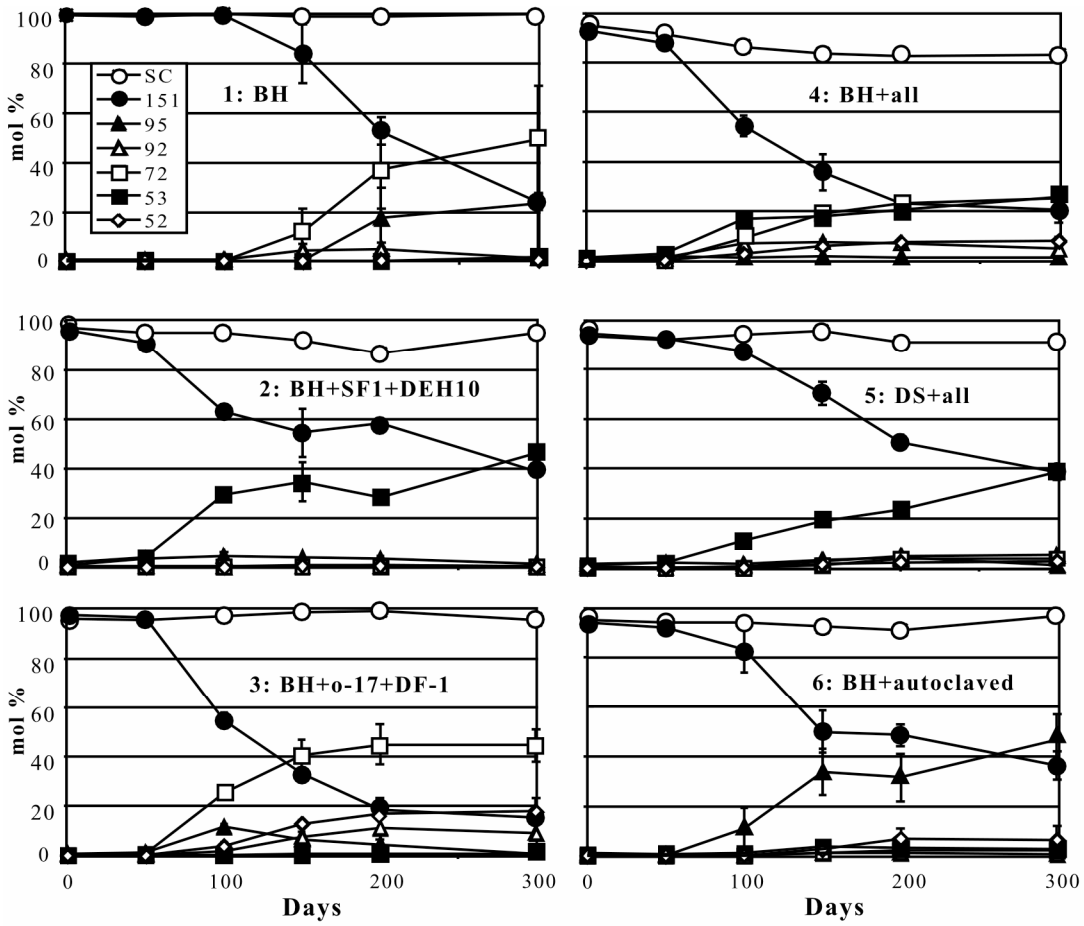


Figure 4.2. Dechlorination of PCB 151 over time with different treatments. The numbers in the legend refers to different PCB congeners or PCB 151 in the sterile control (SC). BH = Baltimore Harbor sediment, DS = Dry sterile sediment.

Treatment 2, with phylotypes SF1 and DEH10 added to fresh BH sediments, exhibited only *meta* dechlorination of PCB 151 through PCB 95 to PCB 53 (25-26), with little (less than 5 mol%) accumulation of PCB 95. On the other hand, there was limited *meta* dechlorination observed in Treatment 3 (*o*-17 plus DF-1), with only 9± 2.5 mol % of PCB 95 at day 300. The major product in these microcosms at day 300 was PCB 72 (25-35) at 44.4± 6.7 mol%. This pathway was the result of two subsequent *ortho* dechlorination steps, with PCB 92 as an intermediate. Some *meta*

dechlorination of the intermediate PCB 92 was observed, as PCB 52 (25-25) was present at 17.9 ± 5.1 mol % on day 300.

The most diverse set of dechlorinating activities of PCB151 was observed in microcosms containing all four phylotypes added to BH sediment (treatment 4), since there was no single PCB congener that was the major product. Although *meta* dechlorination of PCB 151 to PCB 53 (25-26) was detected at 25.8 ± 2.8 mol % on day 300, with little accumulation of PCB 95 (236-25) (less that 2 mol %), PCB 151 was mostly dechlorinated in the *ortho* position to PCB 92 (235-25). However, this intermediate did not accumulate (less than 5 mol %) since it was subsequently dechlorinated mostly in the *ortho* position to PCB 72 (25-35), which was present at 25.2 ± 3.3 mol % on day 300, but also in the *meta* position to PCB 52 (25-25), at 7.7 ±1.9 mol % on day 300.

We used dry, sterile BH sediment to study the effects on dechlorination without competition by the indigenous microbial community present in fresh BH sediments. Treatment 5, exhibited a longer lag time than the fresh BH sediment microcosms and we mainly detected *meta* dechlorination to PCB 53 (25-26) at 38.4 ± 0.8 mol % at day 300 with little accumulation (< 2 mol%) of the intermediate, PCB at 95 (236-25) at day 300. A negative control was added to study the effect of the addition of small amounts of PCB that was carried over with the cultures. Treatment 6, fresh BH sediment with autoclaved cultures added, exhibited mainly *meta* dechlorination of PCB 151 to PCB 95 (236-25), but PCB 95 was not further dechlorinated to PCB 53 (25-26), Figure 4.2.

4.4.3. Effect of bioaugmentation of the dechlorination patterns of Aroclor 1260

Aroclor 1260 is a mixture of PCB congeners and it is a common form of PCB contamination in the environment. All treatments dechlorinated Aroclor 1260 (Figure 4.3), as the total chlorines per biphenyl decreased in all treatments (Figure 4.3, panel A). Treatment 4, BH sediment with all added dechlorinators exhibited, the most extensive dechlorination, with the loss of 1.77 chlorines per biphenyl in 300 days, and treatment 2, BH sediment with SF1 and DEH10, had similar levels of dechlorination, with an average loss of 1.7 chlorines per biphenyl throughout the incubation. Treatment 5, dry BH sediment with all the dechlorinators, exhibited a dechlorination rate similar to the other the treatments but interestingly the dechlorination rate leveled off after 200 days and the treatment resulted in an average loss an of 1.17 chlorines per biphenyl after 300 days of incubation. The remaining treatments went from about 6.4 chlorines per biphenyl to about 5 chlorines per biphenyl after incubation.

The total loss of *meta* chlorines over time looked similar to the loss of total chlorines over time (Figure 4.3, panel B). However, there were differences in the loss of *ortho* chorines between the different treatments. Treatment 3, BH sediment with o-17 and DF-1, and treatment 6, BH sediment with autoclaved microorganisms dechlorinated PCB congeners in Aroclor 1260 in the *ortho* position to a greater degree compared to the rest of the treatments, with average losses of 0.24 and 0.21 *ortho* chlorines per biphenyl over 300 days respectively. In contrast, treatment 4, BH sediment with all added organisms, dechlorinated in the *para* position to a greater

extent than other treatments, however, this might be just a reflection of higher total dechlorination by this treatment.

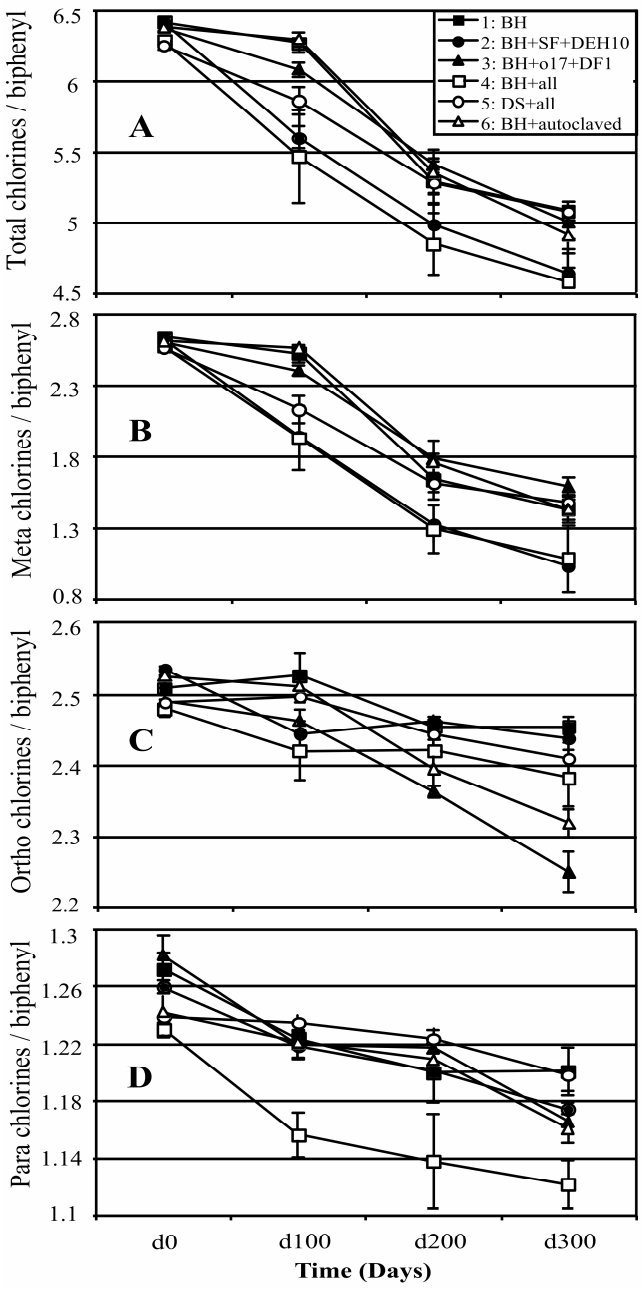


Figure 4.3. Dechlorination of Aroclor 1260 in microcosms with different treatments (see legend). BH = Baltimore harbor sediment, DS = Dry, sterile sediment. Total chlorines (panel A) per biphenyl and meta (panel B), ortho (panel C) and para (panel D) per biphenyl was calculated from triplicate cultures.

4.4.4. Effect of bioaugmentation on the dechlorinating microbial community

We performed DGGE analysis throughout the incubation to investigate whether the bioaugmented microorganisms continued to grow in the microcosm and had an impact on the indigenous microbial community in the microcosm. We used PCR primers targeting all known dechlorinators (42). Figure 4.4 shows a composite DGGE gel of the 16S rRNA gene community in different treatments over time.

Unexpectedly, all DNA extracts for Day 0 contained some substance inhibitory to PCR, which we never observed in the past using the exact same extraction protocol. We suspected that the extraction kit might have been faulty, and subsequent samples were extracted using a different extraction kit, that yielded uninhibited samples. We further purified Day 0 samples and although inhibition was still an issue, we were able to obtain products for DGGE.

We could detect a *Dehalococcoides* species in BH sediments without any addition of microorganisms (treatment 1) at day 0. However, this phylotype was distinct from DEH10 and was similar (472 identity over 478 bp) to another *Dehalococcoides* species, DHC ANAS (acc. number DQ855129). This phylotype was no longer detected after incubation with PCB 151. This was also the case in treatments 3 and 6. Two microorganisms were enriched in treatment 1, dechlorinating PCB 151, the first band at day 200 and 300 was identified as *o*-17 and second, lowermost band was identified as SF1. Thus it appears that both these organisms still inhabit BH sediments after being enriched from the same location (42). Similar DGGE patterns were observed in treatment 3, with *o*-17 and DF1, and treatment 6, with autoclaved microorganisms.

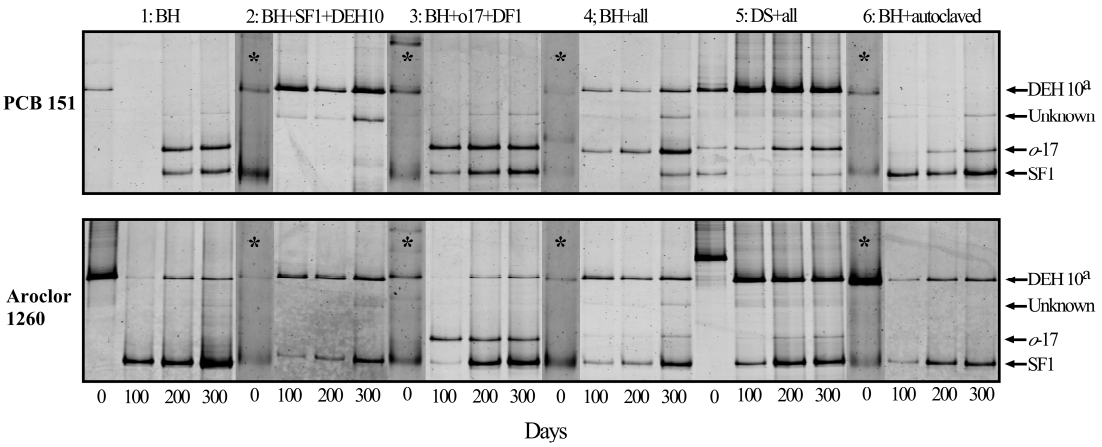


Figure 4.4. Composite DGGE of microcosms with different treatments. *= for some of the day 0 samples the DNA was subjected to PCR with 40 cycles and run on a separate gel from the rest of the time-points. a = the *Dehalococcoides* species in the day 0 samples was not DEH10 but another *Dehalococcoides* species which was 98.7 % identicalto DHC ANAS (DQ855129.1). Unknown = 97 % identical to uncultured Chloroflexi, clone VHS-B3-87 from Victoria Harbor (DQ294968.1). Bands that migrated above those of DEH10 are chimeras.

We could detect SF1 at day 0 in microcosms with SF1 and DEH10 (Treatment 2) dechlorinating PCB 151, but it was mainly DEH10 that was enriched. A second phylotype was enriched in this treatment and this phylotype was 97% (471/478) identical to an uncultured *Chloroflexi*, clone VHS-B3-87 from Victoria Harbor (DQ294968.1). We could also detect this uncultured *Chloroflexi* in treatment 3, 4 and 6, which were dechlorinating PCB 151 but the role of this phylotype is unclear. In treatment 4, with BH sediment with all dechlorinators added, four different phylotypes were enriched at day 300, with DEH 10 and *o*-17 appearing as predominant DGGE bands in the microcosms dechlorinating PCB 151 (Figure 4.4). In treatment 5, dried sediment with all the dechlorinators added, we could detect DEH10, *o*-17 and SF1 at day 0 and DEH10 and *o*-17 appeared to be enriched during incubation.

The DGGE patterns were different for microcosms dechlorinating Aroclor 1260. DEH 10 was enriched in all the microcosms although we could detect the other *Dehalococcoides* species, similar to DHC ANAS described above, in most of the day 0 samples. SF1 was also enriches in all the microcosms dechlorinating Aroclor 1260 and *o*-17 was only enriched in treatment 3, although we could detect faint bands representing *o*-17 in treatments 4 and 5. DF-1 was not detected in any samples.

4.4.5. Enumeration of putative dechlorinating microorganisms

Since DGGE is not quantitative, I also performed competitive PCR (cPCR) (Kjellerup, 2007, in preparation) with normalized DNA from the microcosms as described in Fagervold *et al.* (41) to investigate whether the dechlorinators increased in numbers throughout incubation. The number of 16S rRNA copies per µl normalized DNA (10 µg/ml) generally increased from about 1000 copies, to between 10^5 and 10^6 after 300 days, with small differences (Figure 4.5).

However, two treatments showed different numbers at day 0. Treatment 1, BH sediment alone and treatment 5, dry sediment with all the dechlorinators, exhibited elevated numbers of putative dechlorinators at day 0 in both PCB 151 and Aroclor 1260 incubations. In the microcosms with Aroclor 1260, the numbers were greatly elevated in treatment 1 and 5, starting at 4 x 10^6 16S rRNA gene copies per µl. However, the number of putative dechlorinators in these samples did not increase during incubation and at day 300, the numbers of putative dechlorinators in these treatments were similar to the other treatments.

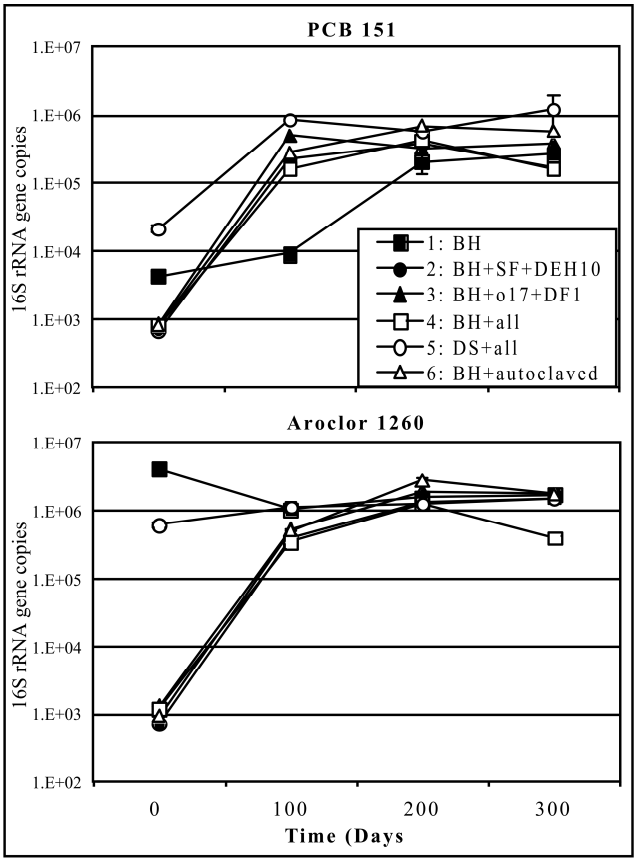


Figure 4.5. Enumeration of PCB dechlorinators by competitive PCR over time in microcosms dechlorinating PCB 151 and Aroclor 1260. Number of 16S rRNA gene copies throughout incubation. BH = Baltimore Harbor sediment, DS = Dry sterile sediment.

As explained above, we had some problems with the day 0 samples and these issues may have interfered with the enumeration of the day 0 samples. There does not seem to be any failure in cPCR protocol, since the DGGE results are consistent with the cPCR results. Also, inadvertent addition of extra amount of dechlorinators seems unlikely, since we see the same results in both PCB 151 and Aroclor microcosms. Dry sediment might not absorb the DNA to same extent as the wet sediment. Thus, DNA extraction might be more efficient in dry sediment microcosms. However this does not explain why we see elevated amounts of putative

dechlorinators in BH sediment alone, treatment 1. If one assumes that 4 x 10^6 cells per 1µl of DNA were present (Figure 4.5) it would mean that the microcosms would have at least 1.3 x 10^8 putative dechlorinators per ml of cultures at day 0. This is highly unlikely, since this would have to come from 1.5 grams of sediment. The 1.5 grams of sediment would then have to contain 8.8 x 10^8, almost 10^9, putative dechlorinators per gram. As a comparison Schippers and Neretin (111) reported maximum numbers of 10^8-10^{10} 16S rRNA gene copies of prokaryotes in marine sediments (per cm^{-3}). Putative dechlorinating *Chloroflexi* in numbers as high as 10^9 is an unreasonable number based upon prior studies that shows putative dechlorinators are only a minor fraction of the microbial community of sediments (97). Also, fresh BH sediments were added to all the other microcosms, except treatment 5, so we should have seen the same number in the other treatments. It is therefore clear that these numbers are artifacts.

4.4.6. Changes in the microbial community in relation to dechlorination patterns

It is clear that the addition of the different microbial populations had an effect on both the dechlorination pattern and the microbial community over time (Figures 4.2 and 4.4) despite the fact that level of PCB 151 was similar for all treatments. In all treatments where SF1 and DEH10 were added (treatments 2, 4 and 5), DEH 10 was further enriched and there was a complete *meta* dechlorination of PCB 151 (2356-25) to PCB 53 (25-26). However, in microcosms where DEH10 was not enriched (treatments 1, 2 and 6), I observed an accumulation of PCB 95 (236-25). I

had previously (41) (Chapter 3) hypothesized that DEH10 is responsible for the *meta* dechlorination of both PCB 151 to PCB 95 and further to PCB 53. However, in contrast to previous results, the initial *meta* dechlorination of PCB 151 to PCB 95 did not appear to be dependent on DEH10.

We did not see any *meta* dechlorination of PCB 151 in treatment 3 microcosms, but we did observe some *meta* dechlorination of PCB 92 (235-25) to PCB 52 (25-25), which did not occur in treatment 1 microcosms with BH sediment alone. We did, however, observe extensive *ortho* dechlorination of PCB 151 in two subsequent steps to PCB 72 (25-35) in microcosms with both of these treatments. We have previously hypothesized that *o*-17 is responsible for the *ortho* dechlorination of PCB 151, but is unknown if *o*-17 or SF1 is responsible for the second *ortho* dechlorination reaction. We could detect less *o*-17 in the treatment 6, with autoclaved organisms, and also less *ortho* dechlorination, which suggests that *o*-17 might be responsible for the *ortho* dechlorination of PCB 151.

We observed that fresh BH sediment also was the source of SF1 and *o*-17, since both treatment 1 and 6 enriched for these microorganisms. DF-1 did not survive incubation with fresh or dry BH sediment. However, the addition of *o*-17 did have an effect, which can be seen from differences in both the dechlorination pattern and the microbial community in treatment 2 and 4 (Figure 4.4). In treatment 4, we observed a more diverse set of dechlorination activities and a more diverse microbial community of putative dechlorinators than in treatment 2. In treatment 2, we observed that DEH 10 dominated the community of putative dechlorinators, so this phylotype appeared to survive. DEH 10 also appeared to be the most predominant

dechlorinator in treatment 5, although we could also detect the other phylotypes at day 300.

In microcosms with Aroclor 1260, the microorganisms have a mixture of different congeners as substrates. It is therefore not surprising that different microorganisms will be enriched. Phylotypes DEH 10 and SF1 were enriched in all microcosms. This is similar to previous results where we showed that these two phylotypes were responsible for most of the dechlorination activities of Aroclor 1260 (41). The addition of SF1 and DEH10 to fresh BH sediment did have an effect as we saw more extensive dechlorination in these microcosms (Figure 4.3). PCB congeners in Aroclor 1260 were also dechlorinated in the *ortho* position to a greater extent (Figure 4.3, panel C) when *o*-17 was added further showing that the addition of *o*-17 and DF-1 did indeed have an effect. We added approximately $2x10^5$ cells to 10 ml, for a final concentration of $2x10^4$ cells per ml. As a comparison, in a bioaugmentation study with KB-1 (77), a mixture of *Dehalococcoides* species (38) used for bioremediation of chlorinated ethenes, the authors used added approximately 3×10^6 cells to a microcosms of 210 ml, for a final concentration of about $1.4x10^4$ cells per ml. This is a similar concentration to what we used.

4.4.7. Did bioaugmentation work?

The results show that bioaugmentation might be a feasible strategy for PCB dechlorination, but more research is needed. The addition of all the dechlorinators decreased the lag time before dechlorination occurred, and we saw a more diverse range of dechlorination activities. However, there were some inconsistencies with

our experiment that are difficult to explain, especially the cPCR results. Another challenge is that the only electron acceptors for these microorganisms are chlorinated compounds. Effective bioaugmentation or biostimulation will require scale-up in batch reactors or *in situ* by the addition of electron acceptors by non-chlorinated compound that are not themselves considered environmental contaminants. DF-1 has been shown to grow with PCE and TCE (82) and this could be a possible method for culturing since these are volatile compounds and can be removed by gas sparging prior to use for bioaumentation. Another limitation is that the 16S rRNA profile of a community does not necessarily predict the activity. As shown with several *Dehalococcoides* species (37, 50, 56), predicting physiology from phylogeny is not always accurate. Although likely, we do not know if the same limitations apply to the *o*-17/DF-group.

Clearly, more research is required before bioaugmentation can be used as a strategy for the bioremediation of PCB contaminated sites. However, I have shown that the addition of PCB dechlorinating microorganisms reduced the lag time and increased the overall extent of dechlorination of Aroclor 1260. The dechlorination patterns were different, and it appears that microorganism DEH10 used for bioaugmentation can successfully compete with the indigenous microorganisms.

Chapter 5: Putative reductive dehalogenases in DF-1

5.1. Abstract

Degenerate PCR primers that target putative reductive dehalogenases similar to those found in the *Dehalococcoides* group were used to identify putative polychlorinated biphenyl (PCB) reductive dehalogenases from phylotype DF-1. Phylotype DF-1 is able to dechlorinate PCBs as well as pentachlorobenzene and chlorinated ethenes. Several putative reductive dehalogenase genes were identified in DF-1 by using published degenerate PCR primers and these genes appear to cluster with putative reductive dehalogenase genes from the *Dehalococcoides* group, even though they are only about 90% identical according to the 16S rRNA gene. Specific primers were designed to target each of the specific putative reductive dehalogenases we identified, and these were used to study differential expression when DF-1 was dechlorinating PCB, pentachlorobenzene and tetrachloroethene (PCE). Very small differences in expression of these genes were observed between the treatments using the specific primers. I made cDNA expression libraries using the same published degenerate PCR primers and several other putative reductive dehalogenases were identified, but I did not perform further expression studies with these. More work is needed to design additional primers and conduct quantitative RT-PCR on the same mRNA that was used in this study.

5.2. Introduction

Halorespiration has been associated with members of several phylogenetic groups including the δ- and ε- *protebacteria,* low G+C Gram positive bacteria and the deeply branching *Dehalococcoides*-like phylotypes within the *Chloroflexi*. These organisms can generally use chlorinated ethenes and some chlorinated phenolic compounds as electron acceptors (113). One such organism, *Desulfomonile tiedjei,* was the first dechlorinating bacterium shown to couple reductive dechlorination to energy conservation and growth (36). Enzymes mediating this transfer of electrons are called reductive dehalogenases. Several reductive dehalogenase genes have been sequenced, the enzymes purified and activity tested. Examples are tetrachloroethene reductive dehalogenase (PceA) from *Dehalospirillum multivorans* (91), chlorophenol reductive dehalogenase (CprA) from *Desulfitobacterium dehalogenans* (122), trichloroethylene reductive dehalogenase (TceA) from *Dehalococcoides ethenogenes* (74) and vinyl chloride reductive dehalogenase (VcrA) from *Dehalococcoides strain VS* (86).

Most of these enzymes contain iron-sulfur clusters and a corrinoid as cofactors. Generally, reductive dehalogenases also contain a twin arginine signal sequence that is thought to be involved in transporting the protein across the cytoplasmic membrane (Figure 5.1). Their catalytic units are about 60 kDa and appear to be membrane associated. Despite their physiological similarities, the reductive dehalogenases have a low overall sequence similarity. However, some regions are conserved, including the region around the TAT signal peptide and the two-iron sulfur clusters and degenerate PCR primers were designed based on these

regions (65, 127) (figure 5.1). A protein that appears to be co-transcribed with reductive dehalogenases has been identified and is thought to act as a membrane anchor for the catalytic subunit.

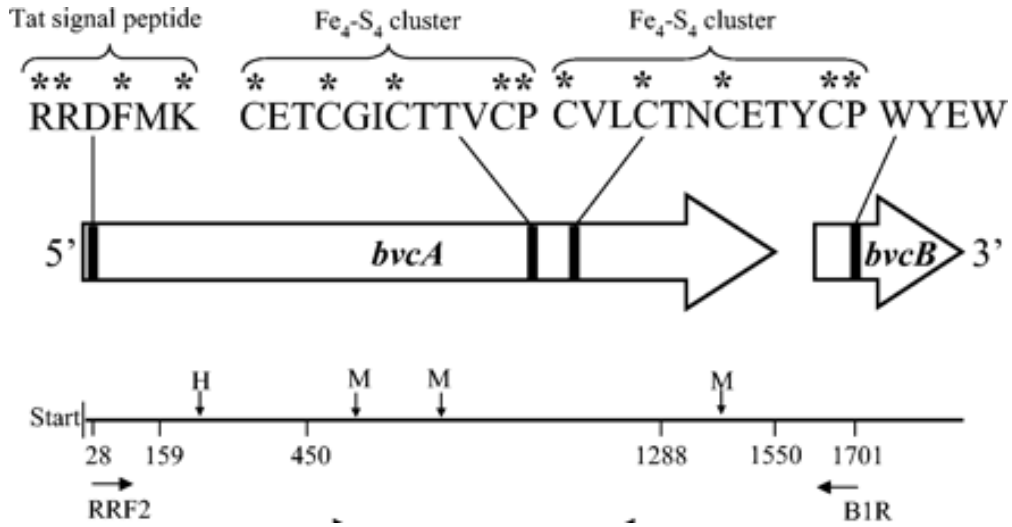


Figure 5.1. Arrangement of the *bvc* gene cluster (65). BvcA is the gene for the catalytic subunit while bvcB is thought to be a membrane anchor. Also shown are the conserved reductive dehalogenase features including the TAT signal peptide and the two-iron sulfur clusters. RRF2 and B1R are the primers that were developed based upon these conserved regions

Only a few microbial reductive dehalogenases within the *Dehalococcoides* group have been identified through biochemical methods because of difficulty in culturing adequate amounts of cell material for protein purification (58, 74). These include the PCE reductive dehalogenase that dechlorinates perchloroethene (PCE) to trichlororethene (TCE) and the TCE- reductive dehalogenase (*Tce*A) (74) that dechlorinates TCE to ethene in *Dehalococcoides ethenogenes* strain 195 (75). Reductive dechlorination of chlorobenzenes has also been identified in cell extracts of CBDB1, but the reductive dehalogenase(s) has not been purified (58). Other *Dehalococcoides* species contain several putative reductive dehalogenase

homologues, including BAV 1 with 7 homologues (65), CBDB1 with 32 (67) homologues and FL2 with 14 homologues (57).

To date, a PCB reductive dehalogenase has not been identified. *Dehalococcoides ethenogenes* has been shown to co-metabolically dechlorinate PCB (45). The *Dehalococcoides ethenogenes* 195 genome has been sequenced (112), and Villemur *et al.* (126) found 17 putative reductive dehalogenase genes based on comparative sequence analysis of identified open reading frames (orfs). However only one reductive dehalogenase gene encoding trichloroethene reductive dehalogenase (*tce*A, DET0079) has been functionally confirmed based on activity (74). Although the other putative reductive dehalogenases might be involved in the dechlorination of PCB congeners, this has not been examined.

The goal of this study was to conduct comparative sequence analyses of putative reductive dehalogenases in bacterium DF-1 with those identified in other dechlorinating species, especially microorganisms within the *Chloroflexi* group. By comparing the putative reductive dehalogenases of DF-1 and *Dehalococcoides* spp. one might be able to determine whether these are conserved among dehalogenating species. This will provide some indication of how well reductive dehalogenases are conserved between these disparate groups of dehalogenators (82). My goal was to tentatively identify the functional role of the putative reductive dehalogenases and possibly identify a PCB reductive dehalogenase by comparing the relative expression of putative reductive dehalogenases during growth of DF-1 on PCE, pentachlorobenzene and PCB.

5.3. Materials and methods

5.3.1. Growth of cultures

DNA from DF-1 co-culture dechlorinating 2,3,4,5-CB (137) (132) was used for the first clone library to identify putative reductive dehalogenases in DF-1. This organism was originally enriched from Charleston Harbor (SC) sediment (136). DF-1 cells used for mRNA extraction were grown in 3- liter cultures with PCB, pentachlorobenzene or PCE by G. Miller in the laboratory of Dr. Hal May at the Medical University of South Carolina.

5.3.2. Clone libraries

The putative reductive dehalogenase gene clone library (n=96) was generated using PCR products generated with primers RRF2 and B1R as described in Krajmalnik-Brown *et al.* (65) with DNA from a dechlorinating co-culture with DF-1. Fragments were ligated into pCR2.1 using the TA Cloning® Kit (Invitrogen, Carlsbad, CA). The library was screened using the same primers (RRF2 and B1R) followed by restriction fragment length polymorphism analysis with restriction endonucleases *Msp*I and *Hha*I (65). Digestion products were discriminated by gel electrophoresis on a 3% (wt/vol) Trevigel at 25V for 3 hours at 4^{o}C. Three other clone libraries were later created as described above, except the source of the template was cDNA from mRNA of DF-1 dechlorinating PCB, pentachlorobenzene and PCE.

5.3.3. Design of primers specific for reductive dehalogenases from DF-1

To specifically target putative reductive dehalogenases in DF-1, different primers sets were designed using Probe Design in the ARB software package (128). The primers were checked for compatibility and possible self-annealing using Primer Express (Applied Biosystems, Foster City, CA) and the products were only approximately 200 basepairs long, which is an optimal length for conducting quantitative PCR (qPCR) at a later time. These primers were checked for specificity and PCR conditions were optimized. PCR conditions were as follows: initial melting at 94ºC for 30 seconds, 30 cycles of 94ºC for 30 seconds, 60ºC for 25 seconds and 72ºC for 30 sec.

5.3.4. Messenger RNA extraction and reverse transcription PCR

Messenger RNA was extracted from cultures grown with PCB, pentachlorobenzene and PCE. The cultures were grown with titanium (III) nitrilotriacetric acid (TiNTA) and most of the harvested pellet consisted of precipitate. The mRNA protocol was optimized to harvest DNA from cells embedded in the precipitate. A 3-liter culture was aliquoted into 250 ml Oakridge centrifugation bottles and centrifuged for 20 minutes at 15,000 rpm. Each pellet was transferred to a 1.5 ml eppendorf tube into an equal volume of RNA*later*® (Ambion, Austin, TX) and the samples were immediately frozen with dry ice and stored anaerobically at -20 ^{o}C until used. Prior to use for RNA preparation, the pellets were slowly defrosted on ice, centrifuged at 18.000 x g at room temperature for 20 min. The pellets were pooled in one 1.5 ml eppendorf tube and resuspended in 150 µl TE,

150 µl phosphate buffer and 150 µl SDS, transferred to a 1.5 ml screw cap tube with silica beads (97), then incubated on ice for 10 minutes. The tubes were subjected to bead beating with a Fastprep Cell Disruptor (Qbiogene, Carlsbad, CA) for 20 seconds at speed 5.5. This was followed by two extractions with 150 µl phenol chloroform (1:1) followed by one extraction with 150 µl chloroform. NaCl (5M, 7 µl) was added before the mRNA was precipitated with 600 µl 100% isopropanol for 1 hour on ice. The sample was centrifuged at 15,850 x g for 20 minutes at 4 ^{o}C and the pellet was washed once with 70% ethanol. The pellet was air dried for 30 minutes and resuspended in 100 µl DEPC treated water. The mRNA was further purified with RNeasy (Qiagen, Valencia, CA) without DNAase treatment followed by treatment with DNAase (TURBO DNA-free, Ambion) according to the manufacturer's instructions.

For reverse transcription of mRNA, an Access RT-PCR system (Promega, Applied Biosystems) was used according to the manufacturer's instruction. The master mix consisted of 1µl primer, 6 µl RNA, 2 µl dNTPs and 3 µl water and the reaction was carried out for 1 hour and 20 minutes at 50 °C. cDNA was then used as a template for PCR amplification as described above, with either the specific putative dehalogenase primers or the degenerate putative dehalogenase primers RRF2 and B1R as described in Krajmalnik-Brown *et al.* (65).

5.3.5. Sequence and phylogenetic analysis

The fragments that were present two or more times in the clone library were sequenced using Big Dye 3.1 kit (Applied Biosystems) and an ABI 3100 (Applied

Biosystems). The sequences were examined for errors and assembled using the software Pregap4 and Gap4 of the Staden software package (http://sourceforge.net/projects/staden). The "find stop codons" tool in Gap4 was used to identify stop codons in the sequences and only the open reading frames of the catalytic subunit of the putative reductive dehalogenases from DF-1 were extracted. A BLAST search was performed to identify all reductive dehalogenases present in GenBank as of April 2005. The DNA sequences of 85 putative reductive dehalogenases were extracted from GenBank and imported into ARB software package (128). Once in ARB, the sequences were translated into proteins and aligned with clustalW (54) using the BLOSUM weighting matrix (53). Putative dehalogenase sequences were excluded from the alignment if they were too different and or truncated, which left 84 sequences remaining for phylogenetic analysis. A second alignment was conducted with putative and confirmed dehalogenases from *D. ethenogenes, D. CBDB1* and DF-1, which resulted in a total of 45 candidates. A filter was created using the "filter by base frequency" tool in ARB that excluded positions in the alignment where gaps were more frequent than characters and positions with ambiguous characters. This resulted in 383 positions for the phylogenetic tree based upon the 85 sequences and 516 positions were used for a phylogenetic tree based on the 45 sequences. Phylogenetic trees were generated using the neighbo joining (110) algorithm in the ARB software. Bootstrap analyses (100 replicates) were performed using the PHYLIP package (44).

5.4. Results and discussion

5.4.1. Initial clone library from DF-1 DNA

Primers for the putative reductive dehalogenases developed by Krajmalnik-Brown and co-workers (65) were used for identifying a putative vinyl chloride reductive dehalogenase (BvcA) in a *Dehalococcoides* species (Figure 5.1). These same primers were used to generate a clone library with primers RRF2 and B1R, which resulted in 14 different fragments with unique RFLP patterns that were present in the clone library (Table 5.1). The most predominant pattern was pattern 3 (Pat 3, Table 5.1) that was present with 11 fragments in the clone library.

Table 5.1. Frequency of different fragments in the DNA clone library of DF-1 as identified with RFLP analysis.

Name	Frequency of fragments in clone library
Pat 1	3
Pat 2	2
Pat 3	11
PatF3	3
Pat 4	8
Pat 5	4
Pat 6	7
Pat 7	5
Pat 8	5
Pat 9	6
Pat 11	3
Pat 12	6
Pat 13	5
Pat 15	3

5.4.2. Phylogeny of putative reductive dehalogenases from DF-1

The open reading frame of the putative reductive dehalogenases was identified and only subunit A was imported into ARB, translated and aligned with other known reductive dehalogenase sequences (Figure 5.2). The bootstrap values shown at each node are relatively low for some major branching points. The reductive dehalogenases from the low G+C gram positive microorganisms cluster together, which includes *cpr*A and *pce*A from *Desulfitobacterium* and *Dehalobacter* species. However, two putative reductive dehalogenases from *Dehalococcoides ethenogenes* cluster together with this group. One of the reductive dehalogenases genes, *pce*A from *Desulfitobacterium hafniense*, has been reported to be within a transposable element.

Four of the reductive dehalogenases from *Dehalococcoides ethenogenes* are also located in transposable elements (DET0079, DET0162, DET0876 and DET1559). These appear to be scattered throughout the phylogenetic tree, so it is difficult to infer clear relationships. However, it was proposed that the reductive dehalogenases were laterally transferred in an ancestor of the *Dehalococcoides* spp. (57), which may explain why the putative reductive dehalogenases are scattered throughout the tree. The reductive dehalogenases from DF-1 are also scattered throughout the phylogenetic tree. The 16S rRNA gene of DF-1 is only 90% identical to *Dehalococcoides* spp., and this might suggest that this common ancestor was even more ancient than previously hypothesized. Another possibility is lateral gene transfer within the *Chloroflexi.* I chose the *pce*A from *Dehalobacter restricus* as the root of the tree because it is least similar to other reductive dehalogenases (76).

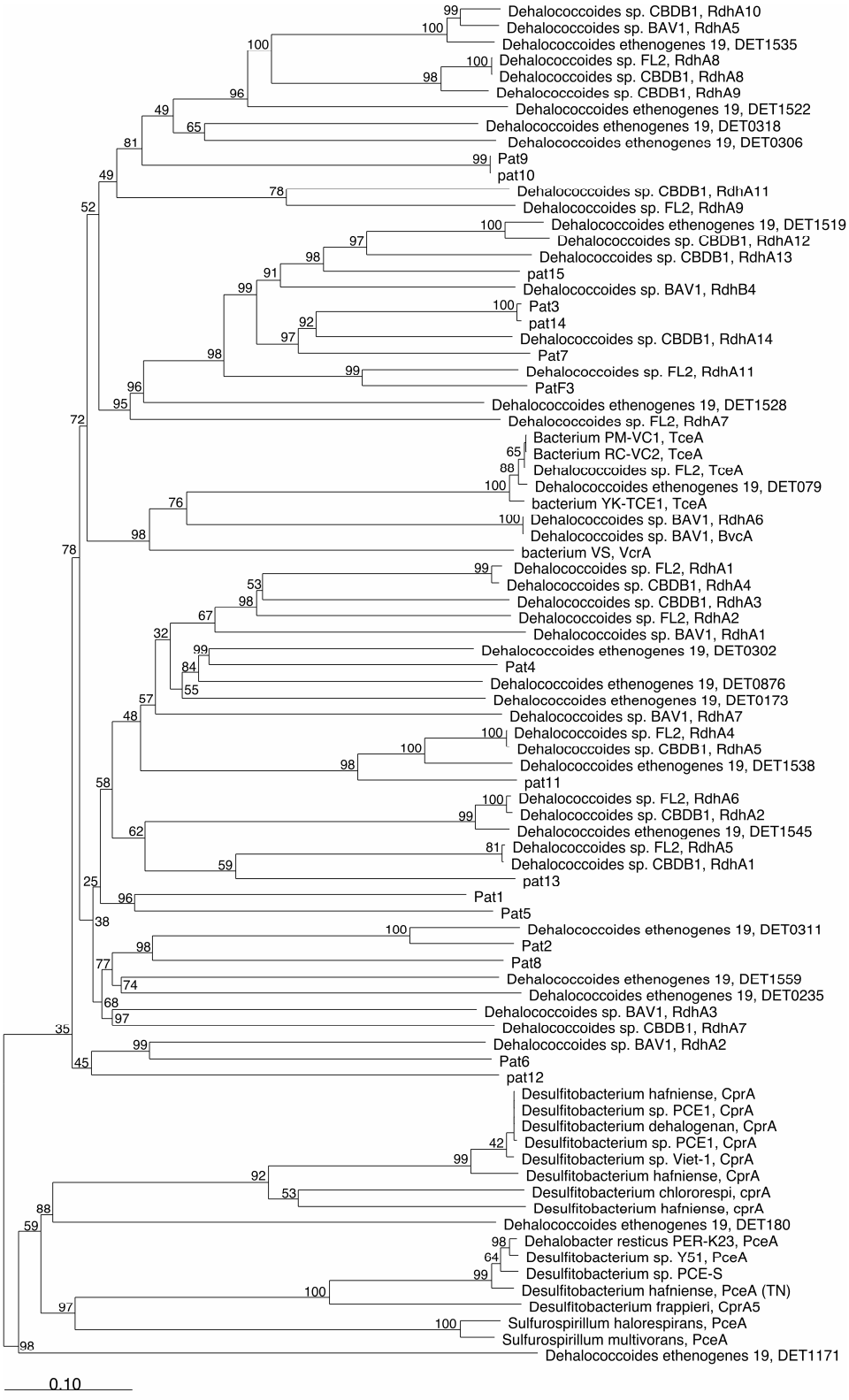


Figure 5.2. Neighbor joining phylogenetic tree of different reductive dehalogenases. Bootstrap analysis was performed using the PHYLIP package with 100 replicates. The scale bar indicates 0.1 substitutions per amino acid position. The root (not shown) was PceA from Dehalobacter restricus.

Figure 5.3 shows a phylogenic tree of all the reductive dehalogenases from *Dehalococcoides ethenogenes* 195 (labeled DET), *Dehalococcoides* CBDB1 (RdhA) and DF-1. Again, the bootstrap values are not high, but this is consistent with other phylogenetic trees of putative reductive dehalogenases in the literature (57). There are no obvious relationships between the reductive dehalogenases.

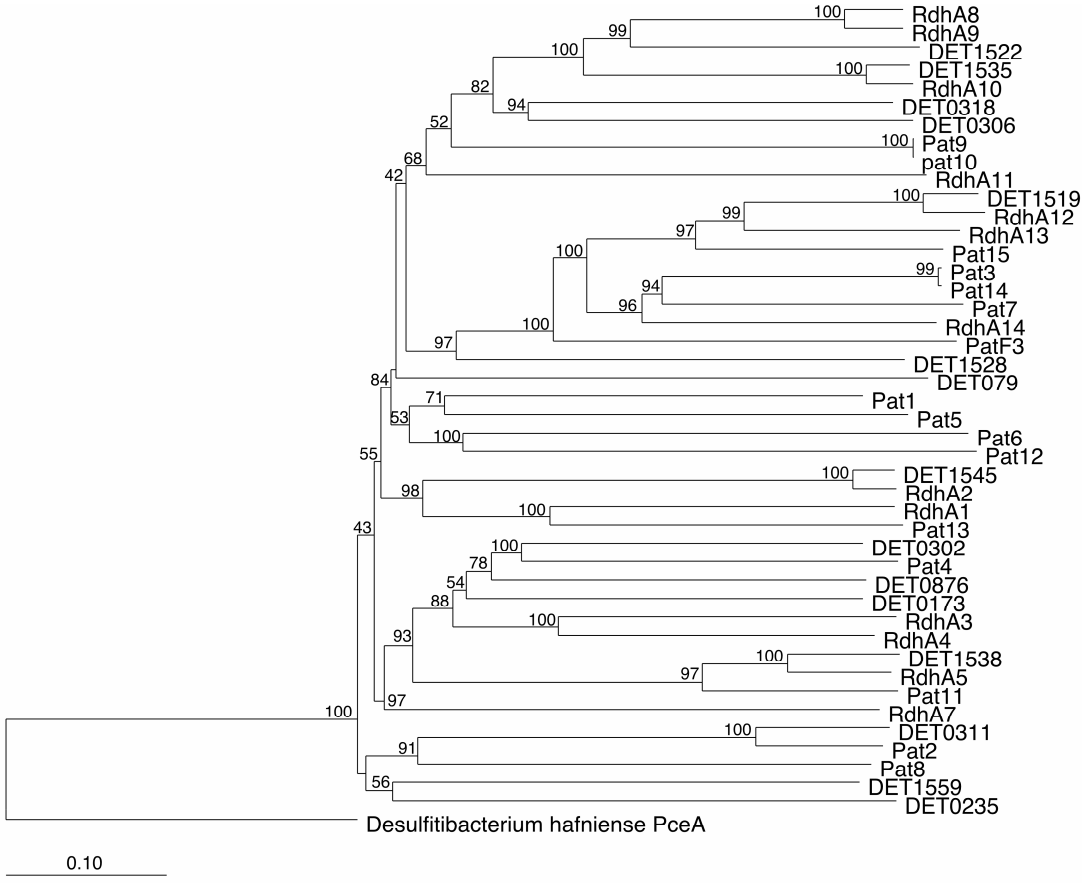


Figure 5.3. Neighbor joining phylogenetic tree of different reductive dehalogenases from *Dehalococcoides ethenogenes* 195 (marked only DET), *Dehalococcoides* CBDB1 (RdhA) and DF-1 (Pat). Bootstrap analysis was performed using the PHYLIP package with 100 replicates. The scale bar indicates 0.1 substitutions per amino acid position.

5.4.3. Expression of putative reductive dehalogenases from DF-1 dechlorinating different chlorinated compounds

Primers specific for each dehalogenases were developed (Table 5.2).

Table 5.2. Primers specific for putative DF-1 reductive dehalogenases. The different patterns refers to RFLP patterns from the clone library.

Pattern	Forward primer	Reverse primer
1	ATGGTTCAATGAGCGGTGCT	TTCCGGAGAGGTGATTGCAT
2	ATCTTATGCCCGCTCAATCG	AAGAAGACTTCGGCTTTGCG
F3	CATGGTGCGTGCTGCTTTAA	TGGTTGCCCCCATGTTATGT
3	GAGGCGTCCTTTTTCTGGAA	TGATAATGCTACCTGCCCCA
4	ATAGTGCGTCTCCGGCATTT	TTCTCTCCGGACCCATCAAT
5	TTAACCAGAAATGGCCCTGC	AGGATTCATGCGGCTAGCTT
6	TTGCCCATTTAACCACCTCC	TCTCACCGAAACCGAGAAGA
7	AAATTACAACCGGCAAACGC	AAGCCTCAGCACACATTCCA
8	TTGGTCTACAGCCTTTGCGT	CTAATTGCCCAAAGCCCACT
9	GACAATGAGGACCCGGAATT	TCCGCCAAAATAACGAACTG
11	TTGCAATCATGTCCCTTCCC	TCTGCACATTTGCGACAGCT
12	TCACCGAAGAAGTTGCATGG	ATCATAACAAGCCGCTTCCG
13	AGCTTGGCATACCTGCGTTT	TTTCGCTGTCAGAAGCAGGA
15	GCCGCCACATCATAATTGAC	CCGCGAATAAACTGCTCAAA

To study the expression of putative reductive dehalogenases, mRNA was extracted from cultures dechlorinating PCB and pentachlorobenzene, and reverse transcription was preformed as described in materials and methods. PCR with the specific PCR primers was performed and there were some differences in the expression of putative reductive dehalogenases responsible for pattern 13 and 15 when DF-1 was dechlorinating PCB, pentachlorobenzene or PCE (Figure 5.4). The bands were slightly more intense when DF-1 was grown with PCB. However these differences were slight and should be confirmed with quantitative PCR to determine whether the differences are significant. Another major finding, however was that not

all the putative reductive dehalogenases appeared to be expressed with either of these substrates. The putative reductive dehalogenases that seemingly were expressed the most, were the ones that were responsible for RFLP patters 7, 9, 13 and 15. However, this also needs to be verified with q-PCR.

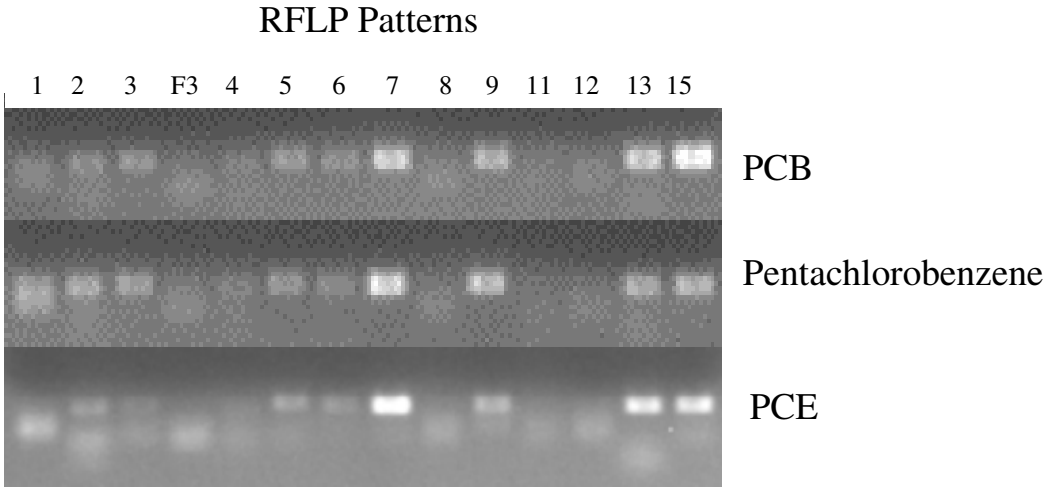


Figure 5.4. Expression of different putative dehalogenases in DF-1 dechlorinating PCB, Pentachlorobenzene and PCB as verified by mRNA extraction and RT-PCR. Each panel shows a agarose gel with PCR products using specific primers for the different putative reductive dehalogenases.

Another approach was tested using a clone library of the mRNA itself (Figure 5.5) to further study possible differences in the expression of different putative reductive dehalogenases when DF-1 was grown on PCB, PCE and pentachlorobenzene. Extraction of mRNA was performed as previously described and reverse transcription was performed with random hexamers. The same putative reductive dehalogenase primers were used with the PCR conditions described in Krajmalnik-Brown *et al.* (65). The clone library was screened as described above. Even though patterns from both the clone library (Figure 5.5) and the PCR-generated

fragments showed that sequences 5, 7, 9, 13 and 15 were differentially expressed based on frequency of detection in the clone library, there were some discrepancies between the clone library and PCR results. However, both these experiments showed that pattern 13 and 15 were expressed at higher levels in DF-1 dechlorinating PCB and PCE. Several additional putative reductive dehalogenases were also identified in the clone library from cDNA.

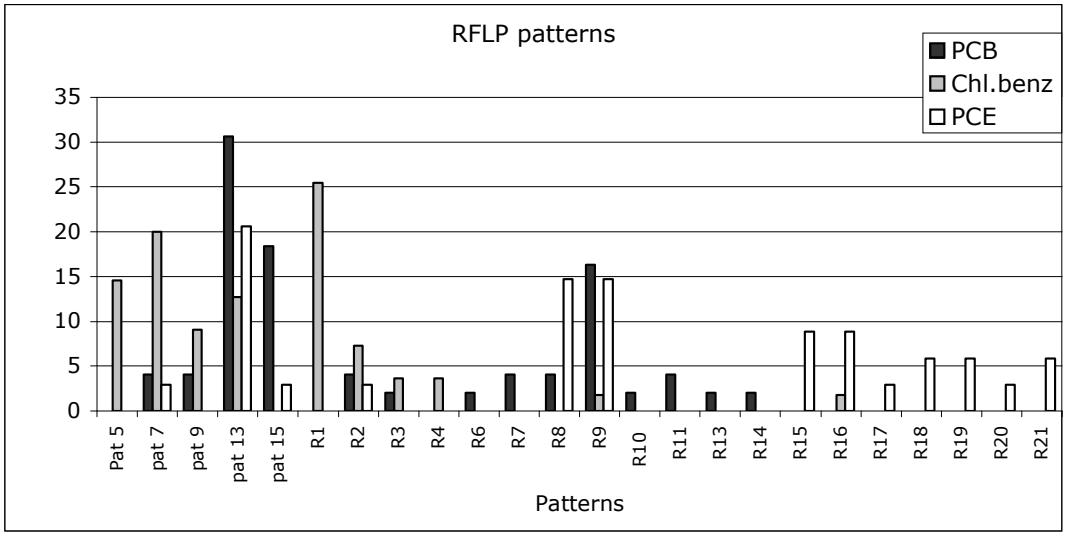


Figure 5.5. Distribution of different patterns in clone libraries produced from mRNA of DF-1 cultures dechlorinating PCB, pentachlorobenzene and PCE.

5.4.4. Conclusions and future perspectives

In conclusion, we have shown that DF-1 has several putative reductive dehalogenase genes with varying degrees of sequence similarity to putative reductive dehalogenase genes within the *Dehalococcoides* group. Indeed figures 5.2 and 5.3 show that the putative reductive dehalogenases from DF-1 cluster with putative reductive dehalogenases from the *Dehalococcoides* group although they are only about 90% identical based on the 16S rRNA gene sequence. Expression studies with mRNA from DF-1 grown with PCB, pentachlorobenzene and PCE showed that there were some differences in expression between the putative reductive dehalogenases in these cultures, and this suggests that these genes might be regulated in response to substrates. Although Krajmalnik-Brown *et al.* (65) could identify a vinyl chloride reductive dehalogenase gene (*bvcA*) using similar experiments, others have shown that multiple reductive dehalogenases are expressed during dechlorination of various chlorinated ethenes in KB-1, a mixed culture of *Dehalococcoides* species (129). Although most of the putative reductive dehalogenases in *Dehalococcoides ethenogenes 195* appear to be regulated by a two-component signal transduction system that might respond to intracellular stimuli, the one identified dehalogenase, TceA, does not seem to be regulated this way (112). In contrast, it has been shown that the expression of reductive dehalogenase genes *tceA* and *vcrA* were induced by the addition of TCE, cDCE and VC (70), but the transcript numbers and activity did correlate. It is yet unknown whether PCB reductive dehalogenases are differentially regulated or constitutively expressed, but these results suggest that some putative reductive dehalogenases are constitutively expressed, while others might be induced.

Chapter 6: Discussion and future perspectives

6.1. Microorganisms influencing the pattern of dechlorination of PCBs

In an attempt to investigate the factors that drive the specific dechlorination patterns observed for Aroclor 1260 the major dechlorination pathways of Aroclor 1260 in Baltimore Harbor microcosms were identified (Fig. 3.2). In these microcosms, most of the dechlorination occurred in the *meta* position and the final products were mainly tetrachlorinated biphenyls with unflanked chlorines. The specific rates of dechlorination for each of the dechlorination steps were compared to various chemical properties of each of the PCB congeners, but a clear relationship was not identified, which suggests that the specific pathways were biologically mediated. In order to identify the microorganisms responsible for the dechlorination pathways, we developed new PCR primers based upon the 16S rRNA of known PCB dechlorinating microorganisms *o*-17 (33), DF-1 (137) and *Dehalococcoides* (45). In conjunction with Denaturing Gradient Gel Electrophoresis, we showed for the first time that a member from both the *o*-17/DF-1 group, called SF1, and a member from the *Dehalococcoides* group, called DEH10, were needed to dechlorinate 2,2',3,3',4,6'CB (PCB 132) (Chapter 2).

The microorganisms responsible for each of the major dechlorination pathways in Aroclor 1260 were identified, and unexpectedly, only a few phylotypes appeared to be involved in the dechlorination of Aroclor 1260 (Chapter 3). Microbes in Baltimore Harbor sediment microcosms preferentially dechlorinated double-flanked chlorines, and most of this double-flanked dechlorination was catalyzed by

phylotype SF1, which is most similar to strain *o*-17. Generally, phylotype SF1 dechlorinated all 2345-substituted chlorophenyl rings preferentially in the double-flanked *meta* position, and 2356-, 236- and 235-substituted chlorophenyl rings in the *ortho*-flanked *meta* position when the other ring contained 245- or 24-substitutions. Phylotype *o*-17 dechlorinated the only 2346-substituted chlorophenyl ring tested (PCB 183) in the double-flanked *meta* position and the 2356-substituted chlorophenyl ring of PCB 151 in the *ortho* position. This is similar to previous reported activities for this phylotype (33, 80). Finally, phylotype DEH10, which belongs to the *Dehalococcoides* group, dechlorinated double-flanked chlorines in 234-substituted chlorophenyl rings. DEH10 also showed a preference for *para*-flanked *meta* chlorines when no double-flanked chlorines were available. Combined, these results indicate that the majority of PCB congeners can be anaerobically dechlorinated to congeners susceptible to aerobic dechlorination.

By identifying the specific dechlorination patterns and rates for different congeners in Aroclor 1260 (Chapter 3), it is now possible to generate models to predict the *in situ* dechlorination potential of sediments. This could be achieved by analyses of the congener distribution combined with enumeration of total PCB dehalogenating populations in specific PCB impacted sites. Finally, I found that dechlorination of Aroclor 1260, as well as most of the individual congeners, was linked to growth of the dechlorinating microorganisms. This suggests that PCB-impacted environments can sustain populations of PCB dechlorinating organisms, which is particularly important for the development of biostimulation or bioaugmentation strategies for PCB bioremediation.

6.2. Implications for bioremediation

Addition of specific organisms to Baltimore Harbor sediments (Chapter 4) showed that bioaugmentation might be a tractable strategy for PCB contaminated sites. The addition of different dechlorinating microorganisms to Baltimore Harbor sediment microcosms had an effect on the pattern of PCB dechlorination in two respects: the lag time before which PCB dechlorination occurred decreased, and the extent of dechlorination of Aroclor 1260 was more extensive in bioaugmented microcosms. However, before bioaugmentation can be used, approaches must be developed to stimulate growth for mass culturing inoculum. DF-1 grows very slowly in isolation, and this is also true for several *Dehalococcoides* species (50, 51, 112), and indeed, the KB-1 culture commercially used for bioremediation is not a pure culture (37). Both DF-1 and *o*-17 are co-enriched with sulfate reducing bacteria and there seems to be a factor that these organism need for growth that they cannot make themselves. Further understanding of the relationship between the dechlorinators and the environment is needed. Also, dechlorinating microorganisms have yet to be enriched on electron acceptors other that chlorinated substrates. Since most chlorinated compounds are toxic to humans and are an environmental concern, more work on finding alternative electron acceptors should be performed, particularly if the final goal in bioaugmentation is to add these cultures back into the environment.

6.3. Bioremediation monitoring

Monitoring PCB dechlorinating microorganisms *in situ* is critical for assessing the effectiveness of bioremedial approaches in the environment. Molecular monitoring is a more rapid approach than activity assays that can take hundreds of days and it allows us to monitor the major microbial populations at a given site over time. 16S rRNA gene monitoring is one way of monitoring the survival of microorganisms in bioaugmented sites (72, 77), biostimulated sites (72) or sites where natural attenuation is occurring (52, 73). However, PCB dechlorinators are a small part of the microbial community in sediments, and it is difficult to detect PCB dechlorinators using universal 16S rRNA primers (97, 133). Thus, the development of the PCR primers specific for the 16S rRNA gene of putative dehalogenating *Chloroflexi* is an important development for assessing the role of natural attenuation at sites contaminated with PCBs However, although detection of 16S rRNA genes is effective for enumeration and differentiation of specific phylotypes, they do not predict the function or specific dechlorination activities (37, 50, 56). An alternative approach that would provide information on function would be monitoring of specific dehalogenases. There have been several studies involving the identification of reductive dehalogenases specific for certain reactions of the dechlorination of chlorinated ethenes (56, 105, 129) to monitor the success of bioaugmentation or biostimulation. Although a number of putative dehalogenases were identified in DF-1 during the course of this research, I could not identify a reductive dehalogenase unequivocally associated with PCB dechlorination. Comparative sequence analysis of putative reductive dehalogenases from DF-1 showed that these clustered with

putative reductive dehalogenases from several *Dehalococcoides* species, which suggests that these genes were conserved among dehalogenating bacteria. Because of the difficulty generating sufficient cell material for reverse genetic identification of dehalogenases, new approaches must be developed to recombinantly express functional dehalogenases to identify specific PCB reductive dehalogenases. Such a breakthrough, in addition to providing activity-specific molecular assays, would enable us to finally understand the enzymology of PCB dechlorination and identify physiological factor that limit or promote the dechlorination process on an organismal and molecular level.

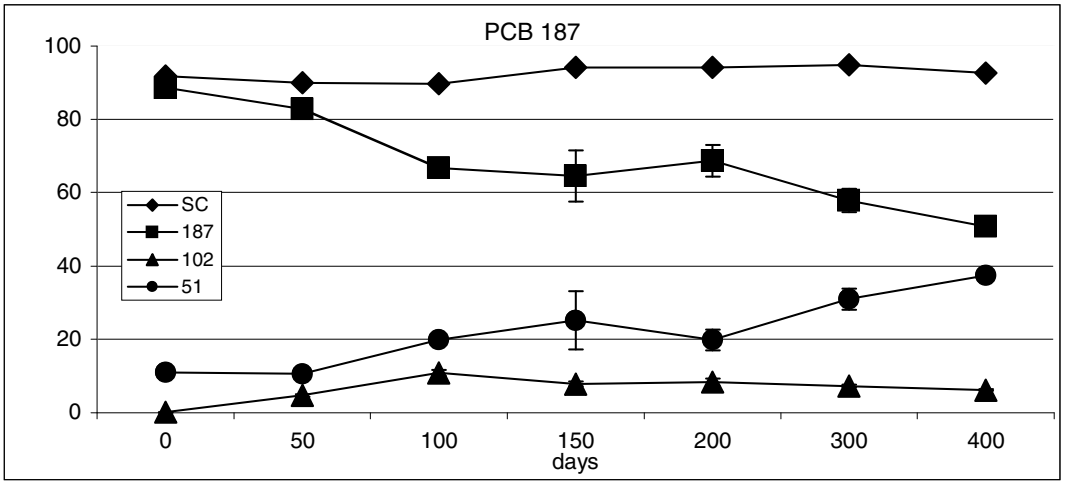
6.4. Final conclusions

The identification of the major dechlorination pathways in Aroclor 1260 and the realization that only a few phylotypes are responsible for these pathways in Baltimore Harbor sediment microcosms are two novel discoveries. We have shown that microorganisms have different PCB dechlorination specificities and the dechlorination pattern can be changed by the addition of specific dechlorinating populations to sediment microcosms. This strengthens the hypothesis that the driving factor behind the different patterns we see in the environment is the presence of different microbial populations. Although we have yet to prove that these results can be extrapolated to other sediments and other Aroclor mixtures, the results have important implications for how we approach risk assessment and bioremediation at PCB contaminated sites. It is now possible to selectively monitor the monophyletic dehalogenating group within the *Chloroflexi* to further study their activities and how

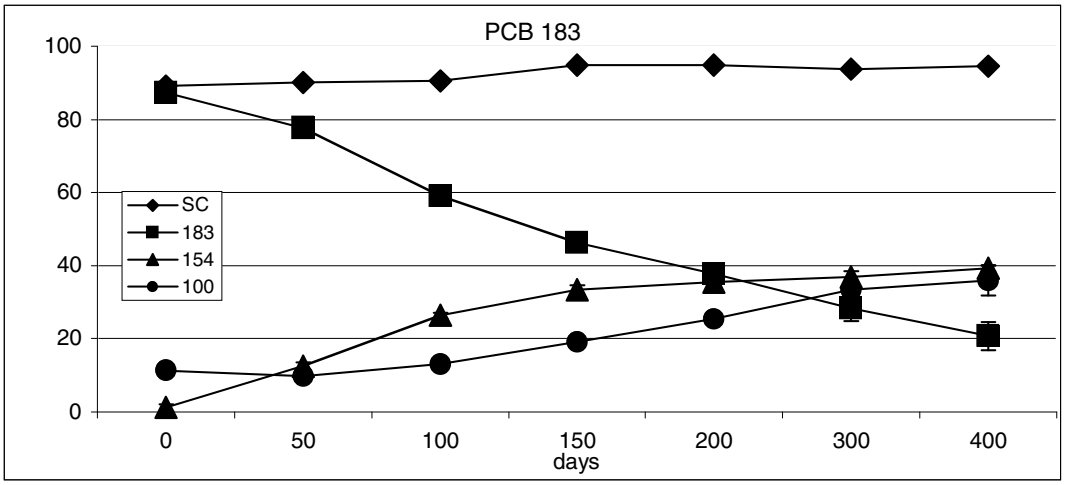
they interact with the complex microbial population present in contaminated sediments. A better understanding of these co-dependent interactions will enable development of solutions for treating PCB contaminated sites.

Appendix 1. Dechlorination curves

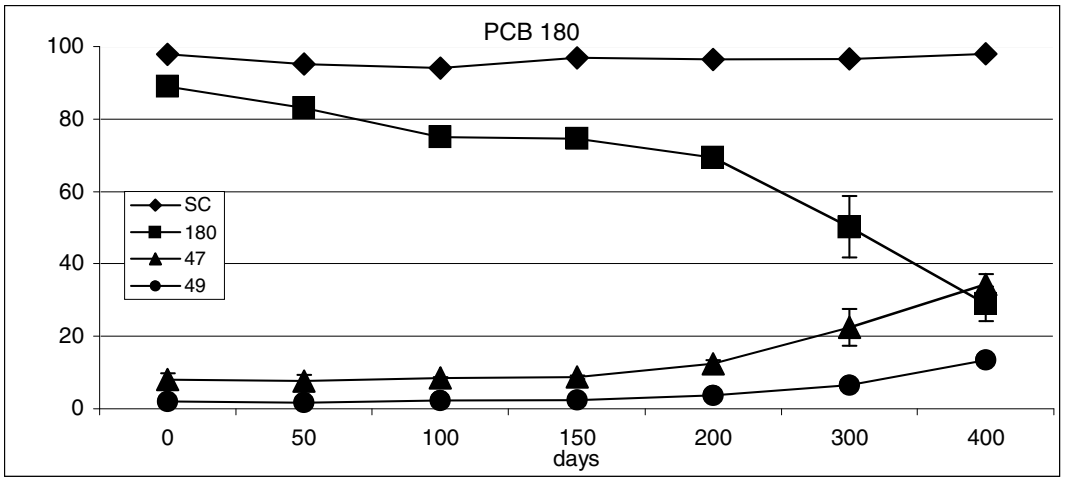
Unless otherwise stated, the dechlorination rates were calculated from the disappearance of the parent compound.



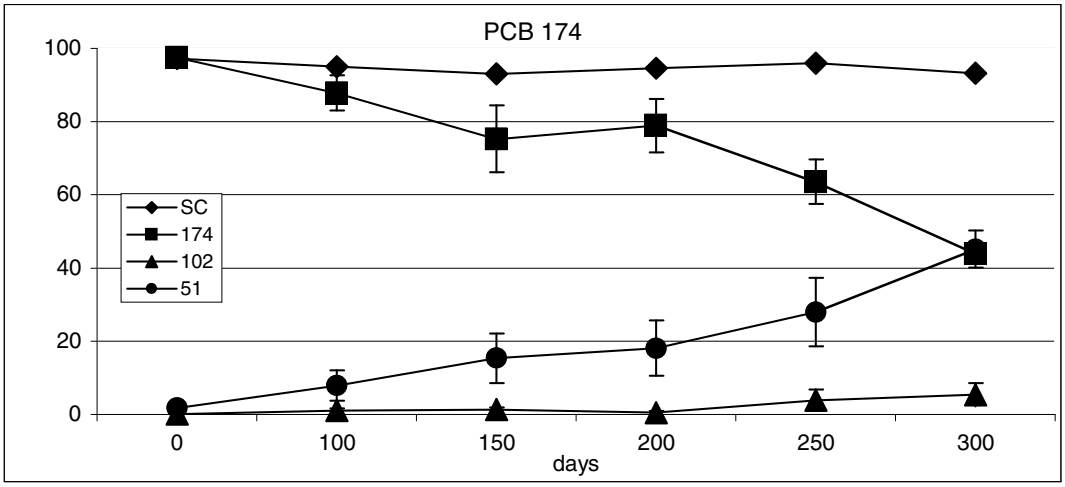
Rate was calculated from day 0 to day100



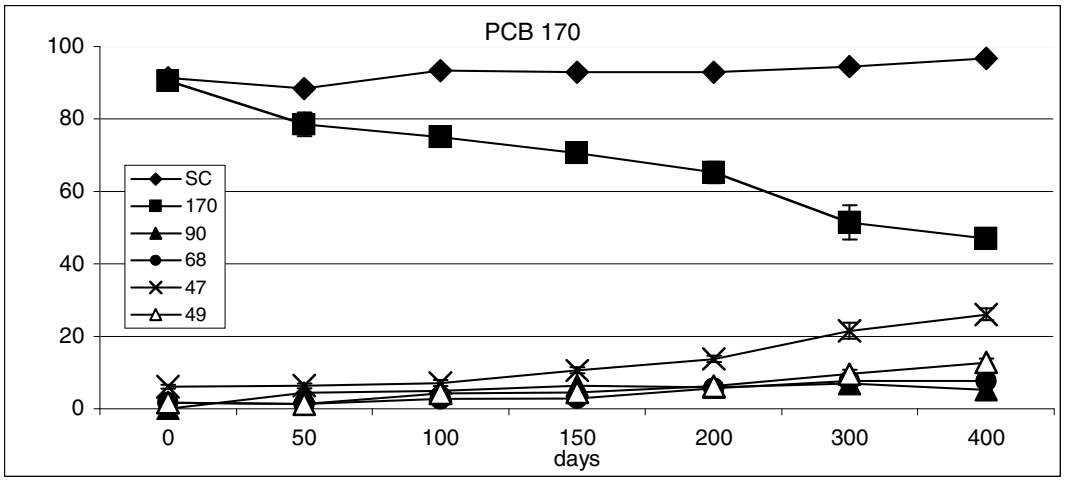
Rate was calculated from day 0 to day 400



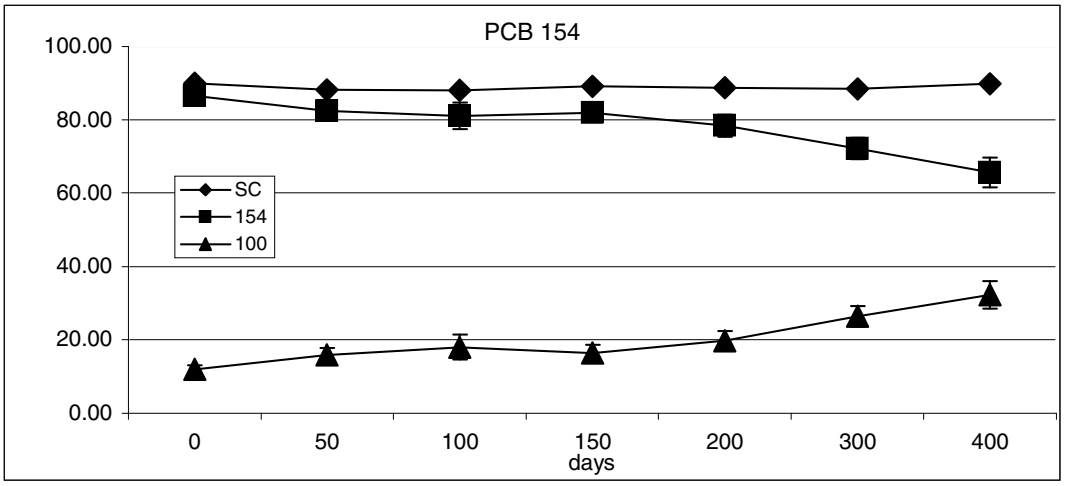
Rate was calculated from the appearance of the dechlorination products.



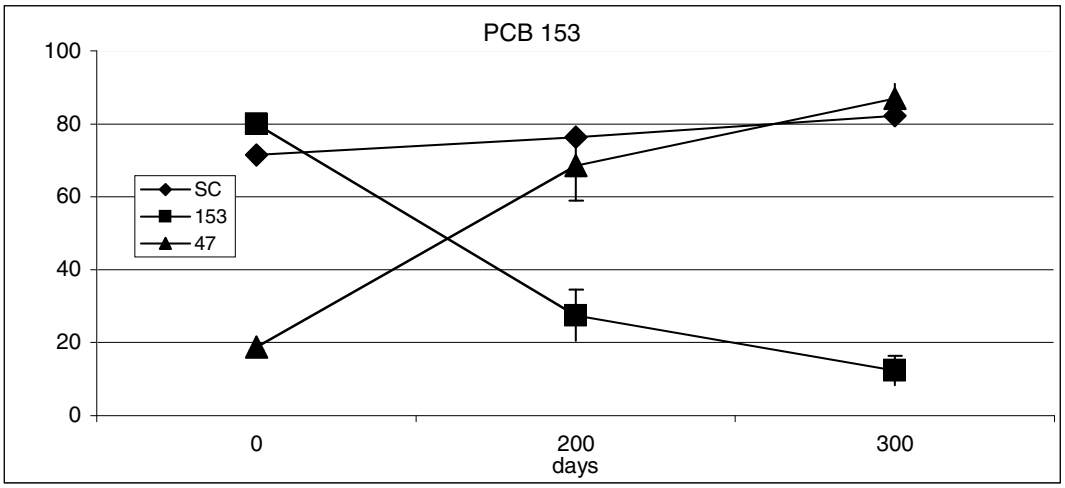
Rate was calculated from day 200 to day 300.



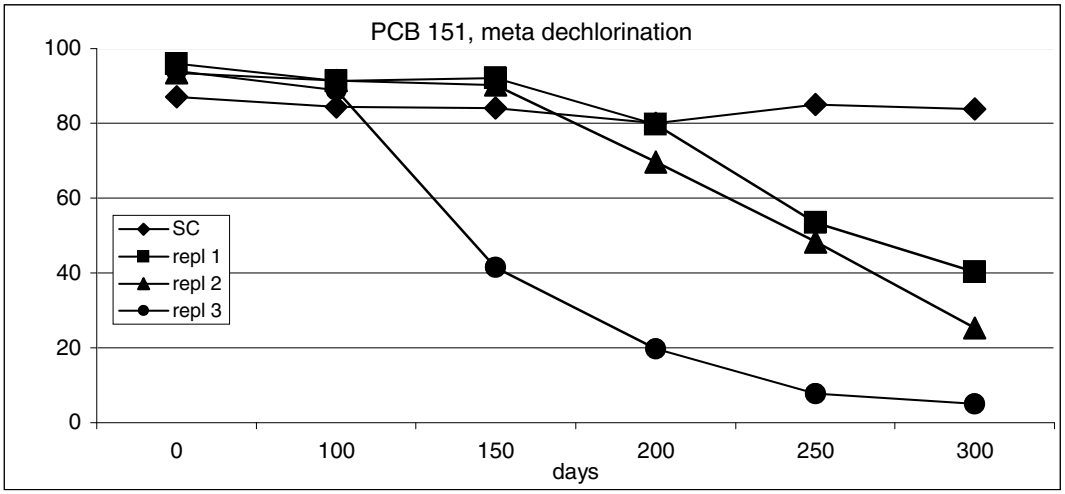
Rate was calculated from day 0 to day 300.



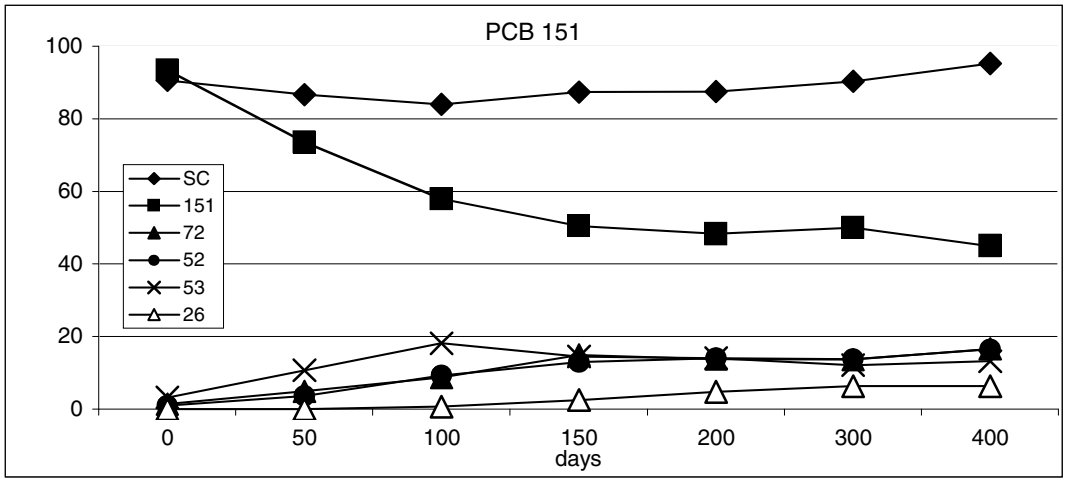
Rate was calculated from day 150 to day 400.



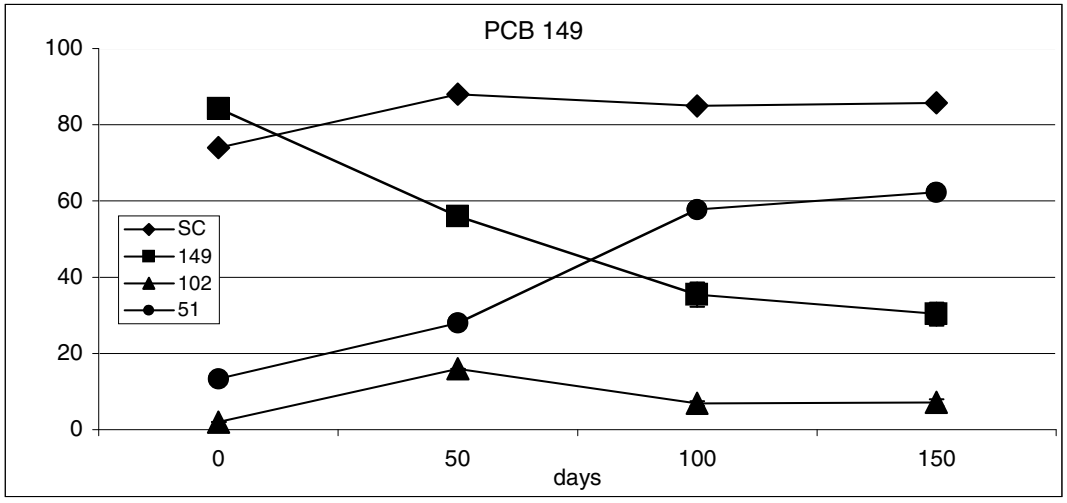
Rate was calculated from day 0 to day 200.



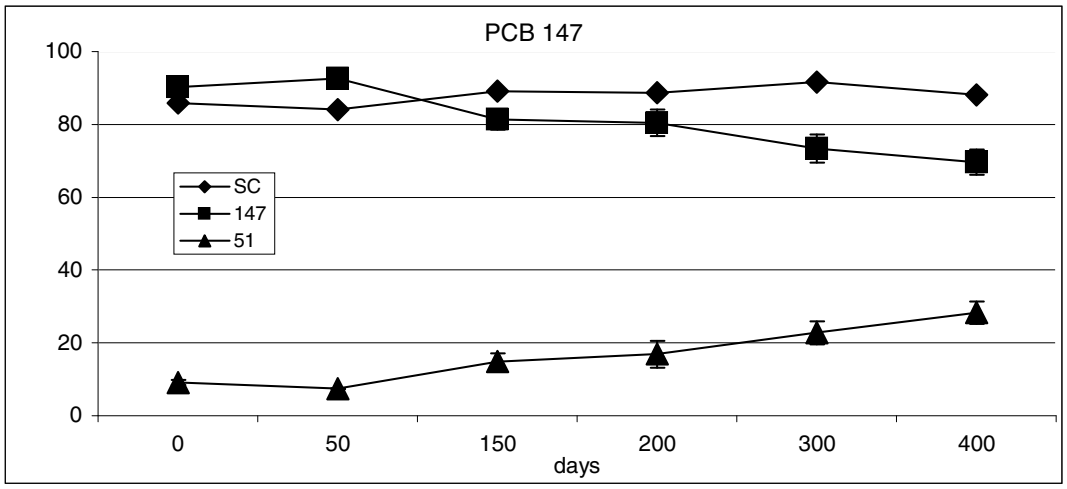
Rate was determined from day from day 150 to day 300 in replicate 1 and 2, while rate was determined from day 100 to day 200 for replicate 3.



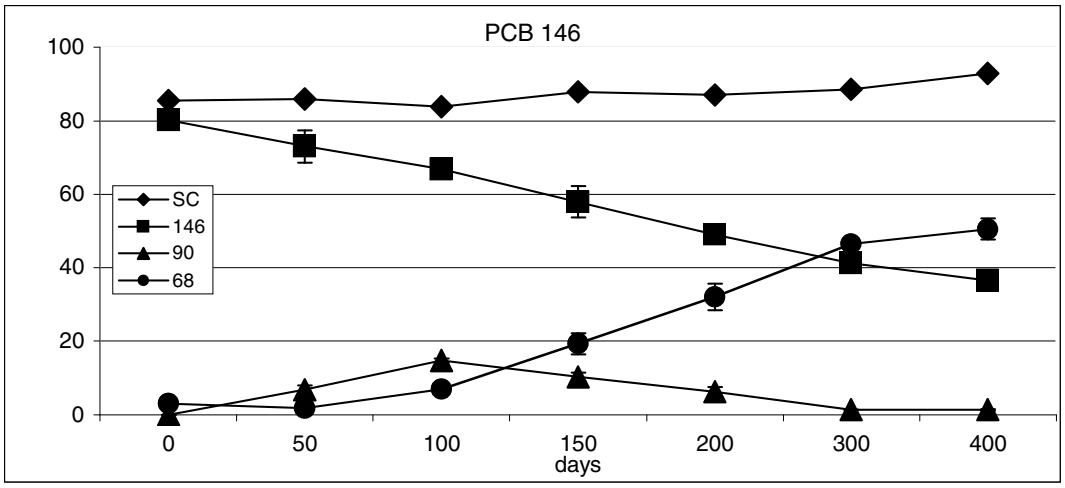
Rate was determined from the appearance of dechlorination products, PCB 72, 52 and 26 from day 0 to day 400.



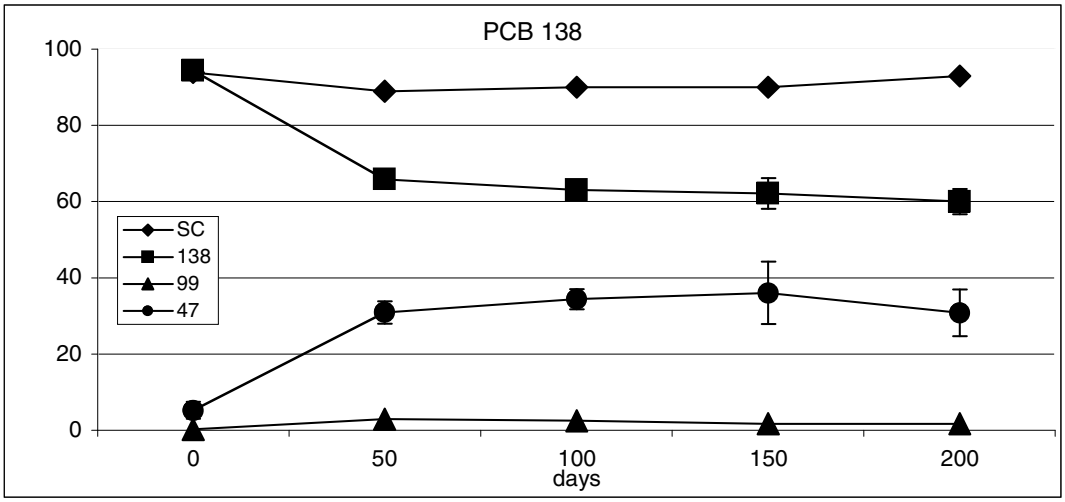
Rate was calculated from day 0 to day 100.



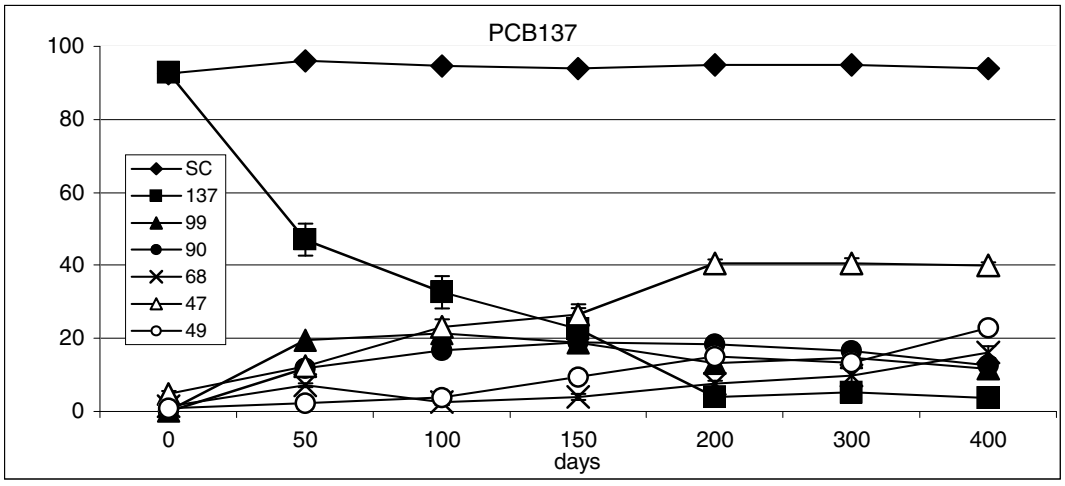
Rate was calculated from day 50 to 400.



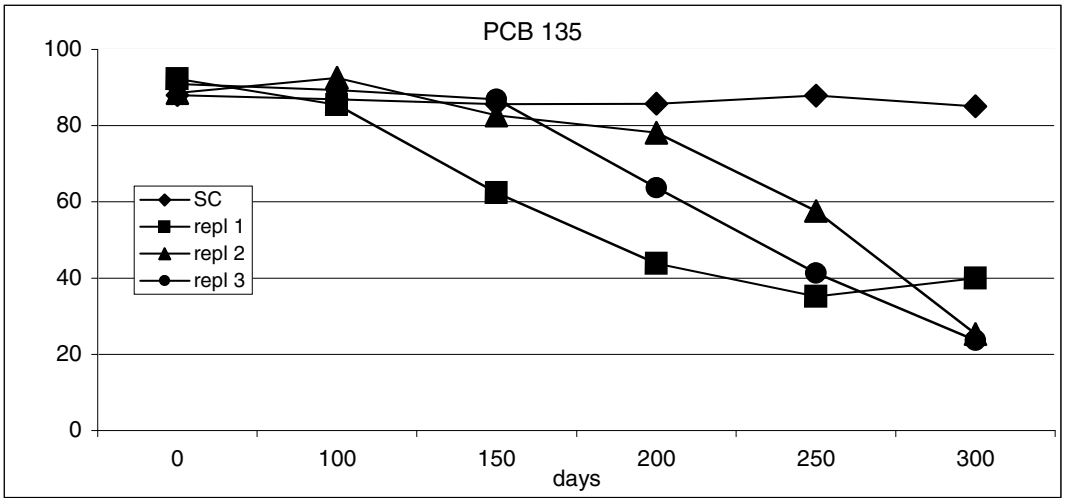
Rate was calculated from day 0 to day 400.



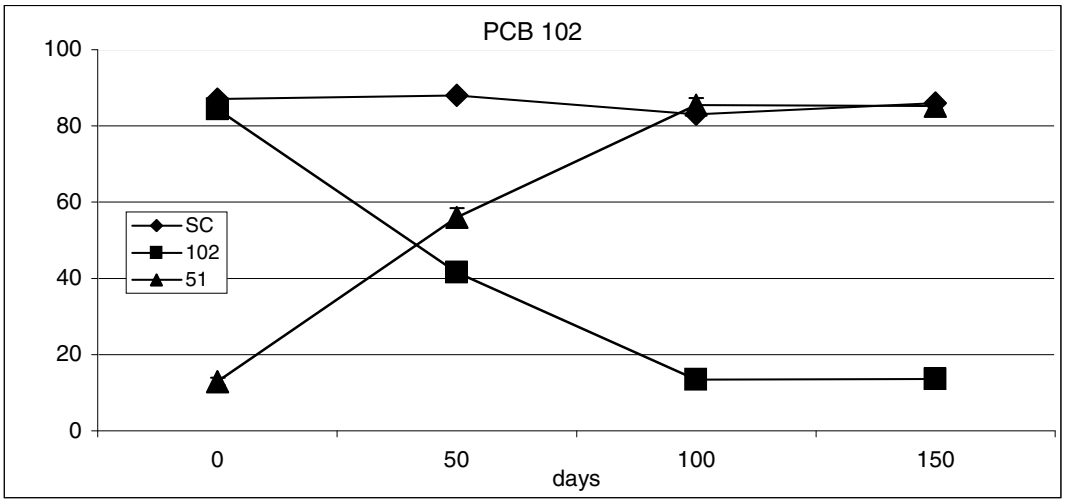
Rate was calculated from day 0 to day 50.



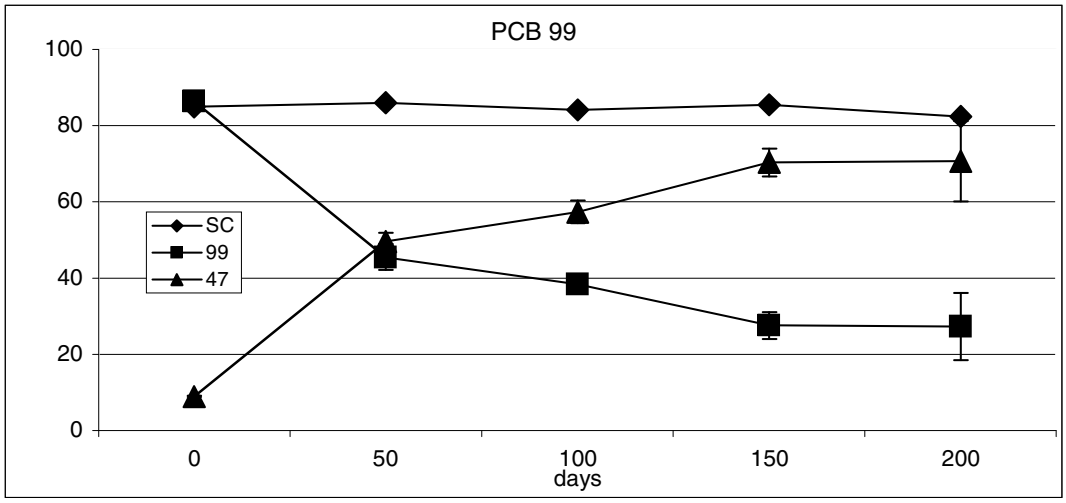
Rates were determined by the appearance of dechlorination products.



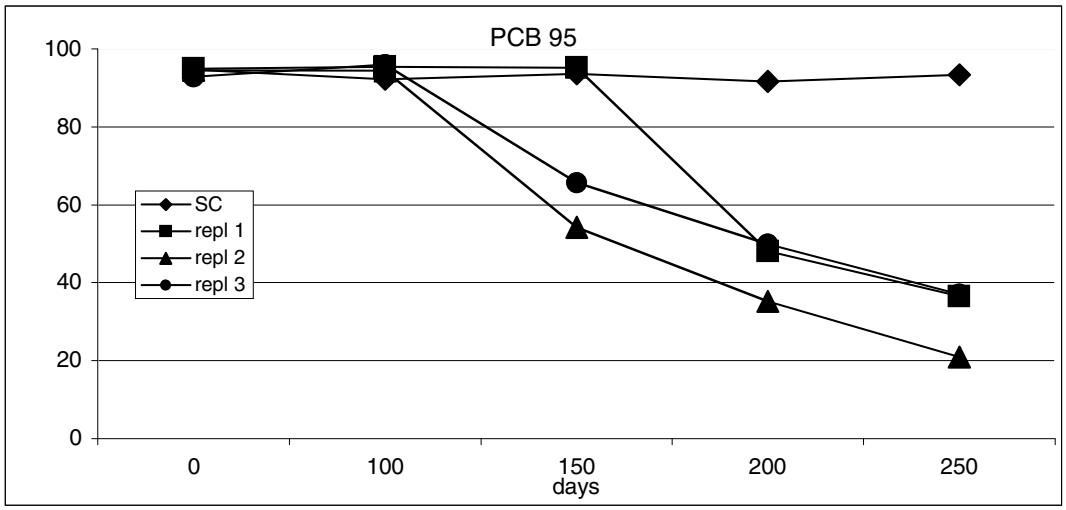
Rate was determined day 100 to day 200 for replicate 1, and from day 150 to 250 for replicate 2 and 3.



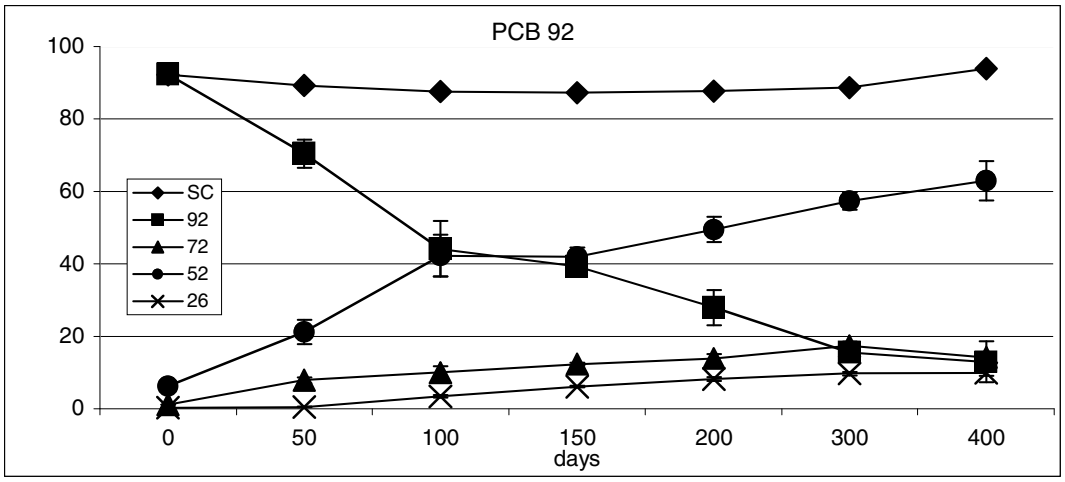
Rate was calculated from day 0 to day 100.



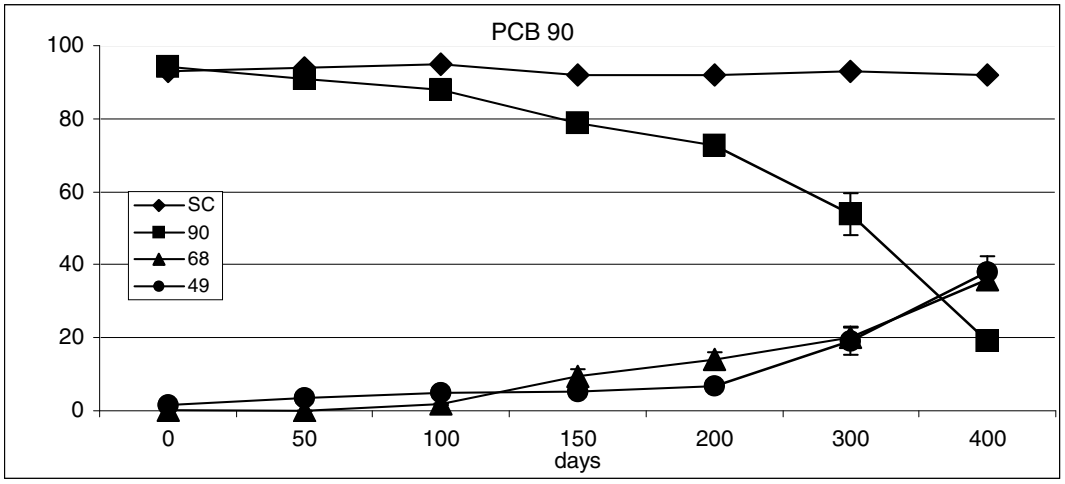
Rate was calculated from day 0 to day 50.



The rate was determined from day 100 to day 150 for replicates 2 and 3, and from day 150 to day 200 for replicate 1.



Rates were determined by the appearance of dechlorination products from day 0 to day 400.



Rate was determined by the appearance of dechlorination products from day 100 to day 400.

Glossary

aa	amino acid
ARDRA	Amplified Ribosomal DNA Restriction Analysis
bp	base pair
cDNA	complementary DNA
DEPC	diethyl pyrocarbonate
DGGE	Denaturing Gradient Gel Electrophoresis
DNTP	dinucleoside triphosphate
Kb	kilobase
PCR	Polymerase Chain reaction
ppm	parts per million
RFLP	Restriction Fragment Length Polymorphism
SDS	sodium dodecyl sulfate
TAE	Tris acetate EDTA

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