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Quantifying Enhanced Microbial Dehalogenation Impacting the Fate and Transport of Organohalide Mixtures in Contaminated Sediments

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14. ABSTRACT

The project investigated techniques and amendments to enhance microbial dehalogenation in sediments contaminated with organohalide mixtures and developed methods and tools to monitor the effectiveness of biostimulation processes. Micro- and mesocosm studies with organohalide-contaminated sediments demonstrated that these contain diverse communities of dehalogenating microorganisms. Dechlorination of historical PCB and PCDD/F contaminant mixtures can be stimulated by addition of amendments and/or bioaugmentation with dechlorinating bacteria. The enhanced dechlorination correlates with increased numbers of dehalorespirer populations and reductive dehalogenase genes. Identification of the specific microbial members associated with PCB- and PCDD/F-dechlorinating activity should allow for better strategies to enhance dehalogenation. The results provide compelling evidence to support further testing and development of biostimulation and bioaugmentation with dehalorespiring bacteria as environmentally less invasive and lower cost alternatives for in situ treatment of PCB impacted sediments.

15. SUBJECT TERMS

organohalide pollutants, PCBs, polychlorinated dioxins, sediments, anaerobic dehalogenation, Dehalococcoides

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List of Acronyms

CDD	chlorodibenzo-*p*-dioxin
CDD/F	chlorodibenzo-*p*-dioxin and chlorodibenzofuran
CDF	chlorodibenzofuran
DGGE	denaturing gradient gel electrophoresis
DHPLC	denaturing high-performance liquid chromatography
ED	electron donor
OCDD	octachlorodibenzo-*p*-dioxin
OCDF	octachlorodibenzofuran
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDD/Fs	polychlorinated dibenzo-*p*-dioxins and dibenzofurans
PCDF	polychlorinated dibenzofuran
PCR	polymerase chain reaction
PeCDF	pentachlorodibenzofuran
PCNB	pentachloronitrobenzene
TeCA	tetrachloroanisole
TeCB	tetrachlorobenzene
TeCDD	tetrachlorodibenzo-*p*-dioxin
TeCDF	tetrachlorodibenzofuran
TRFLP	terminal restriction fragment length polymorphism

List of Keywords

Dechlorination, PCBs, Dioxins, *Dehalococcoides*, bioaugmentation, biostimulation, sediments, compound specific isotope analysis

List of Figures & Tables

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Abstract

Objectives. Halogenated, hydrophobic contaminants of aquatic sediments pose a serious challenge. These toxic, bioaccumulating pollutants include legacy industrial chemicals, such as polychlorinated biphenyls (PCBs), ubiquitous polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), as well as current commercial manufacturing chemicals including brominated flame retardants. To date, remediation of contaminated sediments has mainly involved sediment removal with destruction or sequestration of the compounds. New methods are needed for *in situ* containment and degradation of halogenated contaminant mixtures. Stimulation of natural communities of dehalogenating bacteria and bioaugmentation using specialized dehalogenating bacterial strains holds promise for development of new approaches for sediment remediation. The objectives of this project were to extend techniques and amendments that enhance microbial dehalogenation for placement in sediments contaminated with organohalide mixtures and to develop methods and tools to monitor the effectiveness of the biostimulation process.

Technical Approach. The project focused on developing stimulatory amendment mixtures (e.g., bioaugmented dechlorinating bacteria, organic electron donors and halogenated co-amendments) to sediments and their placement methods in conjunction with capping. Molecular tools to monitor dehalogenating bacteria were developed and refined for use to assess the effectiveness of remediation treatments.

Results. We optimized a suite of molecular methodologies for rapid high-throughput detection, enumeration and diversity characterization of bacterial communities that reductively dehalogenate organohalides. To monitor the activity of dehalogenating bacteria and their response to different bioremediation treatments, we developed phylogenetic analysis targeting dechlorinating *Chloroflexi* including *Dehalococcoides* species and functional analysis targeting putative reductive dehalogenase (*rdh*) genes. Polymerase chain reaction (PCR) assays were developed to quantify and monitor biostimulated and bioaugmented dehalogenation in microcosms and mesocosms, and eventually in the field. We combined complementary molecular approaches including denaturing high-performance liquid chromatography (DHPLC), terminal restriction fragment length polymorphism (TRFLP), and nested PCR denaturing gradient gel electrophoresis (DGGE).

The protocols for assaying dehalogenating potential and the effects of different treatments and amendments were tested in different PCB and PCDD/F-contaminated sediments (Anacostia River, Kearny Marsh, Kymijoki River). Active dehalogenating bacteria are indigenous to these sediments and these communities have PCB and PCDD/F dechlorinating potential. Biostimulation may enhance the activity of both native *Dehalococcoides* spp. and the bioaugmented dehalogenating bacteria, such as *D. ethenogenes* strain 195. Microorganism(s) harboring various *rdh* genes play a key role in dechlorination. Our findings on the identity of species and genes involved in anaerobic PCB dechlorination may be used to evaluate environmental PCB and PCDD/F dechlorination potential and to monitor the dechlorination process under various treatments.

Benefits. Organohalide-contaminated sediments contain diverse communities of dehalogenating microorganisms and addition of appropriate amendments can enhance microbial dehalogenation of historic organohalide contaminant mixtures, including PCBs and PCDD/Fs. The enhanced dechlorination correlates with increased numbers of dehalorespirer populations and reductive dehalogenase genes, supporting our hypothesis that the halogenated co-substrates enhance dechlorination of historic pollutants by supporting growth and activity of dehalogenating bacteria. A combined bioaugmentation/biostimulation approach can be effective in bioremediation of sediments contaminated with organohalide mixtures. Identification of the specific microbial members associated with PCB- and PCDD/F-dechlorinating activity should allow for better strategies to enhance dehalogenation in contaminated environments.

Objective

The overall objectives of this project were to extend techniques and amendments that enhance microbial dehalogenation for placement in sediments contaminated with organohalide mixtures, including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) and chlorinated pesticides (CPs) and to develop methods and tools to monitor the effectiveness of the biostimulation process.

The specific project tasks were to:

1) Develop sensitive complementary molecular methods to quantify and monitor the progress of site-specific indigenous or bioaugmented dehalogenating activity;

2) Characterize the effect of sedimentary conditions on the (bio)transformation rates of organohalide mixtures and their transformation products under various bioremediation scenarios;

3) Apply combined techniques to accelerate dehalogenation including chemical co-amendments, bioaugmentation and capping (active and inactive) to optimize remediation/detoxification and minimize environmental impacts; and

4) Evaluate the fate and transport of organohalide contaminants, co-amendments and (bio)transformation products under various bioremediation scenarios.

The goal was to obtain an understanding of the microbial in-sediment processes involved in biodegradation of organohalide mixtures with the development of rapid screening methods and design of solutions for bioremediation of contaminated sediments. Remediation of organohalide-contaminated sediments *in situ* could avoid the problematic redistribution of contaminants that is associated with dredging and will decrease the overall cost of sediment management. One of the outcomes of this project is toolbox of site assessment and monitoring methods and an initial assessment of the potential for *in situ* stimulation of dehalogenation of organohalide mixtures in sediments. The complement other ongoing activities related to remediation of sediments (e.g., capping) by developing techniques for stimulating *in situ* bioremediation, site characterization methods to measure the activity of dehalogenating bacteria *in situ*, and assessing the partitioning and transport of organohalide contaminants and biotransformation products.

The project focused on developing stimulatory amendment mixtures (e.g., bioaugmented dechlorinating bacteria, organic electron donors and halogenated co-amendments) to sediments and their placement method in conjunction with capping. Molecular tools to monitor dehalogenating bacteria were developed and refined for use to assess the effectiveness of the remediation treatments. The technology development was coupled with model frameworks to provide an understanding of the impacts of stimulated *in situ* bioremediation on contaminant redistribution and transformation product fate.

Our approach was to use historically contaminated site sediments tested at the microcosm scale to identify successful amendment combinations for enhancing dehalogenation of organohalide

mixtures. The approach focused on microcosm studies to refine and confirm the molecular level monitoring tools and to screen potential field sites for the most effective amendment mixtures. Mesocosm tests were conducted to determine how best to accomplish amendment placement in combination or as an integral part of the capping material.

Selected molecular tools/methodologies were developed and refined to provide rapid monitoring of specific microbial populations responsible for respiratory dehalogenation of organohalide contaminants. These tools were designed to detect, enumerate and estimate the activity of specific bacterial populations for extensive bioremediation monitoring in a timely and cost effective manner. Enrichments developed from microcosm studies were used to characterize diverse dehalogenating bacteria found in aquatic sediments and their dehalogenase genes/enzymes. The goal was to link dehalogenase genes to specific strains or rRNA genes within our consortia/microcosms and eventually assess how gene expression related to transformation rates. Identification of diverse dehalogenating bacteria was used for development and optimization of molecular assays for detection and enumeration of dechlorinating bacteria.

Background

Aquatic sediments are ultimate receptors of contaminants from various DoD activities. Contaminated estuarine and marine sediments typically contain complex mixtures of pollutants, including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), chlorinated pesticides (CPs) such as DDT, polynuclear aromatic hydrocarbons (PAHs) and heavy metals. Organohalide contaminants are problematic due to their recalcitrance and toxicity, and furthermore, are often present as complex mixtures. Although microbially mediated reductive dehalogenation of organohalides is well established and bioremediation of contaminated soil and groundwater is already implemented in the field, methods for treating aquatic sediments contaminated with organohalide mixtures are not yet readily available. Developing amendment technologies for enhanced microbial dehalogenation and understanding how amendment placement and mixing stimulates dehalogenation and impacts the fate and transport of organohalide mixtures is a high priority for the successful management of contaminated sediments.

Reductive dehalogenation is a biologically mediated mechanism that could lead to the attenuation of organohalide pollutants when halogens are sequentially removed from the molecule (Häggblom and Bossert, 2003). During dehalorespiration, bacteria couple reductive dehalogenation to a respiratory process for the generation of cellular energy. This energy-yielding process utilizes the halogenated organic compound as the terminal electron acceptor while often molecular hydrogen (H_2) or a short chain organic compound is used as the electron donor (**Fig. 1**). This process occurs under anaerobic conditions and dehalorespiring bacteria compete for electrons with other terminal electron accepting processes such as methanogenesis and sulfate reduction. Dehalogenation is an important part of the biogeochemical cycle for natural and anthropogenic organohalides in the environment and has potential as a bioremediation technology for many compound classes. Despite many other environmental processes affecting pollutant fate and transport in contaminated sediments, anaerobic dehalogenation of organohalides may be the primary transformation mechanism controlling their fate. Lightly halogenated daughter products produced during anaerobic reductive dehalogenation may be aerobically mineralized if they enter a zone where aerobic bacteria are active. Increasing knowledge about the bacteria that mediate reductive dehalogenation of PCBs and PCDD/Fs will enable application of bioaugmentation and biostimulation for remediation of contaminated sediments. Although the occurrence of PCB as wells as PCDD/F dechlorination in the environment appears widespread, the rate, extent and specificity of the observed dechlorination activities vary substantially. An effective method for stimulating the activity

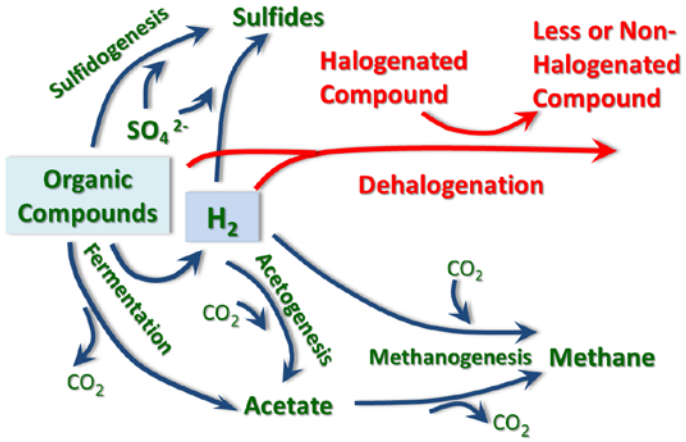


Figure 1. Electron flow in an anaerobic system including reductive dehalogenation.

of indigenous dechlorinating microorganisms would have great potential for eventual application in site remediation.

The addition of alternate halogenated compounds or "haloprimers" was first shown by Bedard and co-workers (Bedard et al., 2008) to enhance dechlorination of PCBs. To utilize dehalogenation as a bioremediation approach for contaminated sediment in the simplest instance, dehalogenating bacteria and biostimulation agents such as electron donors or haloprimers would be added to contaminated sediment (**Fig. 2**). This approach has not been realized yet because of the lack of knowledge about the organisms and because the technologies needed to incorporate the amendments have also not been proven.

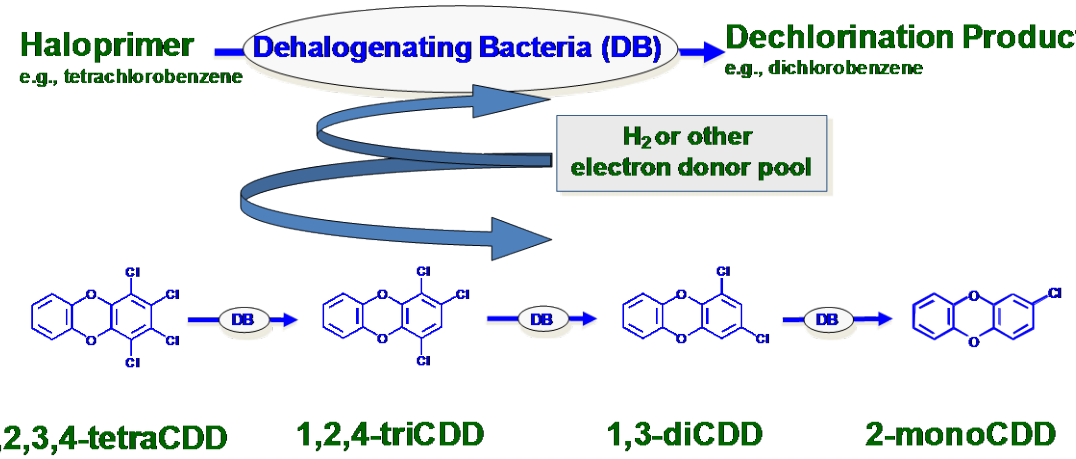


Figure 2. Role of halogenated cosubstrates (haloprimers) for PCDD/F and PCB dechlorination.

Sediment treatment is thus currently limited primarily to dredging with *ex situ* treatment or sequestration. New methods are needed for *in situ* containment and degradation of contaminants. This SERDP-supported project (ER-1492) investigated methods to stimulate the anaerobic biological dechlorination, which offers the most promising approach towards eventual detoxification and complete degradation of halogenated contaminant mixtures. *In situ* bioremediation combined with in-place containment through capping could avoid the problematic redistribution of contaminants that is associated with dredging and offer a more cost effective treatment alternative to dredging, where feasible. Development of *in situ* amendments for enhancing dehalogenation of mixtures, refinement and *in situ* testing of tools and integrated methods for monitoring the effectiveness of amendment placement and mixing, and development of model frameworks to track contaminant fate and transport during *in situ* biostimulation are needed for application of these strategies to contaminated sediments. The research project resulted in an enhanced understanding of the effect of amendments and amendment placement on microbial in-sediment processes involved in biodegradation of organohalide mixtures. This knowledge provides the foundation for the development and design of (site-specific) solutions for bioremediation of contaminated sediments. The use of molecular tools for monitoring microbial activity coupled with the understanding of how redox processes affect dehalogenation can provide a rapid screening method for determining whether a site is a good candidate for bioremediation and to tailor a bioremediation strategy for that site. The work was carried out at the microcosm and mesocosm scale leading to provide an understanding of the impacts of stimulated *in situ* bioremediation on contaminant redistribution and transformation product fate.

Results and Discussion

The following sections report on our findings from a series of microcosms and long-term mesocosm studies aimed at enhancing dechlorination of polychlorinated biphenyls (PCBs) and dibenzofurans (PCDFs) and dibenzo-p-dioxins (PCDDs). For these studies we used sediment collected from the Anacostia River, Washington DC and the Kymijoki River Finland.
The Anacostia River is a freshwater tidal system within the Potomac River Drainage Basin, which empties into the Chesapeake Bay. The lower Anacostia River, downstream of the Washington Naval Yard, is the site of a validation study of active sediment capping technologies (Reible et al., 2006). PCB concentrations in the sediment ranged from 0.4 to 9.1 mg/kg, and the congener profiles are most similar to a mixture of Aroclors 1248, 1254, and 1260.
The Kymijoki River in Finland was heavily contaminated with PCDD/Fs, among them 1,2,3,4,6,7,8- and 1,2,3,4,6,8,9-heptaCDFs and octa-CDF as the dominant congeners (Koistinen et al. 1995; Verta et al. 1999).

The studies integrate the key tasks the development of complementary molecular methods for quantifying and monitoring dehalogenating activity, characterizing the effect of sedimentary conditions on the (bio)transformation rates of organohalides, applying combined techniques to accelerate dehalogenation, and evaluating the fate of organohalide contaminants, amendment chemicals and their (bio)transformation products under various bioremediation scenarios.

We have focused the project on investigating the effectiveness of stimulatory amendment mixtures to accelerate in situ dechlorination of historical PCBs and PCDD/Fs. The range of amendments tested include organic electron donors, halogenated co-amendments (haloprimers), activated indigenous microorganisms (native site-specific sediment populations that have been enriched on haloprimers in ex situ reactors, then returned to the sediment following removal of residual haloprimer daughter products) and bioaugmented dechlorinating bacterial strains. Both the amendments and their placement in conjunction with capping at the micro- and mesocosm experimental scale was assessed. These studies specifically investigated how long the activated indigenous microorganisms and other bioaugmented dehalogenating strains remain active in sediments and the extent of dechlorination of historic contaminants. Treatments being examined at the mesocosm scale included capped and uncapped sediments. The mesocosms were designed using a treated and untreated sediment layer, with sampling efforts that attempted to determine to what extent the treatment exerted an effect on adjoining untreated sediments via diffusive processes.

Active dechlorinating bacterial communities are present in these various sediments and biostimulation may enhance the activity of both native *Dehalococcoides* spp. and the bioaugmented dehalogenating bacteria, such as *D. ethenogenes* strain 195. Our findings on the identity of species and genes involved in anaerobic PCB dechlorination might be used to evaluate environmental PCB dechlorination potential and to monitor the PCB dechlorination process under various conditions. Eventually it will help to understand the anaerobic PCB and PCDD/F dechlorination mechanisms in detail and to accelerate the detoxification and cleanup of contaminated sediments.

1. PCB Dechlorination Enhancement in Anacostia River Sediment Microcosms
(Krumins V, Park J-W, Son E-K, Rodenburg LA, Kerkhof LJ, Häggblom MM, Fennell DE. 2009. Sustained PCB dechlorination enhancement in Anacostia River sediment. Water Research ***43:****4549-4558)*

Abstract

In situ treatment of PCB contaminated sediments via microbial dechlorination is a promising alternative to dredging, which may be reserved for only the most contaminated areas. Reductive dechlorination of low levels of weathered PCB mixtures typical of urban environments may occur at slow rates. Here, we report that biostimulation and bioaugmentation enhanced dechlorination of low concentration (2.1 mg PCBs/kg dry weight) historical PCBs in microcosms prepared with Anacostia River, Washington, DC, sediment. Treatments included electron donors butyrate, lactate, propionate and acetate (1 mM each); alternate halogenated electron acceptors (haloprimers) tetrachlorobenzene (TeCB, 25 µM), pentachloronitrobenzene (PCNB, 25 µM), or 2,3,4,5,6-PCB (PCB 116, 2.0 µM); and/or bioaugmentation with a culture containing *Dehalococcoides ethenogenes* strain 195 (3 x 10^6 cells/mL). Dechlorination rates were enhanced in microcosms receiving bioaugmentation, PCNB and PCNB plus bioaugmentation, compared to other treatments. Microcosm subcultures generated after 415 days and spiked with PCB 116 showed sustained capacity for dechlorination of PCB116 in PCNB, PCNB plus bioaugmentation, and TeCB treatments, relative to other treatments. Analysis of *Chloroflexi* 16S rRNA genes showed that TeCB and PCNB increased native *Dehalococcoides* spp. from the Pinellas subgroup; however this increase was correlated to enhanced dechlorination of low concentration weathered PCBs only in PCNB-amended microcosms. *D. ethenogenes* strain 195 was detected only in bioaugmented microcosms and decreased over 281 days. Bioaugmentation with *D. ethenogenes* strain 195 increased PCB dechlorination rates initially, but enhanced capacity for dechlorination of a model congener, PCB116, after 415 days occurred only in microcosms with enhanced native *Dehalococcoides* spp.

Introduction

Polychlorinated biphenyls (PCBs) are hydrophobic, persistent toxic organic pollutants that accumulate in sediments and biota. Effective, economical methods for remediation of PCB contaminated sediments are lacking, however, progress has been made in understanding potential for biotransformation of PCBs in recent years. Discovery that anaerobic bacteria of the phylum *Chloroflexi*, including the genus *Dehalococcoides* and more distantly-related taxons, can dechlorinate PCBs may offer a promising avenue for bioremediation (Wu et al., 2000; Cutter et al., 2001; Fennell et al., 2004; Watts et al., 2005; Fagervold et al., 2005; Yan et al., 2006; Fagervold et al., 2007; Bedard et al., 2007; May et al., 2008; Kjellerup et al., 2008; and see review by Bedard, 2008). In this study we evaluated whether stimulating native *Dehalococcoides* populations or bioaugmentation could increase dechlorination of low concentrations of weathered PCBs, and whether the treatment effects persist over a relatively long time frame (> 1 year).

Recently, Bedard et al. (2007) reported growth of *Dehalococcoides* spp. coupled to Aroclor 1260 dechlorination and May et al. (2008) showed growth of strain DF-1 a dechlorinating *Chloroflexi*

during dechlorination of 2,3,4,5-PCB. Fagervold (2007) reported that three different phylotypes within the *Chloroflexi* increased in response to Aroclor 1260 in Baltimore Harbor sediment, suggesting that a consortium of dechlorinating bacteria with various specificities may result in more extensive dechlorination of mixed PCBs. In addition to the above studies, the tetrachloroethene dehalorespirer, *Dehalococcoides ethenogenes* strain 195, dechlorinated 2,3,4,5,6-PCB (Fennell et al., 2004), although growth was not tested.

One effective strategy for stimulating PCB dechlorination by sediment microorganisms is addition of alternate halogenated electron acceptors / co-substrates (haloprimers), such as 2,3,4,5,6-PCB (PCB 116) (Van Dort et al., 1997), 2,6-dibromobiphenyl (2,6-DBB) (Bedard et al., 1998), halobenzoates (Deweerd and Bedard, 1999) and chlorobenzenes and chlorophenols (Cho et al., 2002). Wu et al. (1999) used a most probable number method to show that halopriming with 2,6-DBB increased the number of PCB- and PBB- dehalogenators, suggesting that halopriming works by stimulating growth of dehalorespirers.

Dechlorination rates may depend on PCB concentration (Fish, 1996). In sediment microcosms from the Saint Lawrence River spiked with Aroclor 1242, Cho et al. (2003) reported threshold concentrations of 35 to 45 mg/kg PCB, below which PCB dechlorination ceased. Fish (1996) found that the maximum dechlorination rate of spiked Aroclor 1242 in sediment microcosms over a range of concentrations from 10 to 250 mg PCBs/kg increased with increasing initial concentration. The data of Fish (1996) generally follow a first-order trend, though it should be noted that the rates measured at higher initial concentrations were higher than would be expected from a perfectly linear relationship. Thus, it is not known whether biological treatment of sediments with low PCB concentrations could be effective.

We examined biostimulation and bioaugmentation for enhancing dechlorination of low concentration historical PCBs in microcosms developed using sediments from the Anacostia River, Washington DC. The Anacostia River is a freshwater tidal system within the Potomac River Drainage Basin, which empties into the Chesapeake Bay. The Anacostia River is classified as a warm-water stream with mean temperatures ranging from 3°C in January to 26°C in August, and summer temperatures of 18 to 32°C (SRC and NOAA, 2000). The lower Anacostia River, downstream of the Washington Naval Yard, is the site of a validation study of active sediment capping technologies (Reible et al., 2006). The area sampled for this study was downstream from a combined sewer overflow (CSO), and sediments are contaminated with PCBs, polycyclic aromatic hydrocarbons, chlorinated pesticides, and heavy metals, (Horne Engineering Services, 2003). PCB concentrations in the sediment ranged from 0.4 to 9.1 mg/kg, and the congener profiles are most similar to a mixture of Aroclors 1248, 1254, and 1260 (Horne Engineering Services, Inc., 2003). Thus, our study of the Anacostia River site addressed stimulation of PCB dechlorination under conditions of relatively low PCB concentrations arising from a weathered mixture of urban and industrial sources. We used electron donors, haloprimers, and/or bioaugmentation with *Dehalococcoides ethenogenes* strain 195 to stimulate dechlorination of historical PCBs in Anacostia River sediment microcosms. We compared dechlorination rates induced by the treatments, evaluated the persistence of the stimulation after 415 days, and tracked the dechlorinating bacterial population.

Experimental Methods

Microcosm Preparation. Microcosms were constructed using homogenized sediment recovered from the Anacostia River capping site control plot (Horne Engineering Services and Sevenson Environmental Services, 2004) on 7 July 2006, using a Van Veen dredge. The sediment contained 42 to 48% total solids. The organic matter content (7%), and textural analysis (41% sand: 39% silt: 20% clay) were determined by the Rutgers University Soils Testing Laboratory. Microcosms were prepared using 200 mL site sediment in 250 mL stock bottles fitted with rubber stoppers. In order to maintain pore water concentrations as similar as possible to *in situ* conditions, the microcosms were constructed using sediment only—no media or additional site water were added.

Eight treatments were run in triplicate: unamended and killed (autoclaving for 40 min at 121◦C on three successive days) controls; a mixture of electron donors; alternate halogenated electron acceptors (haloprimers) tetrachlorobenzene (TeCB), pentachloronitrobenzene (PCNB), or PCB116; bioaugmentation with a mixed culture containing *D. ethenogenes* strain 195; and PCNB plus bioaugmentation. The electron donor mix containing lactate, propionate, acetate, and butyrate was added to all microcosms to a concentration of 1 mM each, except in the live and killed controls. TeCB and PCNB (both 99%, Sigma-Aldrich, Inc., St. Louis, MO) were added in 100 µL of a 50 mM solution in acetone (B&J Brand, 99.9%, VWR, International, Inc., Pittsburgh, PA). PCB116 (AccuStandard, Inc., New Haven, CT) was added in 100 µL of acetone. The final TeCB and PCNB concentrations were 25 µM; and the final PCB116 concentration was 2 µM (1.5 mg/kg dry weight). Acetone (100 µL, without haloprimer) was added to the electron donor and bioaugmentation microcosms as well, resulting in 6.8 mM acetone in all microcosms except the controls.

The mixed culture containing *Dehalococcoides ethenogenes* strain 195 was grown at 25°C on PCE and butyric acid using methods described previously (Fennell, 1998). The culture contained 2×10^8 gene copies per mL, based on quantitative PCR targeting the *Dehalococcoides* 16S rRNA gene (see Krumins et al. 2009; supporting information). Four mL of the mixed culture was aseptically and anaerobically transferred to the bioaugmented microcosms. Based on the 16S rRNA gene copy numbers in the mixed culture, the bioaugmentation treatments had approximately 3×10^6 copies of *D. ethenogenes* strain 195 added per mL microcosm.

The microcosms were stirred, capped and incubated upright and statically at 26^{o}C, reflective of summer temperatures in the Anacostia (SRC and NOAA, 2000). An anaerobic headspace was maintained by flushing with N_2 during microcosm construction and initial sampling, and by performing subsequent sampling in a disposable N_2 flushed glove bag (Cole-Parmer Instrument Company, Vernon Hills, IL).

Samples (approximately 6 g) were collected on Days 1, 30, 60, 135, 185, 281, and 415 for PCB analysis. An additional 1 mL sample was collected from each microcosm during each sampling event for molecular analyses.

Activity Subcultures. On Day 415, subcultures were prepared to evaluate ongoing dechlorination activity in the microcosms. Ten mL of sediment was removed from each

microcosm (except the killed controls), transferred to sterile 60 mL serum bottles under N_2, and 20 mL of medium (Zinder, 1998) was added. The subcultures were spiked with 10 µL of 9.1 mM PCB116 in acetone, 30 µL of electron donor stock containing 1M each of lactate, propionate, butyrate, and acetate, and briefly shaken to mix. The resulting concentrations in the subcultures were: PCB116, 3.1 µM (6.3 mg/kg sediment dry weight); acetone, 4.5 mM; lactate, propionate, butyrate, and acetate, 1 mM, each. One mL samples were collected from the subcultures on Day 1, 30, 60, and 90 using a sterile, anoxic syringe with an 18 G needle. The subcultures were not amended with any additional treatments such as bioaugmentation or biostimulation with haloprimers, thus any varying treatment effects observed in these subcultures resulted from treatment of the original microcosms on Day 0.

Chemical analytical methods. Sediment samples from microcosms were spiked with a PCB surrogate standard containing 50 ng each of PCB14 (3,5-CB) and PCB166 (2,3,4,4',5,6-CB) (Cambridge Isotope Laboratories, Inc., Andover, MA), allowed to air dry for 24 to 48 hours, and were extracted using a Dionex ASE 200 Accelerated Solvent Extractor (Dionex, Bannockburn, IL, USA) in accordance with EPA method 3545. The average weight of the microcosm samples was 6.4 ± 1.9 g wet weight and 3.1 ± 0.9 g after air drying; (average ± 1 standard deviation (1σ), n = 260). The ASE extraction involved heating at 150^{o}C and 10.3 MPa with hexane as the solvent. Samples from Day 0 through Day 185 had copper turnings added to remove sulfur. The extracts were concentrated to 1 mL under ultra-high purity N_2. Precipitated elemental sulfur was removed by centrifugation. Interfering organic compounds were removed by passing the extract through a 2 mL glass pipette filled with Florisil (Sigma-Aldrich, St. Louis, MO). Remaining sulfur was removed by copper powder, according to EPA method 3660B. Samples were transferred to GC vials and 50 ng each of PCB30 (2,4,6-CB) and PCB204 (2,2',3,4,4'5,6,6'-CB) (Cambridge Isotope Laboratories, Inc., Andover, MA) were added as internal standards. Recoveries of PCB14 and PCB166 averaged 70% and 85%, respectively.

The PCB116-spiked subculture samples (1 mL) were extracted by shaking overnight in 7 mL Teflon®-lined screw-cap vials with 4 mL diethyl ether at room temperature, with 25 µL of 40 mg/L PCB114 (2,3,4,4',5-CB) added as an internal standard. The samples were centrifuged and the solvent was decanted to clean 7 mL vials. Sodium sulfate was added to remove water. Three mL of hexane was added and the extracts were concentrated to 1 mL under a stream of ultra-high purity nitrogen, effectively exchanging the solvent from diethyl ether to hexane. Sulfur was removed from the extracts according to EPA method 3660B, and the samples were transferred to GC vials.

PCBs were analyzed by gas chromatography – electron capture detection (GC-ECD) (HP 6890, Hewlett-Packard Co, Palo Alto, CA) using a DB-5 column (60 m x 0.32 mm ID, 0.25 µm film thickness, J&W Scientific, Folsom, CA). The column was heated at 7°C/min from 70°C to 180°C, followed by a 1.05°C/min increase to 225°C. Retention times for each of the 209 PCB congeners were determined from nine mixtures containing subsets of the congeners. Ambiguities were resolved by analyzing different subsets of congeners and by referring to published relative retention times (Frame, 1997). A calibration mixture containing the internal and surrogate standards and "Mullin's mix" (a mixture of Aroclors 1232, 1248, and 1262, (Swackhamer et al., 1996)) was run with every six samples to determine the response factor of each peak relative to the internal standards. Congener concentrations were not corrected for

surrogate recoveries, because of the possible bias in calculated mole fractions of lesser or more chlorinated congeners in the case of differential recoveries of the different surrogates.

Peaks for some of the 204 PCB congeners were not detected in the calibration mix or in any of the samples, while co-elution further decreased the number of quantifiable peaks to 86. Peaks for the surrogate and internal standards and those that co-eluted with sulfur (which was sometimes not completely removed by the copper cleanup), PCB116, or pentachloronitrobenzene and its dechlorination products were also disregarded. Ultimately 72 peaks, representing 159 congeners were used for further analyses (see **Table 1-1**).

Molecular Analyses. For microcosms, DNA was extracted from 0.3 grams of sediment using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Nested PCR was performed to amplify the DNA of the putative dechlorinating community, and resulting DNA products were analyzed by denaturing gradient gel electrophoresis (DGGE) (see section 1.1). First, DNA samples extracted from microcosms were amplified with primers 338F (Lane, 1991) and Chl1101R targeting a 790 bp region of the *Chloroflexi* 16S rRNA gene (see Accomplishments Section 2). The resulting PCR products were purified using the UltraClean™ PCR Clean-Up Kit (MoBio Laboratories Inc., Carlsbad, CA), and these PCR products were then re-amplified using general bacterial PCR primers 341F-GC and 534R (Muyzer et al., 1993) (**Table 1-2**). All PCR reaction mixtures contained 10 mM Tris–HCl (pH 8.3 at 25 °C), 2.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphates, 10 picomole of each primer, and 1 U of Taq DNA polymerase. The temperature profile for nested PCR with 338F and Chl1101R was 94 °C for 5 min followed by 35 cycles of 94 °C for 20 sec, 55 °C for 45 sec, and 72 °C for 45 sec. A final extension step was carried out for 7 min at 72 °C, after which the DNA was stored at 4 °C. General bacterial PCR conditions were as described previously (Park and Crowley, 2006).

Samples of PCR product (20 µL) were mixed with 2x DGGE dye and loaded onto 8% polyacrylamide gels in 1× Tris-acetate EDTA (TAE) buffer using a DCode™ universal mutation detection system (Bio-Rad Laboratories, Hercules, CA). The polyacrylamide gels were made with a linear denaturing gradient from 40% at the top to 60% denaturant at the bottom. The electrophoresis was run for 14 h at 60°C and 60 V. After electrophoresis, the gels were stained with ethidium bromide and photographed on a UV transilluminator. Image analyses of the DNA profiles and band intensities were conducted using Quantity One® (version 4.5.0; Bio-Rad Laboratories, Hercules, CA) and Scion Image (Beta version 4.02, US National Institutes of Health, Frederick, MD). The smiling of bands near the edges of the DGGE gels was corrected using Adobe® Photoshop® (Adobe Systems Inc., San Jose, CA).

DGGE bands of interest were excised and DNA was eluted overnight in 50 µL of MilliQ H_2O at 4 °C. After centrifuging, DNA in the supernatant was amplified using general bacterial primers as described above. The PCR products were cloned into pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Corp. Carlsbad, CA), according to the manufacturer's instructions. The cloned PCR products were sequenced using a 16-capillary ABI PRISM® 3100 Genetic Analyzer. Homology searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The programs CLUSTAL X (1.64b) and GeneDoc (2.0.004) were used to align the sequences and determine their homologies to known bacterial 16S rRNA gene sequences.

Table 1-1. Gas chromatograph peaks and PCB congeners quantified. Only peaks detected in the calibration standard created by mixing "Mullin's mix" (Swackhamer et al. 1996) and the internal and surrogate standards are listed. Congeners in bold are present in one or more of Aroclors 1242, 1248, 1254, and 1260 at a concentration of at least 0.50 wt.% (based on Frame et al. 1996).

Peak	Retention time (min)	PCB congener(s)	Comment
1	16.7	**1**	not used - low sensitivity
2	16.9	2	not used - low sensitivity
3	18.4	3	not used - low sensitivity
4	19.4	**4** + 10	not used - coeluted with PCNB dechlorination product
5	20.5	7 + 9	
6	21.2	**6**	
7	21.5	5 + **8**	
8	22.3	14	used as surrogate
9	22.7	**19**	
10	23.2	30	used as internal standard
11	23.8	12 + 13	
12	24.0	**18**	
13	24.1	**15** + **17**	
14	25.3	**16** + **32**	
15	26.2	29 + 54	
16	26.7	**26**	not used - coeluted with PCNB dechlorination product
17	26.9	**25**	not used - coeluted with PCNB dechlorination product
18	27.3	**31** + 50	
19	27.4	**28**	
20	28.1	**20** + 21 + **33** + **53**	
21	28.6	51	
22	28.8	**22**	
23	29.9	39 + 46	
24	30.3	**52** + 73	
25	30.7	43 + **49**	
26	30.9	38 + **47** + **48** + 75	
27	32.3	**37** + **42** + **44** + 59	
28	33.0	72	
29	33.3	**41** + **64** + 68 + **71**	
30	33.8	96	
31	34.1	**40** + 57 + 103	
32	34.4	67	not used - coeluted with sulfur

Peak	Retention time (min)	PCB congener(s)	Comment
33	34.5	100	not used - coeluted with sulfur
34	35.3	63	
35	35.7	61 + **74** + 94	
36	36.0	**70** + 76	
37	36.4	**66** + 80 + 93 + **95** + 98 + 102	
38	37.3	**91** + 55	
39	38.0	155	
40	38.4	**56** + **60** + **92**	
41	38.8	**84**	
42	39.1	89 + 90 + **101**	
43	39.7	79 + **99**	
44	41.0	78 + **83** + 108 + 112	
45	41.6	86 + **97**	
46	42.0	81 + **87** + 111 + 115 + 116 + 117 + 125 + 145	not used –PCB 116 used in one treatment
47	42.6	**85** + 120 + 148	
48	43.0	**136**	
49	43.3	**77** + **110** + 154	
50	44.8	**82** + **151**	
51	45.5	**124** + **135** + **144**	
52	46.1	107 + 147	
53	46.5	106 + **118** + 123 + 139 + 140 + **146**	
54	47.4	143	
55	48.0	**114** + 133 + 134	
56	48.2	122 + 131 + 142 + 165	
57	48.9	146 + 188	
58	49.9	**105** + 127 + **132** + **153** + 168 + 184	
59	51.4	**141** + **179**	
60	52.3	**130** + **137** + **176**	
61	53.4	**138** + 160 + **163** + **164**	
62	53.7	**158** + 186	
63	54.4	126 + **129** + **178**	
64	55.1	166 + 175	PCB 166 used as surrogate
65	55.8	159 + 182 + **187**	
66	56.5	162 + **183**	
67	57.1	**128**	
68	57.4	167	
69	57.9	**185**	
70	59.0	**174** + 181	
71	59.7	**177**	
72	60.2	**156** + **171** + 202	

Peak	Retention time (min)	PCB congener(s)	Comment
73	60.8	157 + **173** + 201	
74	61.2	204	used as internal standard
75	61.6	**172** + 192	
76	62.0	**180** + **193**	
77	62.9	191	
78	63.2	200	
79	64.7	170 + **190**	
80	65.1	198	
81	65.4	**199**	
82	65.7	**196** + **203**	
83	68.9	**194**	
84	69.2	205	
85	70.8	**206**	
86	72.1	209	

Table 1-2. Primer sets utilized for nested PCR-DGGE of *Chloroflexi* 16S rRNA genes.

Primers	Sequences (5' to 3')	Target	Reference
338F	ACTCCTACGGGAGGCAGCAG	Bacteria	(Lane, 1991)
Chl1101R	CTCGCKAGAAMATKTAACTAGCAAC	putative dechlorinating *Chloroflexi* spp.	(Park et al. 2011)
341F-GC*	CCTACGGGAGGCAGCAG	Bacteria	(Muyzer et al., 1993)
534R	ATTACCGCGGCTGCTGG	Bacteria	(Muyzer et al., 1993)

* GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3')

Quantification of *D. ethenogenes* strain 195 in the mixed culture used for bioaugmentation was performed by quantitative polymerase chain reaction (qPCR). A standard curve was obtained using 10-fold serial dilutions of plasmid DNA carrying a cloned 16S rRNA gene of D. ethenogenes strain 195. Sample DNA and 10-fold serially diluted 16S rRNA gene standards were amplified in parallel on an iQ5 real-time PCR system (Bio-Rad Laboratories, Hercules, CA). Each 25 µL of reaction contained 1 µL of sample or standard DNA, 1X iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 0.2 µM (each) *Dehalococcoides*-specific 16S rRNA gene primers (1F, 5'-GATGAACGCTAGCGGCG-3' and 259R, 5'-CAGACCAGCTACCGATCGAA-3') (Duhamel et al., 2004; Hendrickson et al., 2002) The temperature profile was: 5 min at 95 °C; 40 cycles of 30 sec at 95 °C; 30 sec at 55 °C; and 30 sec at 72 °C. Each analysis was followed by melt curve analysis which confirmed that only Dehalococcoides specific sequences (same single melting point as the cloned standard) were amplified. A control utilizing genomic DNA from *Pseudomonas fluorescens* resulted in no amplification of the 16S rRNA gene utilizing the primers and conditions indicated.

PCB Data Analysis. Chromatogram peaks were quantified relative to internal standards, with peak areas representing co-eluting congeners divided evenly among the contributing congeners. This was a conservative approach that did not presuppose a specific Aroclor as the predominant source of the contaminants, because the site contains weathered PCBs of mixed origin, or a specific dechlorination pathway. The mole fraction of individual homolog groups, average number of chorines per biphenyl (chlorination level), and the number of chlorines by position (*ortho*, *meta*, or *para*) were determined from the calculated mole fractions of the individual congeners.

The congener data was converted to mole fraction of total PCBs and the average number of chlorines per biphenyl (CL) was calculated. To compare effectiveness of different treatments, the decrease in CL over time was analyzed as a 1st order model:

$$\ln(CL)_{i(j)k} = \alpha_i - \beta_j X_k + e_{ik} \qquad \text{(Equation 1)}$$

where $\ln(CL)_{i(j)k}$ is the measured value of ln(CL) for microcosm i (subjected to treatment j) at time k, α_i is the intercept of ln(CL) at time 0 for microcosm i, β_j is the jth treatment slope (equals 1st order decay coefficient), X_k is the day of sampling, and e_{ik} is normally-distributed random error.

Because the interval between samples was not uniform, a "mixed model," rather than a repeated measures ANOVA, was the appropriate test (Littell et al., 2006). The data were analyzed using SAS (Proc Mixed, SAS v. 9.1.3, SAS Institute, Cary, NC). A restricted/residual maximum likelihood method was used, and the Kenward-Roger adjustment to the denominator degrees of freedom was applied to account for the use of repeated measures (Littell et al., 2006). A similar statistical analysis was applied to the activity subcultures with the chlorination level calculated based on PCB116 and its possible first dechlorination products 2,3,4,5-CB, 2,3,4,6-CB and 2,3,5,6-CB. To determine whether there was an effect of chlorine position (*ortho*, *meta*, or *para*) on dechlorination, a principal components analysis (PCA, Proc Factor, SAS v. 9.1.3, SAS Institute, Cary, NC) was performed using the change in ln(CL) from Day 0 to Day 415 for *ortho*, *meta* and *para* chlorine position as the dependent variables.

Results and Discussion

Dechlorination in microcosms. The mole fraction composition of total PCBs by homolog group over time for each treatment is shown in **Figure 1-1**. The average total PCB concentration for all initial time point samples was 2.1 ± 1.4 mg/kg dry weight (mean ± 1σ; n=24). Homolog-specific data for each treatment are shown in **Table 1-3**. For the PCNB treatment, the mole fraction of less chlorinated congeners (2 to 4 chlorines per biphenyl) increased by 20 ± 1.9 percent (average ± standard deviation) by 415 days (compared to day 0). This change was significant in a paired t-test ($p<0.01$). The mole fraction of di- to tetrachlorinated biphenyls also increased by 15.6 ± 4.8 percent in the bioaugmentation treatment ($p<0.05$) and by 12.1 ± 13.4 percent of total PCBs in the PCNB plus bioaugmentation treatment (not significant). For the killed control, live control, electron donor, TeCB and PCB116 treatments, changes in the fraction of less-chlorinated PCBs were smaller and not statistically significant (-1.4 ± 2.9; 1.9 ± 0.96; , 9 ± 17.4; , 2.1 ± 6, and 2.3 ± 5.6 percent, respectively). Note that most of the PCB dechlorination activity occurred and the TeCB and PCNB were both transformed during the first 135 days.

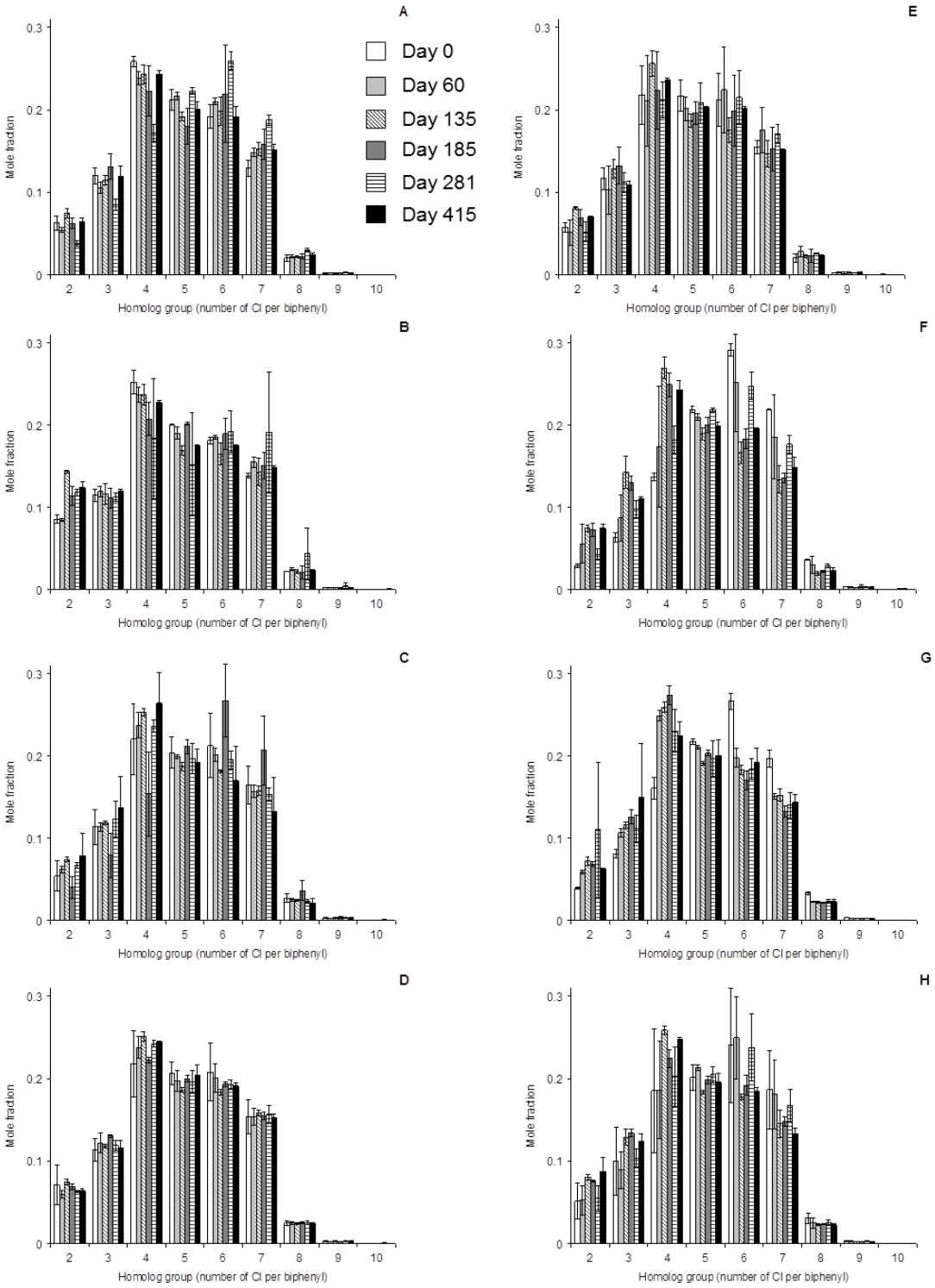
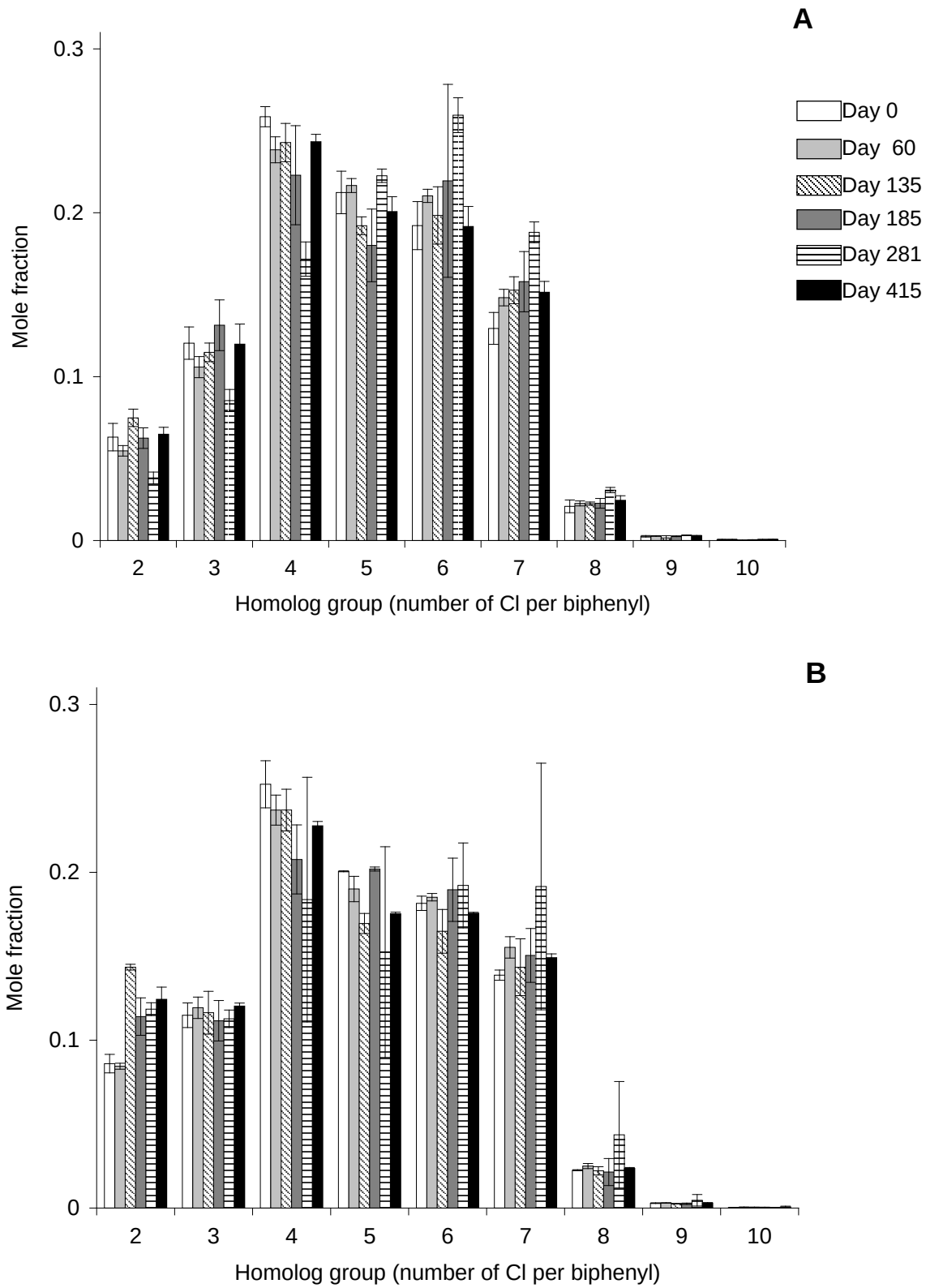
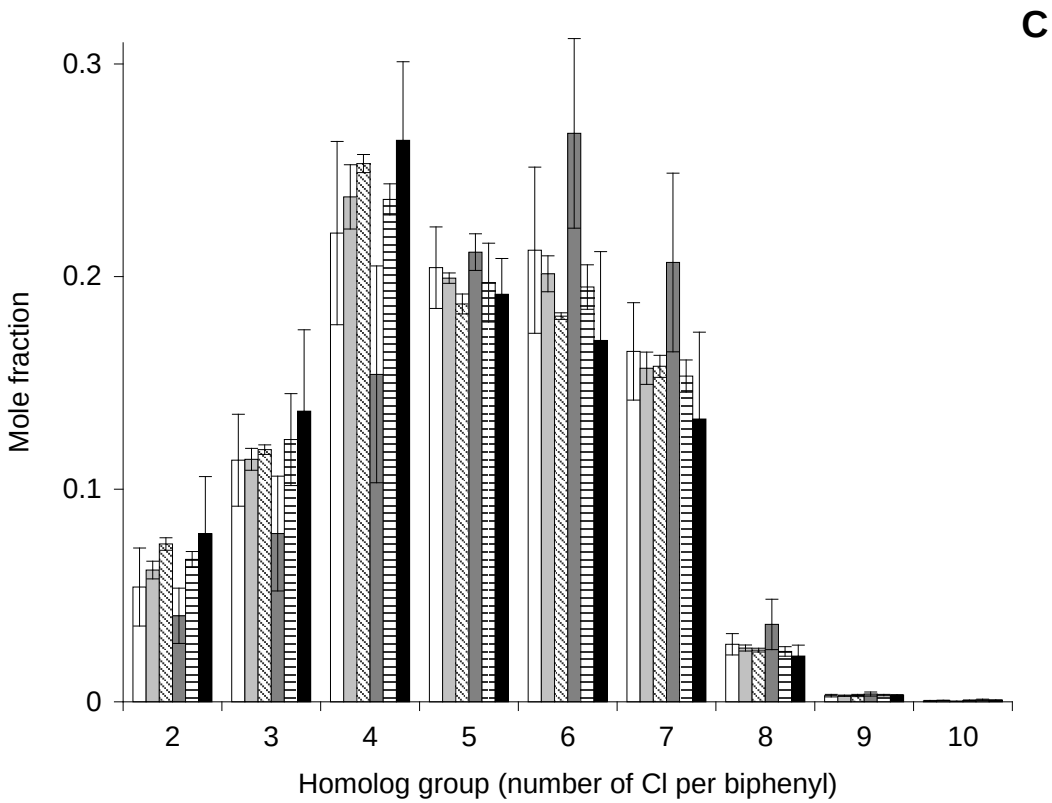
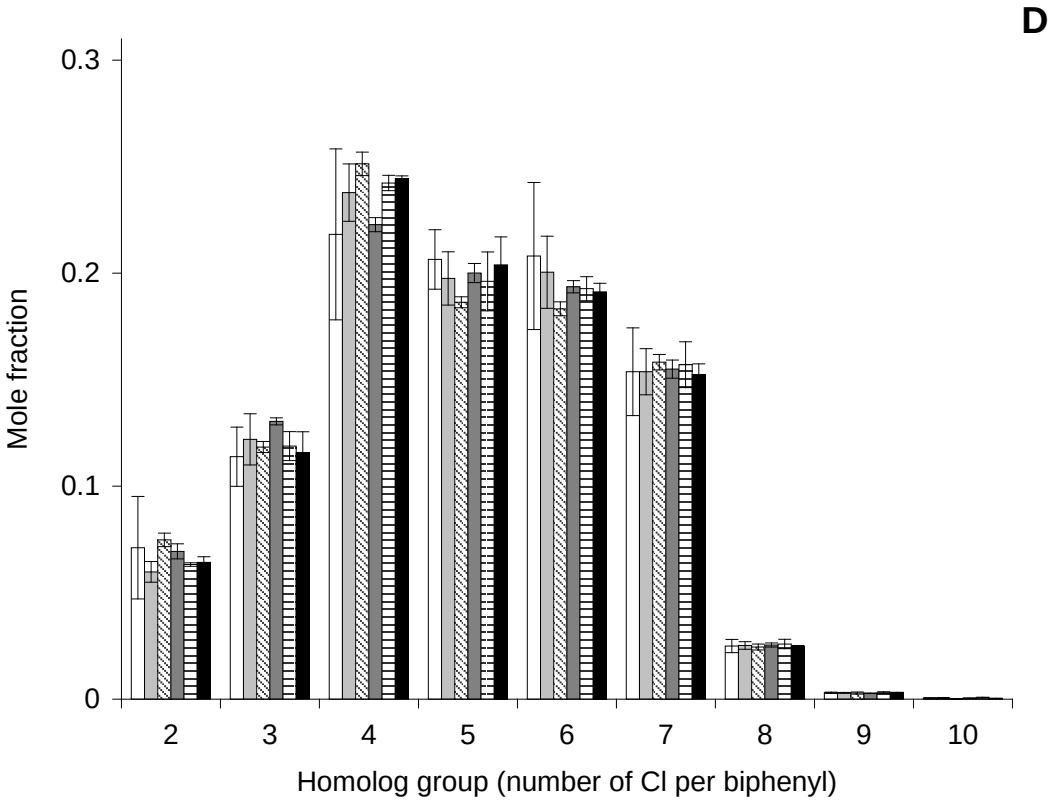
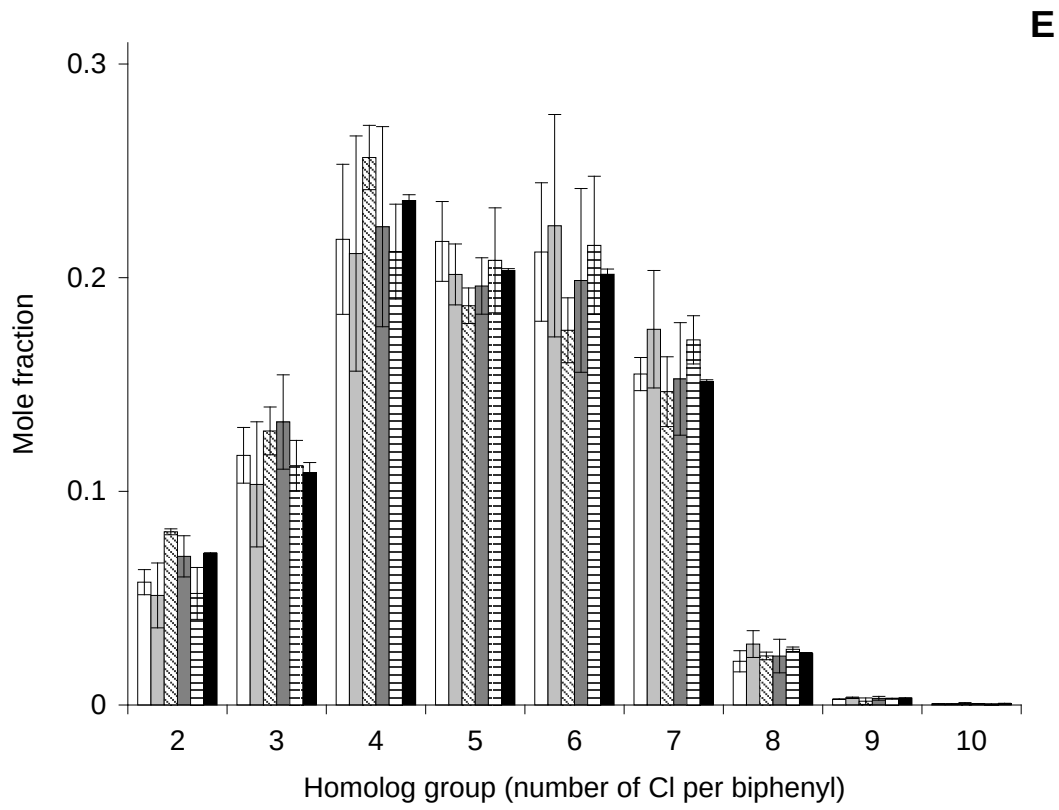


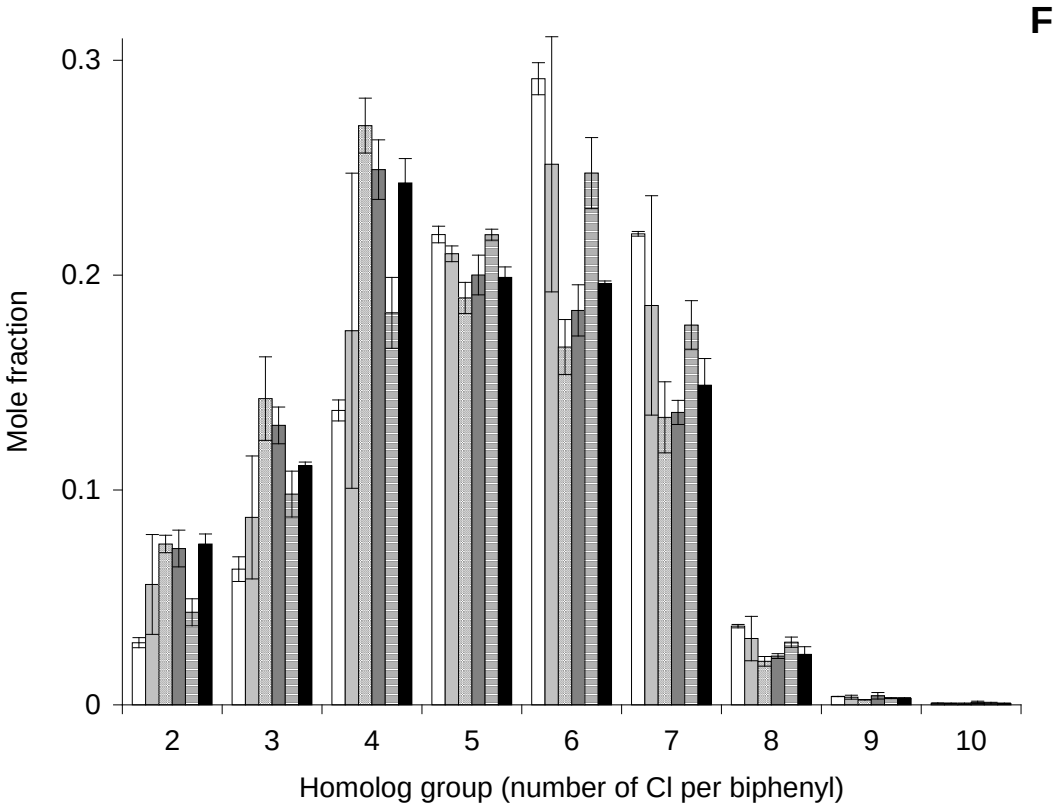
Figure 1-1. PCB distribution over time, by homolog group. **A**: unamended control, **B**: killed (autoclaved) control, **C**: electron donor, **D**: PCB116, **E**: TeCB, **F**: PCNB, **G**: bioaugmentation, **H**: PCNB + bioaugmentation. For each treatment, the six bars represent from left to right, the time points Day 0, 60, 135, 185, 281 and 415. Error bars depict standard deviation of measurements from triplicate microcosms. (Indivudal panels shown on the following pages. See Table 1-3 for data).

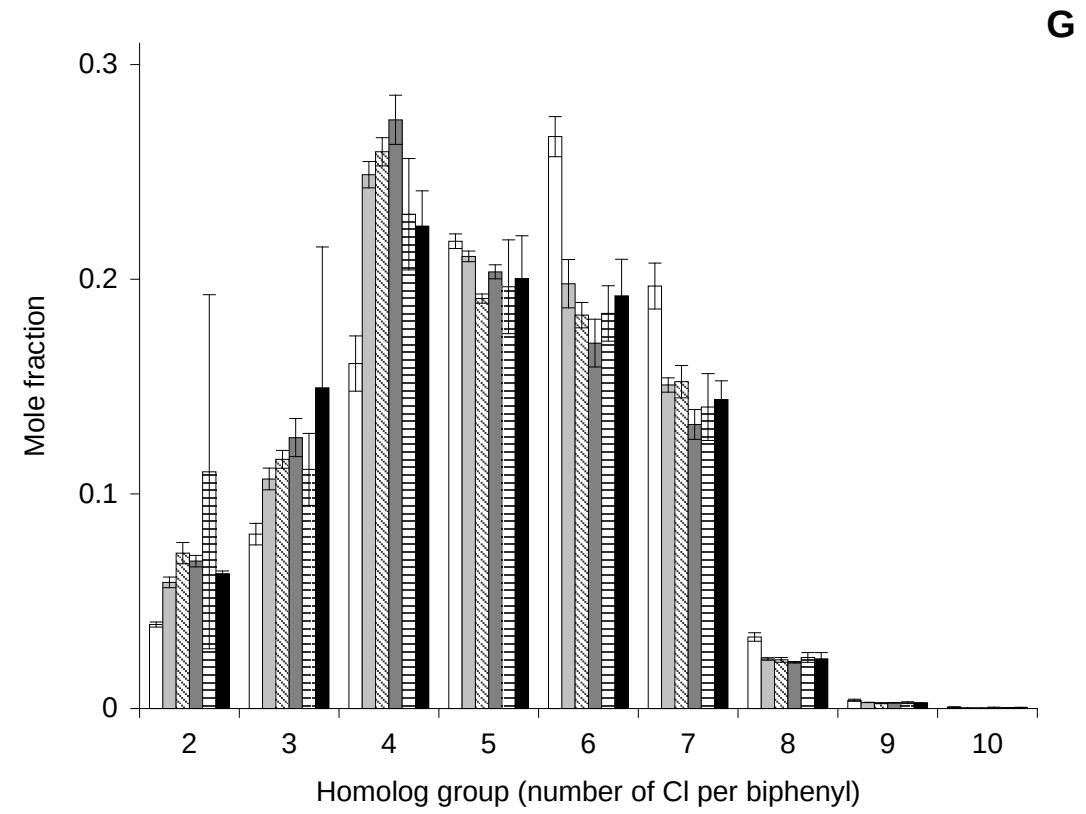

A
Day 0
Day 60
Day 135
Day 185
Day 281
Day 415
Mole fraction
0.3
0.2
0.1
0
2
3
4
5
6
7
8
9
10
Homolog group (number of Cl per biphenyl)
B
Mole fraction
0.3
0.2
0.1
0
2
3
4
5
6
7
8
9
10
Homolog group (number of Cl per biphenyl)


C
0.3
0.2
0.1
0
Mole fraction
2
3
4
5
6
7
8
9
10
Homolog group (number of Cl per biphenyl)


D
0.3
0.2
0.1
0
Mole fraction
2
3
4
5
6
7
8
9
10
Homolog group (number of Cl per biphenyl)


E
0.3
0.2
0.1
0
Mole fraction
2
3
4
5
6
7
8
9
10
Homolog group (number of Cl per biphenyl)


F
0.3
0.2
0.1
0
Mole fraction
2
3
4
5
6
7
8
9
10
Homolog group (number of Cl per biphenyl)


G
0.3
0.2
0.1
0
Mole fraction
2
3
4
5
6
7
8
9
10
Homolog group (number of Cl per biphenyl)

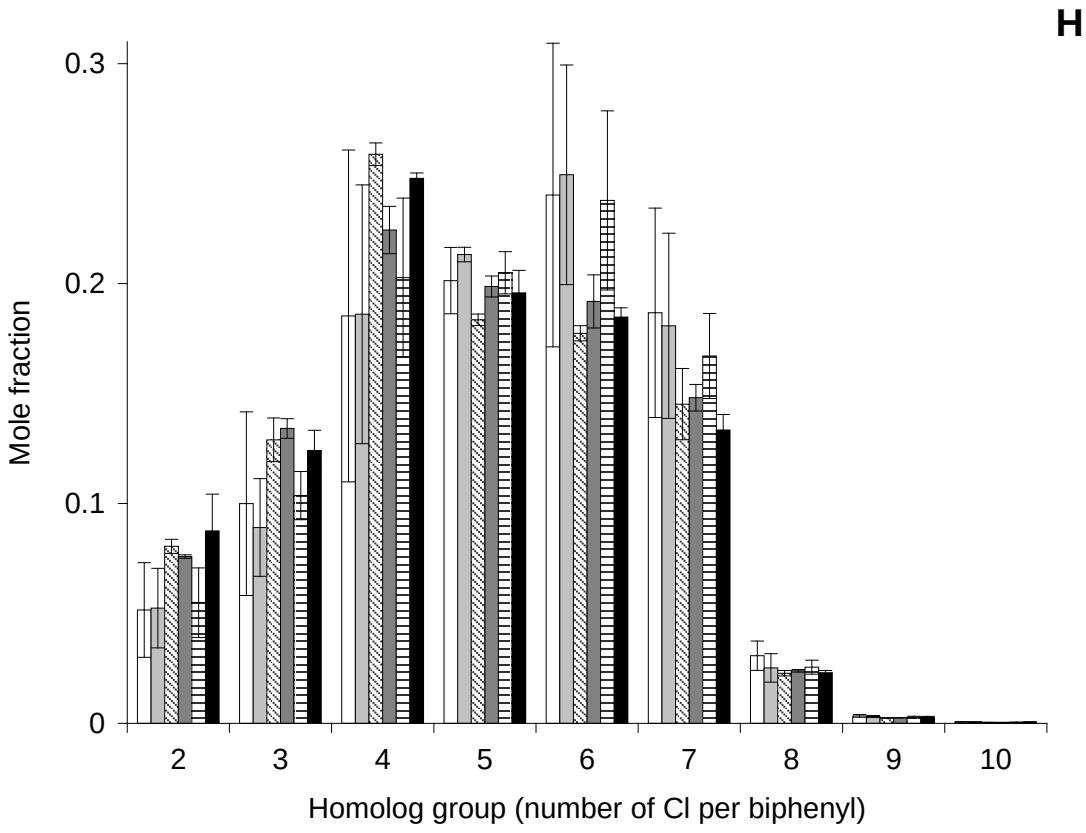

H
0.3
0.2
0.1
0
Mole fraction
2
3
4
5
6
7
8
9
10
Homolog group (number of Cl per biphenyl)

Table 1-3. Mole fraction PCBs by homolog group (number of chlorines per biphenyl) for Anacostia microcosms compared to the sum of all di- through deca- chlorinated biphenyls (homolog groups 2 through 10). Average ± standard deviation (n = 3 samples for each treatment at each time point).

A. Unamended Control.

	Time (day)					
Homolog	**0**	**60**	**135**	**185**	**281**	**415**
2	0.063 ± 0.0084	0.055 ± 0.0032	0.075 ± 0.0053	0.062 ± 0.0063	0.038 ± 0.0036	0.065 ± 0.0042
3	0.12 ± 0.0099	0.106 ± 0.0064	0.115 ± 0.0057	0.131 ± 0.0155	0.085 ± 0.0068	0.12 ± 0.0123
4	0.259 ± 0.0061	0.238 ± 0.0079	0.243 ± 0.0117	0.223 ± 0.0302	0.172 ± 0.0105	0.243 ± 0.0045
5	0.212 ± 0.013	0.217 ± 0.0042	0.192 ± 0.0054	0.18 ± 0.0222	0.223 ± 0.0041	0.201 ± 0.009
6	0.192 ± 0.0147	0.21 ± 0.004	0.198 ± 0.0175	0.219 ± 0.0589	0.26 ± 0.0106	0.192 ± 0.0122
7	0.129 ± 0.0098	0.148 ± 0.0051	0.153 ± 0.0082	0.158 ± 0.0184	0.188 ± 0.0064	0.152 ± 0.0066
8	0.021 ± 0.0039	0.023 ± 0.0015	0.022 ± 0.001	0.023 ± 0.003	0.031 ± 0.0016	0.025 ± 0.0028
9	0.0026 ± 0.00046	0.0028 ± 0.00018	0.0016 ± 0.00139	0.0026 ± 0.00021	0.0032 ± 0.00016	0.003 ± 0.00014
10	0.0005 ± 0.0003	0.0005 ± 0.00029	0.0002 ± 0.00007	0.0005 ± 0.00005	0.0006 ± 0.00009	0.0007 ± 0.00005

B. Killed Control.

	Time (day)					
Homolog	**0**	**60**	**135**	**185**	**281**	**415**
2	0.086 ± 0.0055	0.085 ± 0.0018	0.144 ± 0.0017	0.114 ± 0.0112	0.119 ± 0.0038	0.124 ± 0.0073
3	0.115 ± 0.0073	0.119 ± 0.0064	0.116 ± 0.0127	0.112 ± 0.012	0.113 ± 0.0052	0.12 ± 0.0018
4	0.252 ± 0.014	0.237 ± 0.0089	0.237 ± 0.0124	0.208 ± 0.0206	0.184 ± 0.0729	0.228 ± 0.0026
5	0.201 ± 0.0003	0.19 ± 0.0075	0.17 ± 0.006	0.202 ± 0.0012	0.153 ± 0.0628	0.175 ± 0.001
6	0.182 ± 0.0043	0.185 ± 0.0022	0.165 ± 0.013	0.19 ± 0.0189	0.192 ± 0.0253	0.176 ± 0.0005
7	0.139 ± 0.003	0.155 ± 0.0064	0.143 ± 0.0169	0.151 ± 0.016	0.192 ± 0.0735	0.149 ± 0.0022
8	0.023 ± 0.0003	0.025 ± 0.0015	0.022 ± 0.0025	0.021 ± 0.0081	0.044 ± 0.0317	0.024 ± 0.0005
9	0.0029 ± 0.00019	0.0031 ± 0.00007	0.0026 ± 0.00015	0.0026 ± 0.0004	0.0046 ± 0.00343	0.003 ± 0.00019
10	0.0002 ± 0.000003	0.0004 ± 0.00028	0.0003 ± 0.00024	0.0004 ± 0.0001	0.0004 ± 0.00003	0.0007 ± 0.00044

C. Electron donor.

	Time (day)					
Homolog	**0**	**60**	**135**	**185**	**281**	**415**
2	0.054 ± 0.0183	0.062 ± 0.0042	0.074 ± 0.003	0.04 ± 0.013	0.067 ± 0.0038	0.079 ± 0.0269
3	0.114 ± 0.0216	0.114 ± 0.0052	0.119 ± 0.0023	0.079 ± 0.027	0.123 ± 0.0216	0.137 ± 0.0383
4	0.22 ± 0.0431	0.238 ± 0.0151	0.253 ± 0.0043	0.154 ± 0.051	0.236 ± 0.0074	0.264 ± 0.037
5	0.204 ± 0.0192	0.199 ± 0.0024	0.187 ± 0.0047	0.212 ± 0.0086	0.197 ± 0.0186	0.192 ± 0.0169
6	0.212 ± 0.0391	0.201 ± 0.0084	0.181 ± 0.0015	0.267 ± 0.0446	0.195 ± 0.0105	0.17 ± 0.0418
7	0.165 ± 0.0229	0.157 ± 0.0076	0.158 ± 0.0052	0.207 ± 0.042	0.153 ± 0.0076	0.133 ± 0.0408
8	0.027 ± 0.005	0.025 ± 0.0014	0.024 ± 0.0009	0.036 ± 0.0118	0.024 ± 0.0022	0.022 ± 0.0052
9	0.0029 ± 0.00057	0.0029 ± 0.0003	0.003 ± 0.00053	0.0037 ± 0.00096	0.0032 ± 0.00035	0.0032 ± 0.00017
10	0.0004 ± 0.00023	0.0007 ± 0.00004	0.0003 ± 0.00004	0.0007 ± 0.00016	0.0009 ± 0.00028	0.0008 ± 0.00009

D. PCB116.

	Time (day)					
Homolog	**0**	**60**	**135**	**185**	**281**	**415**
2	0.071 ± 0.0241	0.06 ± 0.0049	0.075 ± 0.0032	0.069 ± 0.0035	0.063 ± 0.0008	0.064 ± 0.0026
3	0.114 ± 0.0139	0.122 ± 0.012	0.118 ± 0.0026	0.13 ± 0.0016	0.119 ± 0.0068	0.116 ± 0.0098
4	0.218 ± 0.0401	0.238 ± 0.0135	0.251 ± 0.0055	0.223 ± 0.0033	0.242 ± 0.0036	0.244 ± 0.0013
5	0.206 ± 0.014	0.198 ± 0.0125	0.186 ± 0.0026	0.2 ± 0.0045	0.196 ± 0.0138	0.204 ± 0.0131
6	0.208 ± 0.0345	0.200 ± 0.0169	0.183 ± 0.0033	0.194 ± 0.0029	0.193 ± 0.0056	0.191 ± 0.004
7	0.154 ± 0.0206	0.154 ± 0.0108	0.158 ± 0.0036	0.155 ± 0.0043	0.157 ± 0.0107	0.152 ± 0.005
8	0.025 ± 0.0031	0.025 ± 0.0018	0.025 ± 0.0014	0.025 ± 0.001	0.026 ± 0.0022	0.025 ± 0.0005
9	0.003 ± 0.00029	0.003 ± 0.00021	0.0029 ± 0.0005	0.0028 ± 0.0001	0.003 ± 0.00044	0.003 ± 0.00023
10	0.0005 ± 0.00022	0.0005 ± 0.00028	0.0002 ± 0.00002	0.0005 ± 0.00006	0.0007 ± 0.00026	0.0003 ± 0.00014

E. TeCB.

	Time (day)					
Homolog	**0**	**60**	**135**	**185**	**281**	**415**
2	0.058 ± 0.0059	0.051 ± 0.0152	0.081 ± 0.0014	0.07 ± 0.0097	0.052 ± 0.0121	0.071 ± 0.0002
3	0.117 ± 0.013	0.103 ± 0.0293	0.128 ± 0.0112	0.132 ± 0.0221	0.112 ± 0.0118	0.109 ± 0.0048
4	0.218 ± 0.0351	0.211 ± 0.0551	0.256 ± 0.015	0.224 ± 0.0468	0.212 ± 0.0222	0.236 ± 0.0027
5	0.217 ± 0.0187	0.202 ± 0.0143	0.187 ± 0.0083	0.196 ± 0.0132	0.208 ± 0.0246	0.203 ± 0.0009
6	0.212 ± 0.0324	0.224 ± 0.0521	0.175 ± 0.0152	0.199 ± 0.043	0.215 ± 0.0323	0.202 ± 0.0025
7	0.155 ± 0.0078	0.176 ± 0.0275	0.147 ± 0.0164	0.153 ± 0.0263	0.171 ± 0.0113	0.151 ± 0.0008
8	0.02 ± 0.0049	0.029 ± 0.0062	0.023 ± 0.0018	0.023 ± 0.0079	0.026 ± 0.0010	0.024 ± 0.0005
9	0.0027 ± 0.00014	0.0033 ± 0.00034	0.0018 ± 0.00153	0.0031 ± 0.00090	0.0029 ± 0.00022	0.0032 ± 0.00018
10	0.0005 ± 0.00014	0.0004 ± 0.00026	0.0007 ± 0.00042	0.0006 ± 0.00012	0.0004 ± 0.00015	0.0005 ± 0.00024

F. PCNB.

	Time (day)					
Homolog	**0**	**60**	**135**	**185**	**281**	**415**
2	0.029 ± 0.0023	0.056 ± 0.0232	0.075 ± 0.0041	0.073 ± 0.0086	0.043 ± 0.0063	0.075 ± 0.0046
3	0.063 ± 0.0058	0.087 ± 0.0286	0.143 ± 0.0194	0.13 ± 0.0086	0.098 ± 0.0107	0.111 ± 0.0018
4	0.137 ± 0.0049	0.174 ± 0.0733	0.27 ± 0.0128	0.249 ± 0.0138	0.182 ± 0.0165	0.243 ± 0.0113
5	0.219 ± 0.0039	0.21 ± 0.0037	0.189 ± 0.0073	0.2 ± 0.0092	0.219 ± 0.0026	0.199 ± 0.0049
6	0.291 ± 0.0074	0.252 ± 0.0594	0.167 ± 0.0128	0.184 ± 0.0119	0.248 ± 0.0165	0.196 ± 0.0012
7	0.219 ± 0.0011	0.186 ± 0.051	0.134 ± 0.0165	0.136 ± 0.0056	0.177 ± 0.0113	0.149 ± 0.0124
8	0.037 ± 0.0008	0.031 ± 0.0103	0.02 ± 0.0022	0.023 ± 0.0011	0.029 ± 0.0024	0.023 ± 0.0036
9	0.0039 ± 0.00007	0.0036 ± 0.00092	0.0024 ± 0.00006	0.0042 ± 0.00151	0.0031 ± 0.00024	0.0030 ± 0.00039
10	0.0007 ± 0.00023	0.0007 ± 0.00021	0.0005 ± 0.00031	0.0012 ± 0.00053	0.0009 ± 0.00032	0.0006 ± 0.00029

G. Bioaugmentation.

	Time (day)					
Homolog	**0**	**60**	**135**	**185**	**281**	**415**
2	0.039 ± 0.0012	0.059 ± 0.0025	0.072 ± 0.0049	0.069 ± 0.0027	0.11 ± 0.0825	0.063 ± 0.0013
3	0.081 ± 0.0050	0.107 ± 0.0050	0.116 ± 0.0042	0.126 ± 0.0089	0.111 ± 0.0168	0.149 ± 0.0656
4	0.161 ± 0.0129	0.249 ± 0.0061	0.259 ± 0.0066	0.274 ± 0.0114	0.23 ± 0.0259	0.225 ± 0.0164
5	0.218 ± 0.0034	0.211 ± 0.0025	0.191 ± 0.0021	0.203 ± 0.0033	0.197 ± 0.0219	0.2 ± 0.0199
6	0.266 ± 0.0094	0.198 ± 0.0113	0.183 ± 0.0059	0.17 ± 0.0111	0.184 ± 0.0129	0.192 ± 0.0171
7	0.197 ± 0.0107	0.151 ± 0.0034	0.152 ± 0.0075	0.132 ± 0.007	0.14 ± 0.0156	0.144 ± 0.0087
8	0.033 ± 0.0020	0.023 ± 0.0006	0.023 ± 0.0011	0.022 ± 0.0004	0.024 ± 0.0023	0.023 ± 0.0029
9	0.0038 ± 0.00049	0.0029 ± 0.00007	0.0025 ± 0.00024	0.0027 ± 0.0001	0.0027 ± 0.00042	0.0026 ± 0.00005
10	0.0007 ± 0.00012	0.0002 ± 0.00003	0.0002 ± 0.00001	0.0004 ± 0.00031	0.0003 ± 0.00014	0.0005 ± 0.00008

H. PCNB + bioaugmentation.

	Time (day)					
Homolog	**0**	**60**	**135**	**185**	**281**	**415**
2	0.052 ± 0.0215	0.052 ± 0.0181	0.081 ± 0.0032	0.076 ± 0.0008	0.055 ± 0.0158	0.088 ± 0.0167
3	0.100 ± 0.0418	0.089 ± 0.0222	0.129 ± 0.0099	0.134 ± 0.0045	0.104 ± 0.011	0.124 ± 0.0092
4	0.185 ± 0.0754	0.186 ± 0.0588	0.259 ± 0.0051	0.224 ± 0.0108	0.203 ± 0.0361	0.248 ± 0.0024
5	0.201 ± 0.0151	0.213 ± 0.0033	0.184 ± 0.0026	0.199 ± 0.0047	0.205 ± 0.0096	0.196 ± 0.0103
6	0.240 ± 0.0691	0.250 ± 0.0499	0.177 ± 0.0035	0.192 ± 0.0121	0.238 ± 0.0407	0.185 ± 0.0042
7	0.187 ± 0.0476	0.181 ± 0.0421	0.145 ± 0.0162	0.148 ± 0.0061	0.167 ± 0.0193	0.133 ± 0.0071
8	0.031 ± 0.0067	0.025 ± 0.0065	0.023 ± 0.0012	0.024 ± 0.0006	0.026 ± 0.0032	0.023 ± 0.0009
9	0.0034 ± 0.00065	0.0031 ± 0.00049	0.0025 ± 0.00009	0.0026 ± 0.00003	0.0028 ± 0.0004	0.0029 ± 0.00025
10	0.0007 ± 0.00006	0.0006 ± 0.00015	0.0003 ± 0.00019	0.0004 ± 0.00003	0.0005 ± 0.00005	0.0005 ± 0.00027

PCB Dechlorination Rates. The average overall chlorination level for the microcosms at the initial time point was 5.1 ± 0.28 (average ± 1σ, n = 24) chlorines per biphenyl. The initial chlorination level varied across microcosms despite our effort to homogenize the sediment (**Fig. 1-2**). Mixed model analysis indicated significant (p = 0.0006) differences in slope of ln(CL) vs. time among the different treatments (β_j in the mixed model). In support of the broad conclusion that PCNB enhanced dechlorination with or without bioaugmentation, the rate constants for the PCNB, bioaugmentation, and PCNB plus bioaugmentation treatments were significantly ($p < 0.01$) different from zero, whereas the rate constants for the controls, the electron donor only and the TeCB treatments were not statistically different from zero (**Table 1-4, Fig. 1-3** – error bars on the figure indicate the standard error (calculated from the e_{ik}) for the mixed model analysis). Furthermore, the dechlorination rate constants for PCNB, bioaugmentation, and PCNB plus bioaugmentation treatments were also significantly different from all other rate constants [with the exception of the electron donor treatment, whose rate constant was not statistically different than zero ($p > 0.1$)].

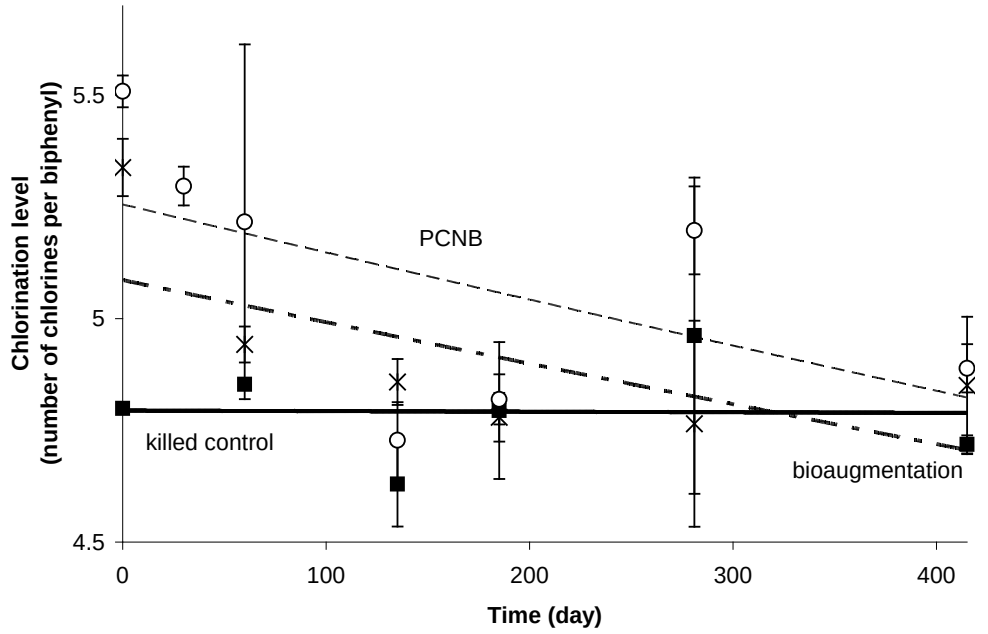


Figure 1-2. Chlorination level over time in microcosm samples. Killed control (■), PCNB (○) and bioaugmentation (×). Trend lines are exponential best fits.

Dechlorination pathway. No statistically significant difference in dechlorination for different chlorine positions (*ortho*, *meta* or *para*) could be elucidated. Principal components analysis of the difference in numbers of chlorines by position between Day 0 and Day 415 indicated that one principal component explained more than 90% of the variability in chlorine removal by position, and it was evenly weighted across the three positions (factor weights of +0.94, +0.98 and +0.92 for *ortho*, *meta* and *para* chlorines, respectively). The other principal components were not informative (eigenvalues < 1). Position analysis was, however, complicated by our approach of evenly splitting coeluting peaks. Identification of the exact dechlorination pathway was not the primary objective of the present study, but will garner more attention in further investigation.

Table 1-4. First order dechlorination rates (*k*) from sediment microcosms and Day 415 subcultures.

Treatment	Sediment Microcosms		Day 415 Subcultures	
	k **(yr^{-1})**	**p**	***k*** **(yr^{-1})**	**p**
Unamended control	-0.028^{a}	0.27	0.001^{a}	0.91
Killed control	$0.002^{a,b}$	0.95	NP	NP
Electron donor	$0.040^{b,c}$	0.10	0.002^{a}	0.80
PCB116	$0.004^{a,b}$	0.84	0.002^{a}	0.73
Tetrachlorobenzene	$0.007^{a,b}$	0.80	**0.022^{b}**	0.0009
Bioaugmentation (*D. ethenogenes* strain 195)	**0.069^{c}**	0.007	0.004^{a}	0.52
Pentachloronitrobenzene (PCNB)	**0.077^{c}**	0.002	**0.118^{c}**	< 0.0001
PCNB plus bioaugmentation	**0.062^{c}**	0.007	**0.098^{d}**	< 0.0001

Dechlorination in sediment microcosms was of historical PCBs; dechlorination rates presented for Day 415 subcultures are for spiked PCB116. The p value is for the effect of time (day of sampling) from the mixed model analysis and indicates whether the slope of ln(chlorination level) vs. time (β in the mixed model, equal to a first order constant *k*) is significantly different from zero. Chlorination level in the sediment microcosms calculated from the mole fraction of all di- through deca-chlorinated biphenyls was quantified. For the Day 415 subcultures, chlorination level is calculated only from PCB116 (2,3,4,5,6-CB) and its three possible first dechlorination products: 2,3,4,5-CB, 2,3,4,6-CB and 2,3,5,6-CB.

^{a,b,c,d} - Treatments not sharing a letter have significantly different slopes at the p = 0.1 level.
NP: Day 415 subcultures not prepared for killed controls.

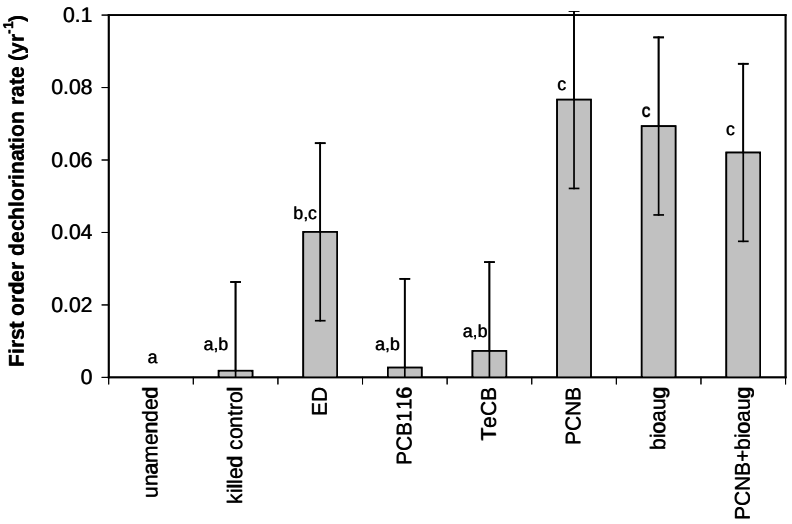


Figure 1-3. First order dechlorination rate of different treatments in Anacostia microcosms Day 0 to 415. Error bars show the standard error calculated from mixed model analysis. Only treatments with PCNB, PCNB plus bioaugmentation and bioaugmentation alone exhibited rates of dechlorination that were significantly different than zero. $^{a.,b,c}$ Treatments not sharing a letter are significantly different at the p = 0.1 level.

Sustained capacity for dechlorination of a model PCB congener. The capacity of the microbial populations stimulated by amendment with PCNB, PCNB plus bioaugmentation and TeCB for sustained dechlorination was demonstrated by dechlorination of spiked PCB116 in microcosm subcultures established at 415 days (**Fig. 1-4)**. Only media, electron donor and PCB116 were added to the subcultures. PCB116 (2,3,4,5,6-CB) was dechlorinated first to 2,3,4,6-CB and then to 2,4,6-CB in the TeCB, PCNB and PCNB plus bioaugmentation treatments. Dechlorination occurred with no lag, and resulted in greater than 10 % dechlorination of the added PCB116 within 90 days. The TeCB, PCNB and PCNB plus bioaugmentation treatments exhibited statistically significant ($p < 0.001$) PCB116 dechlorination rates compared to the unamended control, while the bioaugmentation alone and electron donor alone treatments did not (**Table 1-4)**. The rate for PCNB > PCNB plus bioaugmentation > TeCB. The calculated first order dechlorination rates in the subcultures were higher than in the initial microcosms, most likely because of the higher concentration and higher bioavailability of the freshly-spiked single congener compared to the historical PCBs. Note that the relative observed differences in dechlorination activity between treatments were solely caused by a sustained effect from the amendments that were added 14 months earlier.

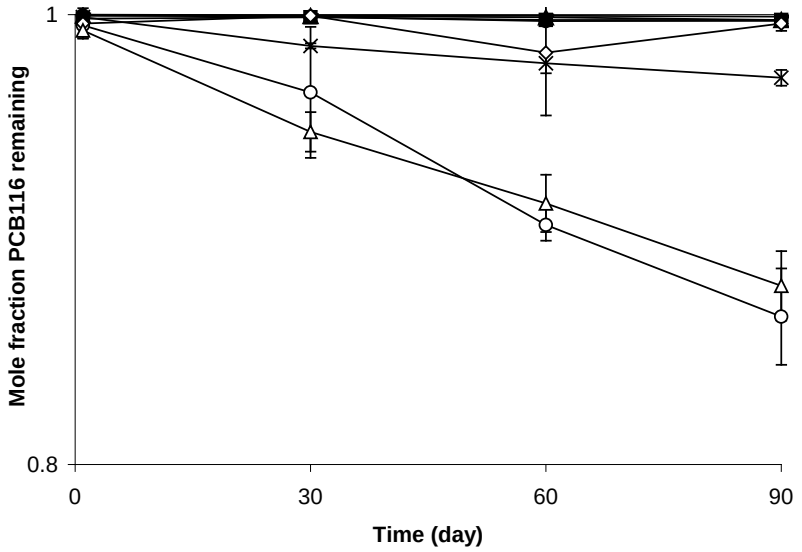


Figure 1-4. PCB116 dechlorination in Day 415 subcultures spiked with 3.1 µM PCB116 and an electron donor mix. The mole fraction is calculated from the ratio of PCB116 (2,3,4,5,6-CB) remaining to the sum of PCB116 and its possible first dechlorination products, 2,3,4,5-CB, 2,3,4,6-CB, and 2,3,5,6-CB. Live (unamended) control (■), electron donor (▲), PCB116 (●), TeCB (×), PCNB (○), bioaugmentation (◊), and PCNB + bioaugmentation (△). Error bars show standard deviation of three subcultures per treatment.

Response of dechlorinating bacteria. Dechlorinating bacterial strains were detected by PCR-DGGE at all time points and changes in relative DGGE band intensities for some strains were observed in TeCB- and PNCB-amended microcosms over the duration of the study. The results of nested PCR-DGGE for microcosm samples collected through Day 281 are shown in **Figure 1-5**. We performed this analysis for triplicate microcosms and the results shown reflect those observed in the other replicates. Bands A and B which exhibited the greatest band intensity changes over time were selected for sequencing. Band A was detectable at low intensities in unamended sediment but band B (strain 195) was not (data not shown). The 16S rRNA gene from band A, observed in most microcosms, was consistent with the Pinellas subgroup of

Dehalococcoides spp., which contains strains CBDB1, BAV1, FL2 and GT among others (Adrian et al., 2000; Sung et al., 2006; He et al., 2003, 2005). The 16S rRNA gene from band B, observed only in bioaugmented microcosms, was identical to that of *D. ethenogenes* strain 195 used in the bioaugmentation treatments and eluted at the same distance on the gel as an amplicon produced from strain 195 and used as a positive control (**Fig. 1-5**). The intensity of band B was initially strong after bioaugmentation, but it decreased by Day 185 and was not detected by Day 281. TeCB and PCNB appeared to stimulate native *Dehalococcoides* spp. (band A), leading to an increase in the DGGE band intensity that was sustained through Day 281. In the PCNB plus bioaugmentation treatment, the increase in the band intensity representing native *Dehalococcoides* spp. (band A) was delayed until Day 60. PCNB stimulated PCB dechlorination in the microcosms and in PCB116-amended subcultures tested at Day 415. PCB116 and electron donor alone did not significantly enhance native PCB dechlorinators; an observation supported by insignificant PCB dechlorination and the lack of increase in band A intensity in electron donor alone and PCB116 amended microcosms (**Fig. 1-5**). The *Dehalococcoides* population dynamics suggested by the DGGE results fits the observed dechlorination activities in the microcosms and Day 415 subcultures. Increases in both the stimulated native strain (band A) and the strain used for bioaugmentation (band B, *D. ethenogenes* strain 195) corresponded to increased dechlorination in the microcosms. However, because strain 195 disappeared over the first 281 days, subcultures taken from the bioaugmented microcosms at Day 415 were not able to respond to the challenge of spiking with PCB116 (**Fig. 1-5**).

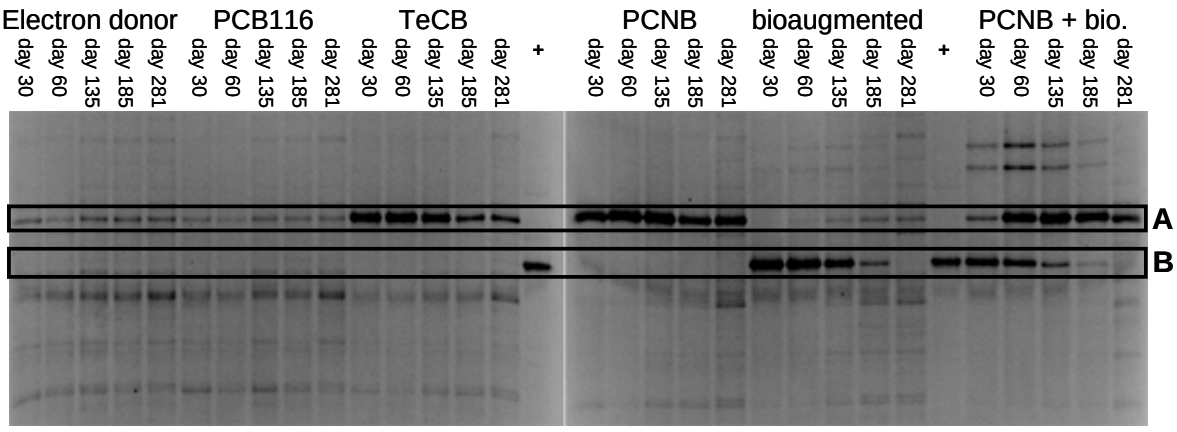


Figure 1-5. DGGE gel from initial microcosms, Days 0 through 281. DNA product from nested PCR using primers for *Chloroflexi*, followed by general bacterial primers. +: positive control (*Dehalococcoides ethenogenes* strain 195). Band **A**: 100% identity with Pinellas subgroup *Dehalococcoides* spp., band **B**: *D. ethenogenes* strain 195, used for bioaugmentation. The smiling of bands near the edges of the DGGE gels was corrected using Adobe® Photoshop® (Adobe Systems Inc., San Jose, CA).

Implications. Historical PCBs are likely to be strongly sorbed to sediment and less bioavailable than recently introduced (e.g., experimentally introduced) PCBs. Further, if dechlorination has occurred *in situ*, congeners most amenable to dechlorination will have been preferentially dechlorinated. The enhanced dechlorination rates (0.062 to 0.077 yr^{-1}) for low concentration PCBs (2 mg/kg dry wt) observed in sediment microcosms from Anacostia River undergoing

biostimulation or bioaugmentation are similar to those reported for higher concentrations of aged PCBs. For example, Alder et al. (1993) observed dechlorination of historical PCBs in New Bedford Harbor sediment (400 to 500 mg/kg PCBs) with a first order dechlorination rate constant of 0.10 yr^{-1}; and Abramowicz et al. (1993) measured dechlorination of pre-existing PCBs (20 mg/kg) in Hudson River sediment spiked with 500 ppm PCB105 (2,3,3',4,4'-CB) at a rate of 0.29 yr^{-1}. Magar et al. (2005) estimated an *in situ* dechlorination rate of approximately 0.022 yr^{-1} using analysis of sediment cores from Lake Hartwell, SC (1 to 60 mg/kg dry weight PCBs).

Because of high cost, dredging of contaminated sediment for *ex situ* treatment or disposal may be reserved as a remedial treatment option for only the most highly PCB-contaminated sediment at a particular site. For example, in the Fox River, WI, sediments containing up to 10 mg/kg total PCBs may be capped according to the Record of Decision (USEPA, 2008). Sediments with lower PCB concentrations thus left in place may be targets of *in situ* remedial efforts, perhaps in conjunction with capping. The dechlorination rates reported here, derived from a freshwater system with low concentrations (2 mg/kg) of mixed source, weathered PCBs may be predictive of those expected for residual PCBs following removal of hotspots undergoing *in situ* biostimulation and/or bioaugmentation treatment options for sediment left in place.

The effect of halopriming on native *Dehalococcoides* spp. for Anacostia sediments are similar to those of Ahn et al. (2007, 2008), who found that addition of TeCB to River Kymijoki, Finland sediment increased the prevalence of a native *Dehalococcoides* spp. with a 16S rRNA gene sequence identical to strain CBDB1. In that study however, both halopriming and bioaugmentation with *Dehalococcoides ethenogenes* strain 195 led to increased dechlorination of spiked 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (Ahn et al. 2007, 2008). For Anacostia River sediment microcosms stimulating native populations resulted in more sustained capacity for PCB dechlorination, as measured by dechlorination of spiked PCB116, over 415 days when compared with bioaugmentation by *D. ethenogenes* strain 195, which is known to dechlorinate PCB116. A slight lag in the response of native *Dehalococcoides* spp., as evidenced by observing band intensity in DGGE of 16S rRNA genes (**Fig. 1-5**), in the PCNB plus bioaugmentation treatment, coupled to the significantly lower PCB116 dechlorination activity after 415 days when compared to PCNB alone, suggests that bioaugmentation may have suppressed native *Dehalococcoides* spp. This could have been caused by competition for electron acceptor or electron donor as suggested by modeling studies of competition between chloroethene dechlorinators by Becker (2006).

Himmelheber et al. (2007) detected and quantified *Dehalococcoides* and reductive dehalogenase genes *tceA*, *vcrA* and *bvcA* in PCE-enriched Anacostia River sediments. Although Himmelheber et al. (2007) did not determine specific 16S rRNA gene sequences for *Dehalococcoides* spp., they speculated that the *tceA* gene could be associated with strains 195 and FL2, the *vcrA* gene with strain GT and the *bvcA* gene with strain BAV1. We did not detect *D. ethenogenes* strain 195 in non-bioaugmented Anacostia sediment; however the native *Dehalococcoides* spp. 16S rRNA gene sequence detected was consistent with the Pinellas subgroup which includes strains FL2, BAV1 and GT (Sung et al., 2006).

Although TeCB and PCNB appeared to increase the population of the native *Dehalococcoides* spp., only PCNB led to biostimulation of PCB dechlorination. This suggests that, for practical applications, detecting a simple increase in *Dehalococcoides* spp. (not strain-specific) may not ensure increases in PCB dechlorination rates *in situ*. Because the gel imaging technique used here is not quantitative, we do not know whether biostimulation with PCNB resulted in a greater enhancement of the native *Dehalococcoides* population than TeCB did. Because different species in the Pinellas subgroup have the same 16S rRNA gene sequence, it is possible that different native *Dehalococcoides* spp., perhaps with identical 16S rRNA gene sequences, but with differing functional capabilities, were stimulated by the different haloprimers. PCNB and TeCB appear to have enriched for different *Dehalococcoides* sub-populations that differ in their dechlorination activity (see Accomplishments Section 2).

For *in situ* bioremediation of PCB contaminated sediments, the persistence of the proposed biostimulatory or bioaugmentation effect should be considered, and monitored over time to determine if the enhancement persists. This would provide guidance on whether or when additional stimulation is needed. The dechlorination extent observed in this experiment was relatively modest, ranging from 0.4 to 0.6 chlorines per biphenyl (7 to 8% of total Cl^-) over 415 days for the PCNB, bioaugmentation, and PCNB plus bioaugmentation treatments. We also noted that much of this stimulation was achieved after the first 135 days which implies that repeated stimulation might be needed to avoid a plateau in dechlorination. While haloprimers such as PCNB and TeCB show promise, because of environmental concerns, they would likely not be applied *in situ*, but rather used to develop activated dechlorinating bacteria for bioaugmentation. Even in the case of bioaugmentation using *ex situ* enrichments of dechlorinating bacteria, the fate of residual haloprimers will need to be considered. Eventual field use will require that dechlorination of the haloprimer results in a benign product, or that the haloprimer (or its dechlorination products) can be removed, for example, by volatilization before application. We are investigating this approach at the mesocosm scale.

Conclusions

- Dechlorination of low-concentration weathered PCBs was significantly enhanced in Anacostia River sediment microcosms receiving bioaugmentation, PCNB and PCNB plus bioaugmentation, compared to other treatments receiving electron donor only TeCB, or PCB 116.
- Microcosm subcultures generated after 415 days and spiked with PCB 116 showed sustained capacity for dechlorination of PCB116 in PCNB, PCNB plus bioaugmentation, and TeCB treatments.
- TeCB and PCNB amendment increased native *Dehalococcoides* spp. from the Pinellas subgroup.
- *D. ethenogenes* strain 195 added as a bioaugmentation agent was detected only in bioaugmented microcosms and appeared to decrease over 281 days.
- Bioaugmentation with *D. ethenogenes* strain 195 increased PCB dechlorination rates initially in sediment microcosms, but dechlorination of PCB116 after 415 days occurred only in subcultures of microcosms with enhanced native *Dehalococcoides* spp.
- Our study suggests that enhanced dechlorination may be obtained by increasing the population and activity of specific dechlorinating bacteria, but that 16S rRNA-based detection alone is not sufficient for predicting PCB dechlorination activity.

2. The Effect of Cosubstrate-Activation on Indigenous and Bioaugmented PCB Dechlorinating Bacterial Communities in Sediment Microcosms

(*Park J-W, Krumins V, Kjellerup BV, Fennell DE, Rodenburg LA, Sowers KR, Kerkhof LJ, Häggblom MM. 2011. The effect of co-substrate activation on indigenous and bioaugmented PCB-dechlorinating bacterial communities in sediment microcosms. Appl. Microbiol. Biotechnol 89:2005-2017*)

Abstract

Microbial reductive dechlorination by members of the phylum *Chloroflexi,* including the genus *Dehalococcoides*, may play an important role in natural detoxification of highly chlorinated environmental pollutants, such as polychlorinated biphenyls (PCBs). Previously, we showed the increase of an indigenous bacterial population belonging to the Pinellas subgroup of *Dehalococcoides* spp. in Anacostia River sediment (Washington DC, USA) microcosms treated with halogenated co-substrates ("haloprimers"), tetrachlorobenzene (TeCB) or pentachloronitrobenzene (PCNB). The PCNB amended microcosms exhibited enhanced dechlorination of weathered PCBs, while TeCB amended microcosms did not. We therefore developed and used different phylogenetic approaches to discriminate the effect of the two different haloprimers. We also developed complementary approaches to monitor the effects of haloprimer treatments on 12 putative reductive dehalogenase (*rdh*) genes common to *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain CBDB1. Our results indicate that 16S rRNA gene-based phylogenetic analyses have a limit in their ability to distinguish the effects of two haloprimer treatments and that two of *rdh* genes were present in high abundance when microcosms were amended with PCNB, but not TeCB. *rdh* gene-based phylogenetic analysis supports that these two *rdh* genes originated from the Pinellas subgroup of *Dehalococcoides* spp., which corresponds to the 16S rRNA gene-based phylogenetic analysis.

Introduction

Polychlorinated biphenyls (PCBs) are toxic and widespread pollutants in soils and sediments. Furthermore, these organohalides are problematic because of their greater stability compared to less chlorinated compounds, which are more readily degraded by aerobic microorganisms (Häggblom, 1992; Wittich, 1998). However, under anaerobic conditions, certain dechlorinating bacteria can use a variety of polychlorinated compounds as a terminal electron acceptor in the process of respiratory reductive dehalogenation or dehalorespiration (Bedard and Quensen, 1995; Löffler et al., 2003; Bedard, 2008; Hiraishi, 2008). This respiratory activity may play an important role in detoxification of organohalides released into the environment. Several groups of bacteria involved in anaerobic dechlorination process have been reported, including members of low G+C Gram positive *Desulfitobacterium,* gamma-, delta-, and epsilon-*Proteobacteria*, and *Dehalococcoides* within the phylum *Chloroflexi* (Löffler et al., 2003). A unique feature of the genus *Dehalococcoides* and other species within the phylum *Chloroflexi* is the ability to respire by reductive dehalogenation of PCBs in addition to other polychlorinated aromatic compounds. Several studies suggest a role in the natural dechlorination of PCBs by *Dehalococcoides* spp. and closely related species (Cutter et al., 2001; Fagervold et al., 2005, 2007; Watts et al., 2005; Bedard et al., 2006, 2007; Yan et al., 2006a, b; Bedard, 2008; Field and Sierra-Alvarez, 2008; Kjellerup et al., 2008; Adrian et al., 2009). Two groups in the phylum *Chloroflexi* are known for

their ability of respiratory dehalogenation; one is the o-17 (Cutter et al., 2001) and DF-1 strain clade (group III) (May et al., 2008) and the other is the *Dehalococcoides* clade (group II) (Bedard, 2008). Group II is further separated into three subgroups based on the variable regions 2 and 6 of their 16S rRNA genes; the so-called Cornell, Victoria, and Pinellas subgroups (Hendrickson et al., 2002). *D. ethenogenes* strain 195 belongs to the Cornell subgroup, while *Dehalococcoides* sp. strains CBDB1, BAV1, and FL2 belong to the Pinellas subgroup. Both strains 195 and CBDB1 have been shown to dechlorinate polychlorinated aromatic compounds, including PCBs (Adrian et al., 2000, 2009; Fennell et al., 2004; Liu and Fennell, 2008). Application of halogenated co-substrates as priming compounds ("haloprimers"), bioaugmentation with *Dehalococcoides* strains, and combined application of haloprimer plus bioaugmentation have been reported to stimulate rates of anaerobic dechlorination (Wu et al., 1997; Bedard et al., 1998, 2005, 2007; Vargas et al., 2001; Ahn et al., 2005, 2007, 2008; Shiang Fu et al., 2005; Winchell and Novak, 2008). However, it is unclear whether different halogenated co-substrates stimulate the same dehalogenating communities, and whether the stimulated community will exhibit the desired function, *viz*., increased dehalogenation of the target contaminant.

PCR-based 16S rRNA gene assays have been described for monitoring indigenous dehalogenating communities within the phylum *Chloroflexi*, including *Dehalococcoides* species (Hendrickson et al., 2002; Fagervold et al., 2005; Watts et al., 2005; Yoshida et al., 2005). A single-nucleotide primer extension assay is available also for the three subgroups in group II *Dehalococcoides* species involved in anaerobic dechlorination processes (Nikolausz et al., 2008; Yohda et al., 2008). However, a limitation of these 16S rRNA gene based assays is that *Dehalococcoides* species typically represent a small fraction of the total bacterial community (Major et al., 2002; Lendvay et al., 2003; Amos et al., 2008) and their 16S rRNA genes are highly conserved (Hendrickson et al., 2002; Duhamel et al., 2004; He et al., 2005; Kube et al., 2005; Sung et al., 2006). As a result, it is difficult to detect closely related strains within the dechlorinating community by monitoring changes in 16S rRNA phylotypes alone. Several *Dehalococcoides* genomes have been sequenced and found to contain multiple putative reductive dehalogenase (*rdh*) genes (Hölscher et al., 2004; Kube et al., 2005; Seshadri et al., 2005: McMurdie et al., 2009), which can be used to refine the identification of species/strains with near-identical 16S rRNA gene sequences. This also creates an opportunity to determine which *rdh* genes are involved in enhanced dechlorination processes.

We previously demonstrated that biostimulation and bioaugmentation can enhance dechlorination of low concentration historical PCBs in microcosms of sediments from the Anacostia River, Washington DC (see Results and Discussion Section 1; Krumins et al. 2009). Although previous analysis of 16S rRNA gene phylotypes demonstrated the enrichment of *Dehalococcoides* species with either 1,2,3,4-tetrachlorobenzene (TeCB) or pentachloronitrobenzene (PCNB) as co-substrates, only PCNB stimulated significant reductive dechlorination of weathered PCBs. To address this apparent discrepancy we conducted a comprehensive analysis of the dechlorinating communities. Specifically, we report here on the in-depth analysis of the PCB dechlorinating communities by using complementary phylogenetic methods and by monitoring 12 putative *rdh* genes common to two known PCB dechlorinating bacteria; *D. ethenogenes* strain 195 and *Dehalococcoides* sp. strain CBDB1. Our results demonstrate that two *rdh* genes were detected only in microcosms that previously showed the

dechlorination of weathered PCB (Krumins et al. 2009). The techniques demonstrated here suggest the potential for use of PCR-based *rdh* gene assays for monitoring *in situ* bioremediation at PCB contaminated sites.

Materials and Methods

Microcosm preparation. Anacostia River sediment was collected from a capping site control plot (Horne Engineering Services and Sevenson Environmental Services, 2004) on 7 July 2006, using a Van Veen dredge. Microcosms were setup as previously described (Krumins et al., 2009). Briefly, in addition to non-amended and autoclaved controls, the microcosm conditions consisted of: 1) mixture of electron donors only, 2) electron donors plus 2,3,4,5,6-pentachlorobiphenyl (PCB116), 3) electron donors plus 1,2,3,4-tetrachlorobenzene (TeCB), 4) electron donors plus pentachloronitrobenzene (PCNB), 5) electron donors plus bioaugmentation with a mixed culture containing *D. ethenogenes* strain 195, and 6) electron donors plus PCNB plus bioaugmentation with a mixed culture containing *D. ethenogenes* strain 195. All microcosm treatments were prepared in triplicate. The mixed culture containing *D. ethenogenes* strain 195 was grown at 25 °C on tetrachloroethene and butyric acid using methods described previously (Fennell et al., 2004; Krumins et al., 2009). The mixed culture containing *D. ethenogenes* strain 195 was first described in Fennell et al. (1997). This culture was obtained from Professor James M. Gossett (Cornell University) in 2003 and was cultivated and maintained by Fennell at Rutgers since 2003. Four mL of the mixed culture was aseptically and anaerobically inoculated to the 200 mL bioaugmented microcosms, resulting in an estimated initial *D. ethenogenes* strain 195 population of 3×10^6 cells per mL (Fennell et al., 2004; Krumins et al., 2009). The microcosms were stirred, capped and incubated upright and statically in the dark at 26 °C. These samples were previously analyzed for PCB dechlorination activity (Krumins et al., 2009).

DNA extraction. Sediment samples were collected from each of triplicate microcosms under nitrogen purge at selected time points using a sterile spatula. DNA was extracted from 0.3 g sediment samples using the PowerSoil™ DNA Isolation Kit according to the manufacturer's instructions (MoBio Laboratories Inc., Carlsbad, CA). DNA extracts were stored at −20 °C prior to analysis.

Community analysis by denaturing gradient gel electrophoresis (DGGE) and nested PCR-DGGE. The DCode TM system (Bio-Rad Laboratories, Hercules, CA) was used for DGGE analysis. All triplicate microcosms were analyzed by this method. PCR reaction conditions and cycles were as described previously (Krumins et al., 2009). After electrophoresis, the gels were stained with ethidium bromide and photographed on a UV transilluminator. Image analyses of the DNA profiles and band intensities were conducted using Quantity One® (version 4.5.0; Bio-Rad Laboratories, Hercules, CA).

Community analysis by terminal restriction length polymorphism (TRFLP). TRFLP analyses were performed using 27F and Chl1101R for putative dechlorinating *Chloroflexi* communities in one replicate set from triplicate microcosms (**Table 2-1**). The fluorescent dye, 6-carboxyfluoroscein [6-FAM] was linked to the 5' end of primer 27F for TRFLP analysis. PCR reaction conditions and cycles were the same as for amplification with 338F and Chl1101R for DGGE analysis. PCR products were purified with the PCR purification kit (MoBio Laboratories

Inc., Carlsbad, CA) and digested for 6 h at 37 °C with *MnlI* (New England Biolabs, Beverly, MA). Ten ng of labeled PCR product was separated on an ABI 310 genetic analyzer (Perkin-Elmer) with Genescan software and internal standards. Peak detection limits were set at 50 fluorescent units and fragment size was restricted to 50-500 bp. For the *Chloroflexi* profiles positive amplifications representing 10 ng were digested, while equal volumes of the negative amplifications were also screened.

Table 2-1. Primer sets for PCR, TRFLP, DGGE, DHPLC, and *rdh* genes.

Primers	Sequences (5' to 3')	Target (expected size‡)	Reference
Chl1101R	CTCGCKAGAAMATKTAACTAGCAAC	putative dechlorinating *Chloroflexi* spp.	This study
27F-FAM*	AGAGTTTGATCMTGGCTCAG	bacteria-specific	Lane, 1991
338F	ACTCCTACGGGAGGCAGCAG	bacteria-specific	Lane, 1991
1525R	AAGGAGGTGWTCCARCC	bacteria-specific	Lane, 1991
341F	CCTACGGGAGGCAGCAG	bacteria-specific	Muyzer et al., 1993
341F-GC†	CCTACGGGAGGCAGCAG	bacteria-specific	Muyzer et al., 1993
534R	ATTACCGCGGCTGCTGG	universal	Muyzer et al., 1993
RDH01F	TGGCTTATGGCTGTTCCAA	DET0180, cbdb_A187	This study
RDH01R	TATCTCCAGGGAGCCCATTC	(148 bp)	This study
RDH02F	GCCGAATTCTGCCCTGT	DET0235, cbdb_A243	This study
RDH02R	CAGRRARCCATARCCAAAGG	(281 bp)	This study
RDH03F	CAAGATGGATAGGCCTGCAT	DET0302, cbdb_A238	This study
RDH03R	ATGGTGCTATCCTGACCGAG	(192 bp)	This study
RDH04F	GATGATACGATTTATGGCAATC	DET0306, cbdb_A1495	This study
RDH04R	CCRAACGGGAARTCTTCTTC	(215 bp)	This study
RDH05F	AAGGATATCAAGTCCAGTATCC	DET0311, cbdb_A88	This study
RDH05R	ATACCTTCRAGCGGCCARTAT	(133 bp)	This study
RDH06F	CACCCCGGTTCGTTCATACA	DET0318, cbdb_A1588	This study
RDH06R	AGTCATCCACTTCRTCCCAC	(102 bp)	This study
RDH07F	TGTCCGGCACTCTTAAACC	DET1171, cbdb_A1092	This study
RDH07R	GCYGCCGCYGGCAGTTACTG	(200 bp)	This study
RDH08F	GGAAAGGCCATCATCAAAC	DET1519, cbdb_A1575	This study
RDH08R	GTCTTRCMGGRGTAACCYTG	(184 bp)	This study
RDH09F	GGTGAGATTTAAAATTGTTGGC	DET1522, cbdb_A1570	This study
RDH09R	CTGGGTGCGGTWGCCGCAKC	(136 bp)	This study
RDH10F	TCCTGAGCCGACAGGGT	DET1535, cbdb_A1595	This study
RDH10R	TTTCATTCMACACTYTCMCG	(223 bp)	This study
RDH11F	ATTTACCCTGTCCCATCC	DET1538, cbdb_A1627	This study
RDH11R	TTTCACASTAGYCTKAGCCGMAG	(235 bp)	This study
RDH12F	GCCCGTCATGGCGTTCCATC	DET1545, cbdb_A1638	This study
RDH12R	GAGCAAGTTTCATTCMATGG	(187 bp)	This study

*FAM (5'end-labeled with carboxyfluorescine)
†GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3')

Community analysis by denaturing high pressure liquid chromatography (DHPLC). DHPLC analyses were performed using a WAVE 3500 HT system (Transgenomic, Omaha, NE) equipped with an ultraviolet detector as described previously (Kjellerup et al., 2008). The same replicate sample set used for TRFLP was analyzed by this method. PCR was conducted in 50 µL reaction volumes using GeneAmp reagents (Applied Biosystems, Foster City, CA). PCR amplicons were obtained using the same nested PCR approach as described previously for the nested DGGE (Krumins et al., 2009). Briefly, DNA samples were amplified with 338F and Chl1101R (**Table 2-1**) with the same reaction conditions and cycles as for PCR with 338F and Chl1101R for DGGE, purified with the PCR purification kit (MoBio Laboratories Inc., Carlsbad, CA), and re-amplified with 341F and 534R as described previously (Park and Crowley, 2006) to analyze the putative dechlorinating *Chloroflexi* community. PCR products of the correct length were confirmed by electrophoresis using a 1.5% agarose gel prior to analysis by DHPLC. The 16S rRNA gene fragments were analyzed in a 20 µL injection volume by DHPLC with a DNASep® cartridge packed with alkylated nonporous polystyrene-divinylbenzene copolymer microspheres for high-performance nucleic acid separation (Transgenomic, Omaha, NE). The oven temperature was 62.8 ºC and the flow rate was 0.5 ml per min with a gradient of 55% to 35% Buffer A and 45% to 65% Buffer B from 0-13 minutes. The analytical solutions used for the analyses were: Buffer A (0.1 M triethylammonium acetate (TEAA), pH 7), Buffer B (0.1 M TEAA and 25% acetonitrile, pH 7), Solution D (25% water and 75% acetonitrile) and Syringe Wash Solution (Transgenomic, Omaha, NE). Analysis was performed using the Wavemaker version 4.1.44 software. An initial run was used to identify individual PCR fragments and determine their retention times. Individual peaks were eluted for sequencing from a subsequent run and collected with a fraction collector based on their retention times. The fractions were collected in 96 well plates (Biorad, Hercules, CA) and dried using a Savant SpeedVac system (Thermo Electron Corporation, Waltham, MA) followed by dissolution in 30 µL nuclease free water. Re-amplification was performed following the protocol described above and PCR amplicons purified for sequencing.

Bacterial identification and phylogenetic analysis. DGGE bands were excised from the gel and eluted overnight in 50 µl of MilliQ H_2O at 4 °C. Eluted DGGE bands were re-amplified with 341F-GC and 534R and purity was confirmed by DGGE. The eluted single DGGE bands were amplified with 341F and 534R, purified using a PCR purification kit (MoBio Laboratories Inc., Carlsbad, CA), and used as a template for DNA sequencing. In cases where direct sequencing of the PCR fragments was unsuccessful, they were cloned into pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Corp. Carlsbad, CA) according to the manufacturer's instructions. DNA sequencing was performed by Genewiz, Inc. (North Brunswick, NJ). DHPLC peaks were collected and reamplified for sequencing as described earlier. The PCR amplicons were electrophoresed in a 1.5% low melt agarose gel and the excised fragment was purified for sequencing using Wizard® PCR Preps DNA Purification Resin/ A7170 (Promega Corp., Madison, WI). Each DHPLC fraction was sequenced in the 5' direction with 250 pM of primer 341F using the BigDye® Terminator v3.1 kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing of purified DNA was performed on an ABI 3130 XL automated capillary DNA sequencer (Applied Biosystems, Foster City, CA). Homology searches were performed using BLAST (Altschul et al., 1997). CLUSTAL X, version 1.64b (Thompson et al., 1997) and GeneDoc version 2.0.004 (Nicholas, 1997) were used to align the sequences and determine their homologies. Phylogenetic and molecular evolutionary analyses

were conducted using MEGA version 4 (Tamura et al., 2007). The partial 16S rRNA gene sequences are deposited in GenBank under accession numbers GU055492 to GU055496.

PCR and intensity analysis of reductive dehalogenase genes. Twelve sets of candidate reductive dehalogenase gene homologs were selected by comparing putative *rdh* genes in the genomes of *D. ethenogenes* strain 195 (Seshadri et al., 2005) and *Dehalococcoides* sp. strain CBDB1 (Kube et al., 2005). PCR primer sets (**Table 2-1**) were manually designed for specific hybridization with each set of genes. Primer specificities were confirmed by performing a sequence homology search using BLAST (Altschul et al., 1997). These twelve sets of primers were designed to detect *rdh* genes in *Dehalococcoides* strain 195 and strain CBDB1, but other orthologous *rdh* genes would most likely also be recognized by the primers, including DehaBAV1_0173 (RDH01F/R), DehaBAV1_0121 (RDH03F/R), DehaBAV1_0104 (RDH05F/R), and DehaBAV1_1302 (RDH12F/R) in strain BAV1; KB1RdhB13 (RDH06F/R) in strain KB1 and RdhA13 (RDH08F/R) in strain FL2. Oligo-Analyzer 3.1 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/), an online service of IDT Biotools (Coralville, IA), was used to ensure minimal self-complementarity and to prevent the presence of secondary structures. PCR conditions were optimized by using DNA from *D. ethenogenes* strain 195 as a positive control. The temperature profile for all 12 sets was 94 °C for 5 min followed by 35 cycles of 94 °C for 20 s, 52 °C for 60 s, and 72 °C for 60 s. A final extension step was carried out for 7 min at 72 °C, after which the DNA was stored at 4 °C. PCR products were loaded in one 1.5% agarose gel with same well size for electrophoresis and a resulting gel image was used to measure relative band intensities by using the ImageJ quantification software (ver. 1.33u, National Institutes of Health, USA), according to the manufacturer's protocol (http://rsbweb.nih.gov/ij/).

Sequencing and phylogenetic analysis of reductive dehalogenase genes. The amplified *rdh* gene PCR products were purified using a PCR purification kit (MoBio Laboratories Inc., Carlsbad, CA) and used as a template for DNA sequencing. DNA sequencing was performed by Genewiz, Inc. (North Brunswick, NJ) and analyzed with BLAST (Altschul et al., 1997). CLUSTAL X, version 1.64b (Thompson et al., 1997), GeneDoc version 2.0.004 (Nicholas, 1997), and MEGA version 4 (Tamura et al., 2007) as described above. The sequences of *rdh* genes analyzed here are deposited in GenBank under accession numbers HQ010285 to HQ010291.

Real-time quantitative PCR of four reductive dehalogenase genes. Four sets of reductive dehalogenase genes (*rdh* 01, 04, 06, and 12) were analyzed by real-time quantitative PCR (qPCR) to measure the relative amount of target *rdh* gene in triplicate microcosms. SYBR Green real-time quantitative PCR was performed on an iCycler IQ thermocycler (Bio-Rad Laboratories, Hercules, CA) using a QuantiTect™ SYBR Green PCR Kit (Qiagen, Valencia, CA). Amplification was carried out in a total volume of 20 µL containing 10 µL of 2X QuantiTect™ SYBR Green PCR mixtures, 10 pM of each primer, and 1 µL of sample DNA. The temperature profile was 5 min at 94 °C followed by 45 cycles of 20 s at 94 °C, 60 s at 52 °C, and 60 s at 72 °C. Data analysis was carried out with iCycler software (version 3.0a; Bio-Rad Laboratories, Hercules, CA). Each of *rdh* genes amplified from *D. ethenogenes* strain 195 were serially diluted to generate a standard curve. The linear correlation coefficient for the standard curve was 0.98 or higher.

Results

***Dehalococcoides* population enrichment versus PCB dechlorination activity.** Our previous study showed that biostimulation with PCNB and bioaugmentation with *D. ethenogenes* strain 195 enhanced dechlorination of historical PCBs in Anacostia River microcosms (Krumins et al., 2009). The extent of chlorine removal in PCNB, bioaugmentation, and PCNB plus bioaugmentation treatments were statistically greater than those in other treatments. After 135 days of incubation approximately 10% removal of the total chlorines per biphenyl was observed in bioaugmentation treatments and 15% in PCNB treatments or in PCNB plus bioaugmentation treatments (**Fig. 2-1**; see details in section 1). In comparison, removal of the total chlorines per biphenyl in samples treated with solvent, electron donor, PCB116, or TeCB was 5% or less (**Fig. 2-1**). The rates of dechlorination over the total 415 days of incubation were also greater in microcosms treated with PCNB, bioaugmentation with *D. ethenogenes* strain 195, or combined application, relative to other treatments (Krumins et al., 2009). Considering that the TeCB-amended microcosms did not show substantially increased dechlorination of weathered PCBs compared to the controls while PCNB-amended microcosms exhibited greater dechlorination, our previous phylogenetic analysis was not able to discriminate the effect of two different haloprimers on the *Chloroflexi* community since the same Pinellas subgroup *Dehalococcoides* phylotype was enriched regardless of PCB dechlorination activity (Krumins et al., 2009). To address this discrepancy, we conducted a more comprehensive analysis as follows.

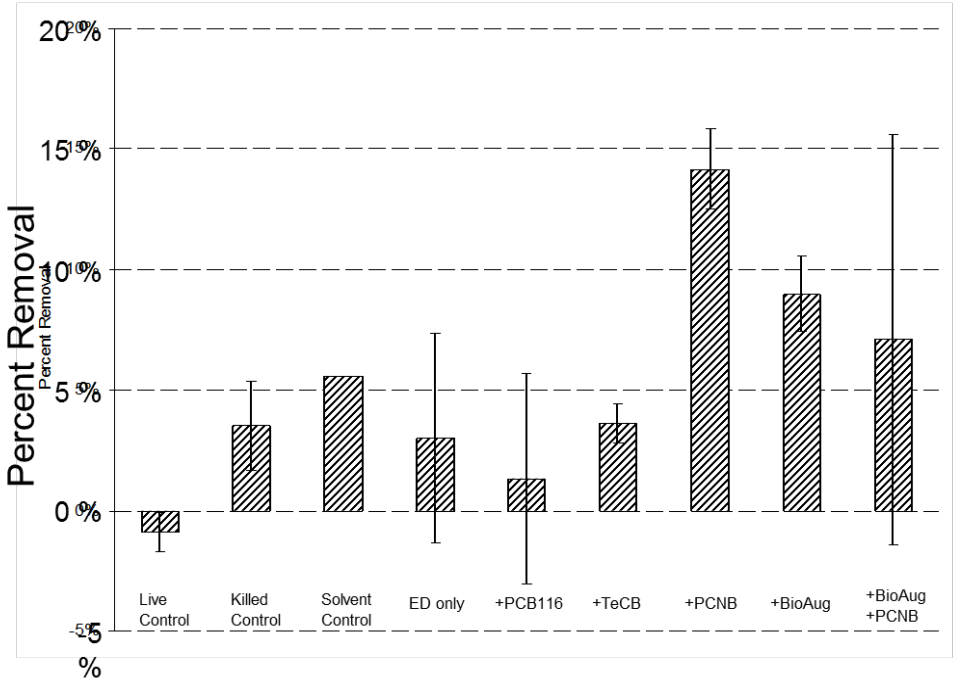


Figure 2-1. Percent chlorine removal based on the number of chlorines per biphenyl measured on day 135 in Anacostia microcosms. Error bars show the standard deviation of three microcosms per treatment. Only treatments with PCNB and/or bioaugmentation exhibited significantly different dechlorination rates. ED: electron donors, TeCB: tetrachlorobenzene, PCNB: pentachloronitrobenzene, BioAug: *D. ethenogenes* strain 195 mixed culture.

16S rRNA gene-based phylogenetic analysis of putative dechlorinating community. The effects of treatments on community profiles after 135 days of incubation were assessed by DGGE using nested PCR with a *Chloroflexi*-specific primer set (**Table 2-1**.) A number of *Chloroflexi* phylotypes were detected in all microcosms indicating a diverse *Chloroflexi* community in the Anacostia River sediments (**Fig. 2-2**). Most of the bands appeared to be minor with little change after the various treatments. However, a predominant band was present in microcosms treated with TeCB, PCNB, or PCNB plus bioaugmentation (**Fig. 2-2A, Band 1**), which corresponds to that observed previously (Krumins et al., 2009). Band 1 from these three microcosms was 100% identical to the 16S rRNA gene of the Pinellas subgroup of group II *Dehalococcoides*. Band 2, the sequence of which was identical to *D. ethenogenes* strain 195 and belonged to the Cornell subgroup of group II *Dehalococcoides*, was detected only in bioaugmented (with or without PCNB) microcosms (**Fig. 2-2A**). The intensity changes of the two *Dehalococcoides* phylotypes (Band 1 and Band 2) were monitored in Anacostia microcosms over the course of 415 days (**Fig. 2-3**). Since PCR primers could not be designed to distinguish between the two phylotypes, the relative changes in DGGE band intensity were used as a proxy to monitor the effect of treatments on the dechlorinating community. The intensity of Band 1 (the Pinellas subgroup of *Dehalococcoides* species) gradually increased in PCNB-treated

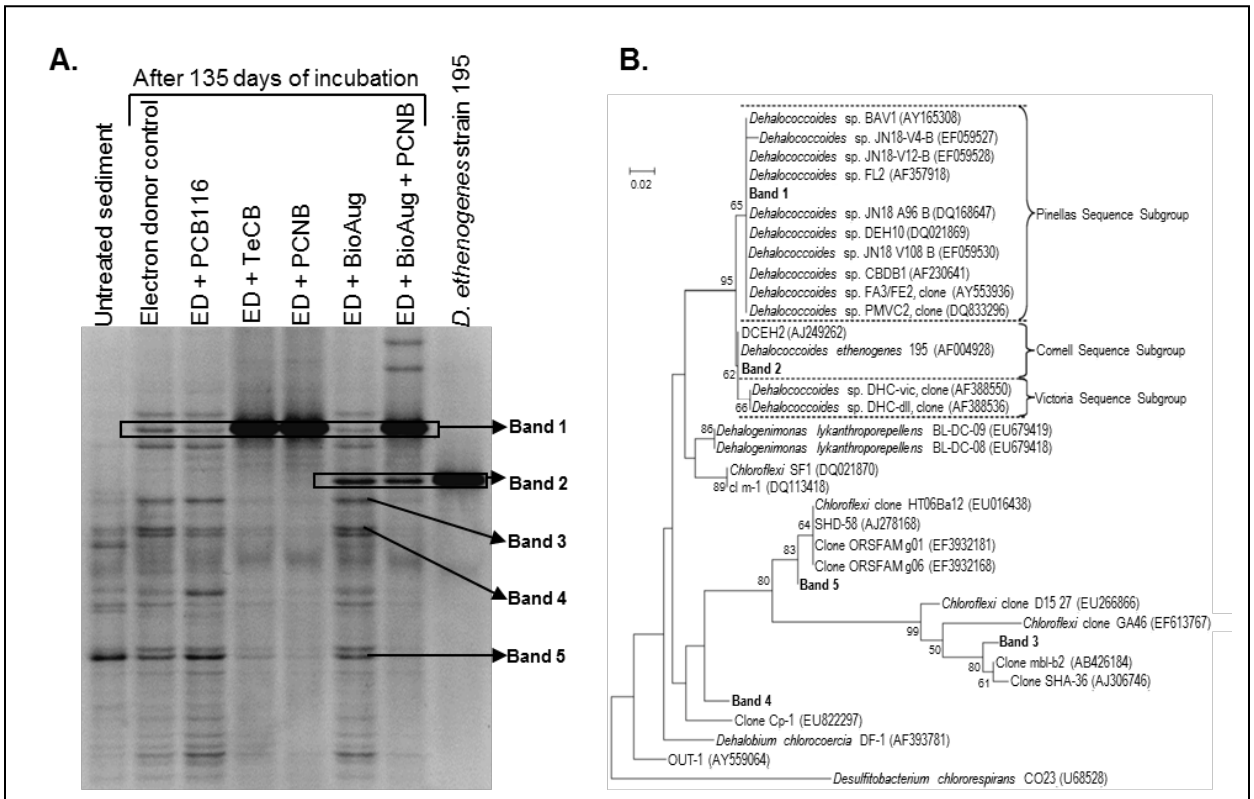


Figure 2-2. Phylogenetic analysis of putative dechlorinating *Chloroflexi* Community in Anacostia River sediment microcosms after 135 days of incubation. (A) nested-PCR DGGE analysis and (B) neighbor-joining tree based on DNA sequences of five DGGE bands and related 16S rRNA genes. Excluding primer regions, 104 bp of DNA sequences were used for analysis. Bootstrap values (1,000 replicates) higher than 50% are indicated at the branch points. The tree was derived from variable region 3 of the 16S rRNA gene. ED: electron donor, TeCB: tetrachlorobenzene, PCNB: pentachloronitrobenzene, BioAug: *D. ethenogenes* strain 195 mixed culture.

microcosms, while Band 2 (bioaugmented *D. ethenogenes* strain 195) intensity gradually decreased in both sets of microcosms over time. Phylogenetic analysis based on 16S rRNA gene sequences eluted from DGGE bands indicated that the *Chloroflexi* community in Anacostia River microcosms contained representatives of different subgroups of *Dehalococcoides* and other potential dehalogenating *Chloroflexi* (**Fig. 2-2B**), but was unable to discriminate the effect of two different haloprimers on this population.

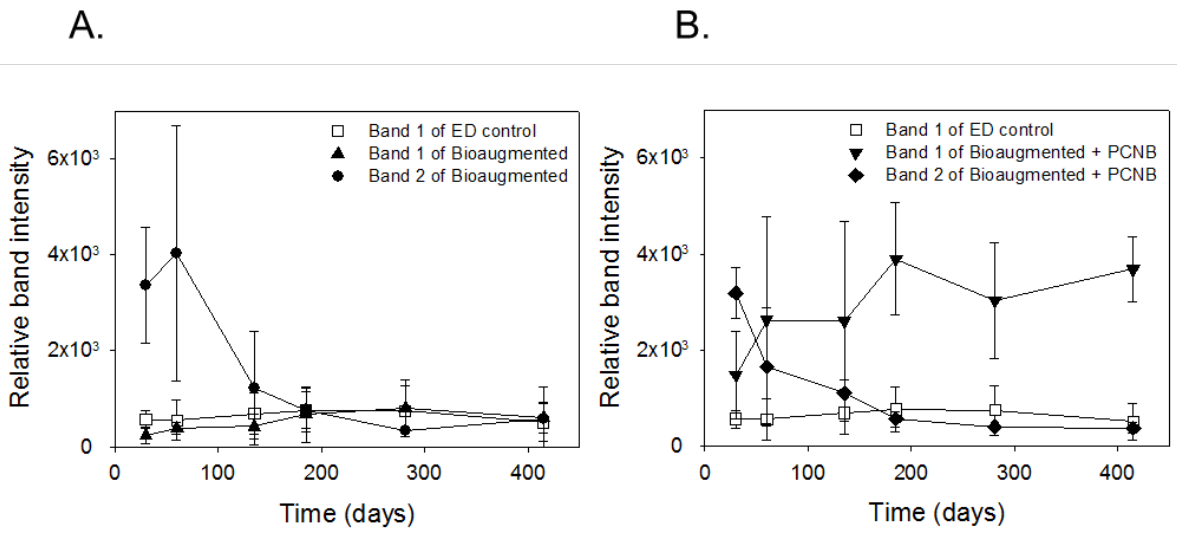


Figure 2-3. Relative intensity of DGGE Band 1 and Band 2 in Anacostia microcosms. Putative dechlorinating *Chloroflexi* community was analyzed by nested PCR-DGGE in bioaugmented (A) or in bioaugmented plus PCNB-treated microcosms (B). Data points represent the mean +/- standard deviation DNA extracted from three independent microcosms. ED: electron donors, PCNB: pentachloronitrobenzene, bioaugmented: *D. ethenogenes* strain 195 mixed culture.

TRFLP and DHPLC analyses of putative dechlorinating bacterial community. The effects of amendment and bioaugmentation treatments on the microbial communities were also determined by TRFLP analyses of the 16S rRNA genes in one replicate set from triplicate microcosms. The bacterial community profiles obtained with putative dechlorinating *Chloroflexi*–specific primers after 135 days of incubation are shown in **Fig. 2-4A**. The terminal restriction fragment (TRF; 105 bp) corresponding to *Dehalococcoides* spp., including strain 195, was observed in microcosms treated with haloprimers, bioaugmented with *D. ethenogenes* strain 195, or in the combined application (**Fig. 2-4A**, arrow). This particular TRF was observed only in low abundance in the electron donor control, solvent control, or in microcosms amended with PCB116. The overall bacterial community profiles obtained with general bacterial primers were similar between treatments (**Fig. 2-5**). This methodology demonstrated the enhancement of *Chloroflexi* in response to the haloprimer amendments, although it was not able to distinguish the effect of two different haloprimers as all have the same 105 bp TRF.

The effects of amendment and bioaugmentation treatments on microbial communities were also monitored by DHPLC using nested PCR with *Chloroflexi*-specific primers followed by amplification with universal bacterial primers. The results illustrate seven to ten *Chloroflexi*

phylotypes in the microcosms, where the electron donor control microcosm had the lowest diversity and the bioaugmented microcosm had the highest diversity (data not shown). Four to five peaks were observed in microcosms treated with electron donor in combination with TeCB, PCNB or PCNB plus bioaugmentation (**Fig. 2-4B**). A common peak eluting at 5 min was present in these microcosms (**Fig. 2-4B**, arrow). When the peaks were collected and sequenced, the peak present at 5 min (GenBank accession number GU048809) showed 100% identity with the sequence of Band 1 detected by DGGE (**Fig. 2-2A**), which belongs to the Pinellas sequence subgroup of *Dehalococcoides* species. Since we could not find a peak exclusively present in the PCNB amended microcosm (**Fig. 2-4B**), our DHPLC analysis was unable to distinguish the effect of PCNB from other treatments.

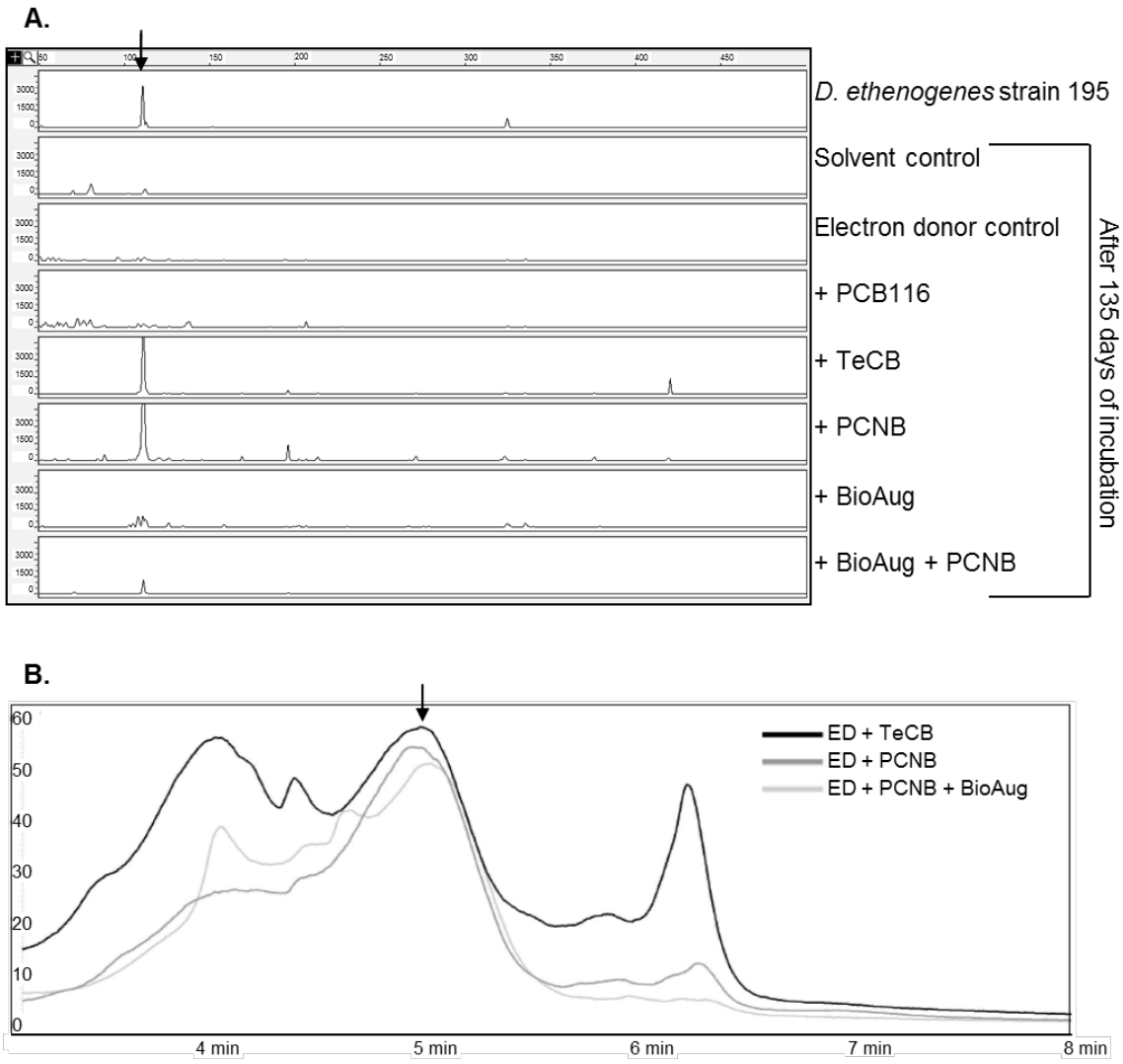


Figure 2-4. TRFLP analysis (A) and DHPLC analysis (B) of putative dechlorinating *Chloroflexi* community in Anacostia River microcosms after 135 days of incubation. In TRFLP analysis, X-axis is the length of terminal restriction fragment (TRF) and Y-axis is fluorescent intensity. In DHPLC analysis, X-axis is time (min) and Y-axis is the absorbance (mV). Arrows indicate the peak of putative dechlorinating *Dehalococcoides* species. TeCB: tetrachlorobenzene, PCNB: pentachloronitrobenzene, BioAug: *D. ethenogenes* strain 195 mixed culture.

Effect of microcosm treatments on *rdh* gene patterns. We analyzed twelve putative *rdh* genes common to *D. ethenogenes* strain 195 and *Dehalococcoides* sp. strain CBDB1 in order to complement the 16S rRNA gene based community analysis and to resolve the apparent discrepancy between haloprimer treatments on *Dehalococcoides* population enrichment and PCB dechlorination activity. PCR priming sets were designed to selectively amplify 12 putative *rdh* genes that are common to both *D. ethenogenes* strain 195 and *Dehalococcoides* sp. strain CBDB1. These primers were tested against *D. ethenogenes* strain 195 and each amplified only the *rdh* gene for which it was designed under the conditions described (data not shown). In microcosm samples, nine putative *rdh* gene amplicons were detected with high intensity in TeCB, PCNB, bioaugmented, or PCNB plus bioaugmented microcosms (**Fig. 2-6** and **Fig. 2-7**). Interestingly, two other putative *rdh* gene amplicons, designated *rdh*04 (targeting DET0306 in strain 195 and cbdb_A1495 in strain CBDB1) and *rdh*05 (targeting DET0311 in strain 195 and cbdb_A88 in strain CBDB1), were detected in microcosms amended with PCNB and/or bioaugmented with *D. ethenogenes* strain 195. However, the intensity of these latter two *rdh* gene amplicons was low in TeCB amended microcosms (**Fig. 2-6**). The intensity of control amplifications of the bacterial 16S rRNA gene, representing the total bacterial community population, did not substantially change with different treatments (data not shown). The sequence of each amplified putative *rdh* gene had over 90% identity with the corresponding *rdh* gene in *Dehalococcoides* spp. strain CBDB1 or strain 195, supporting the specificity of the primer design.

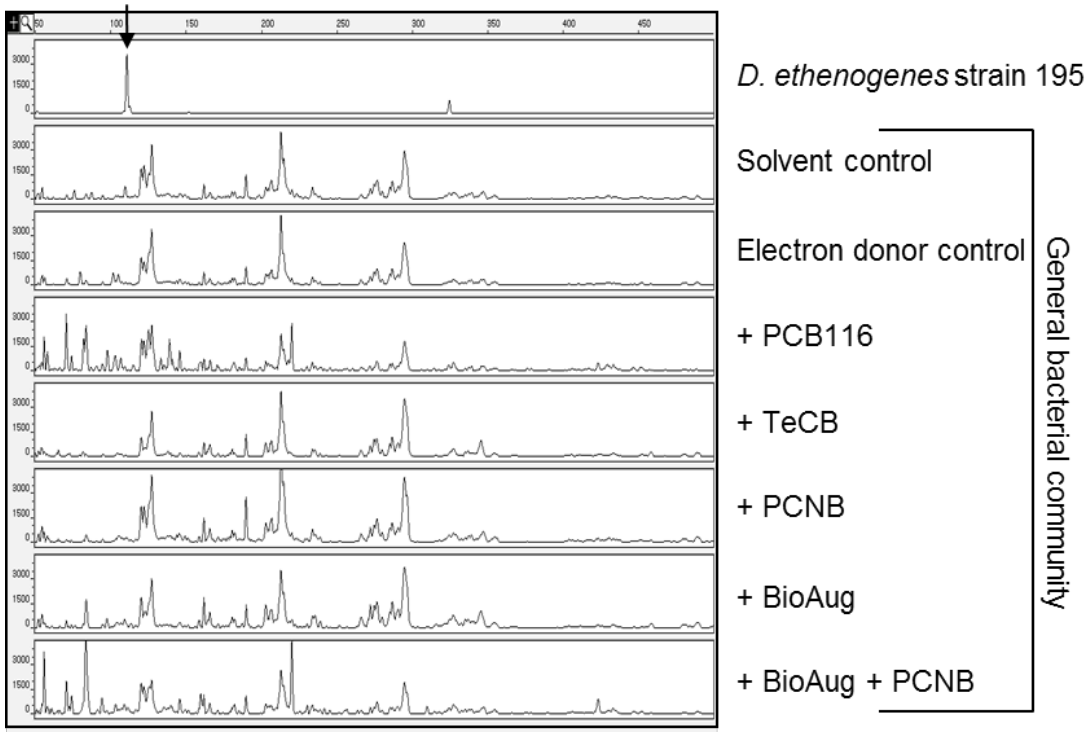


Figure 2-5. TRFLP analysis of general bacterial (27F-FAM/1525R) communities in Anacostia River sediment microcosms after 135 days of incubation. Arrow indicates the peak of putative dechlorinating *Dehalococcoides* species. TeCB: tetrachlorobenzene, PCNB: pentachloronitrobenzene, BioAug: *D. ethenogenes* strain 195 mixed culture.

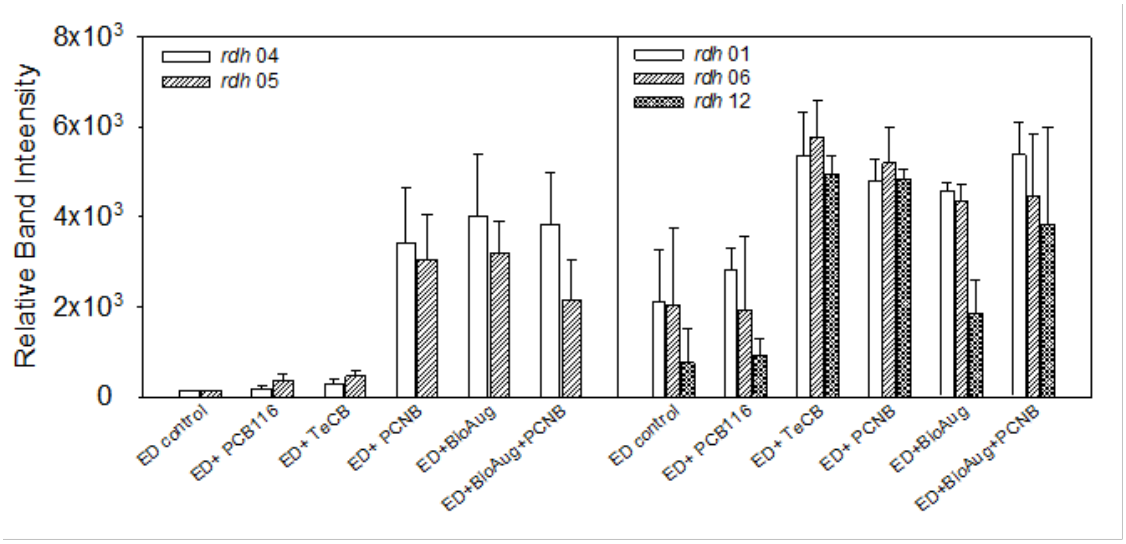


Figure 2-6. PCR band intensity of 5 putative *rdh* genes in Anacostia River microcosms after 135 days of incubation. Putative *rdh* gene amplicons correspond to the following *D. ethenogenes* strain 195 and *Dehalococcoides* sp. CBDB1 genes: *rdh* 01 (DET0180 and cbdb_A187), *rdh* 04 (DET0306 and cbdb_A1495), *rdh* 05 (DET0311 and cbdb_A88), *rdh* 06 (DET0318 and cbdb_A1588), and *rdh* 12 (DET1545 and cbdb_A1638). ED: electron donor, TeCB: tetrachlorobenzene, PCNB: pentachloronitrobenzene, BioAug: *D. ethenogenes* strain 195 mixed culture.

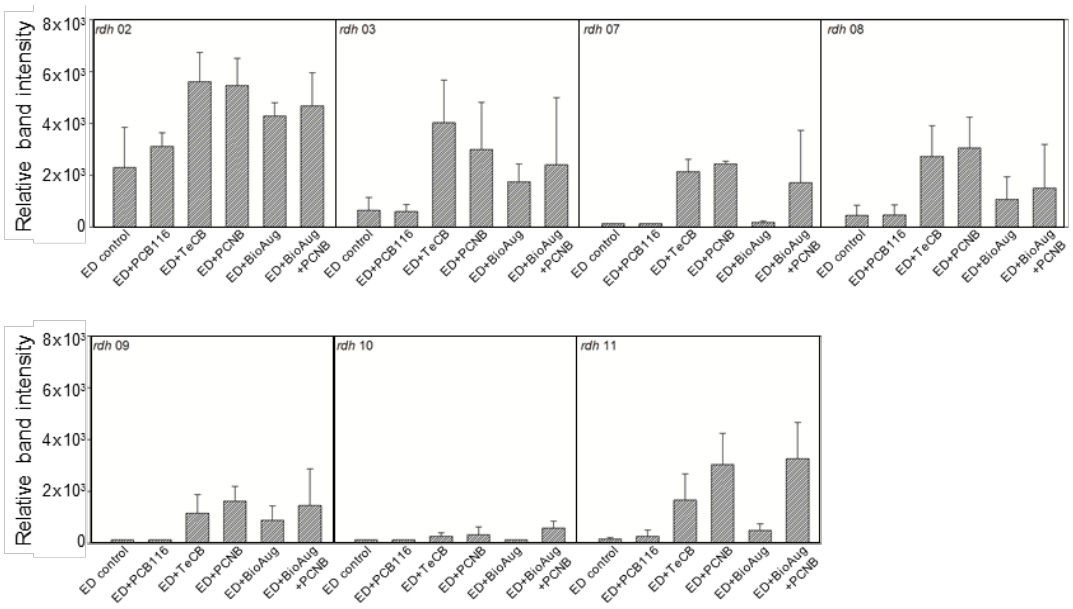


Figure 2-7. Quantification of 7 putative rdh genes in Anacostia River microcosms after 135 days of incubation based on relative intensity of rdh gene amplicons. Putative *rdh* gene amplicons correspond to the following *D. ethenogenes* strain 195 and *Dehalococcoides* sp. CBDB1 genes: rdh 02 (DET0235 and cbdb_A243), rdh 03 (DET0302 and cbdb_A237), rdh 07 (DET1171 and cbdb_A1092), rdh 08 (DET1519 and cbdb_A1575), rdh 09 (DET1522 and cbdb_A1570), rdh 10 (DET1535 and cbdb_A1595), and rdh 11 (DET1538 and cbdb_A1627). D. ethenogenes strain 195 DNA was used as a positive control to confirm primer specificity. ED: electron donor, TeCB: tetrachlorobenzene, PCNB: pentachloronitrobenzene, BioAug: *D. ethenogenes* strain 195 mixed culture.

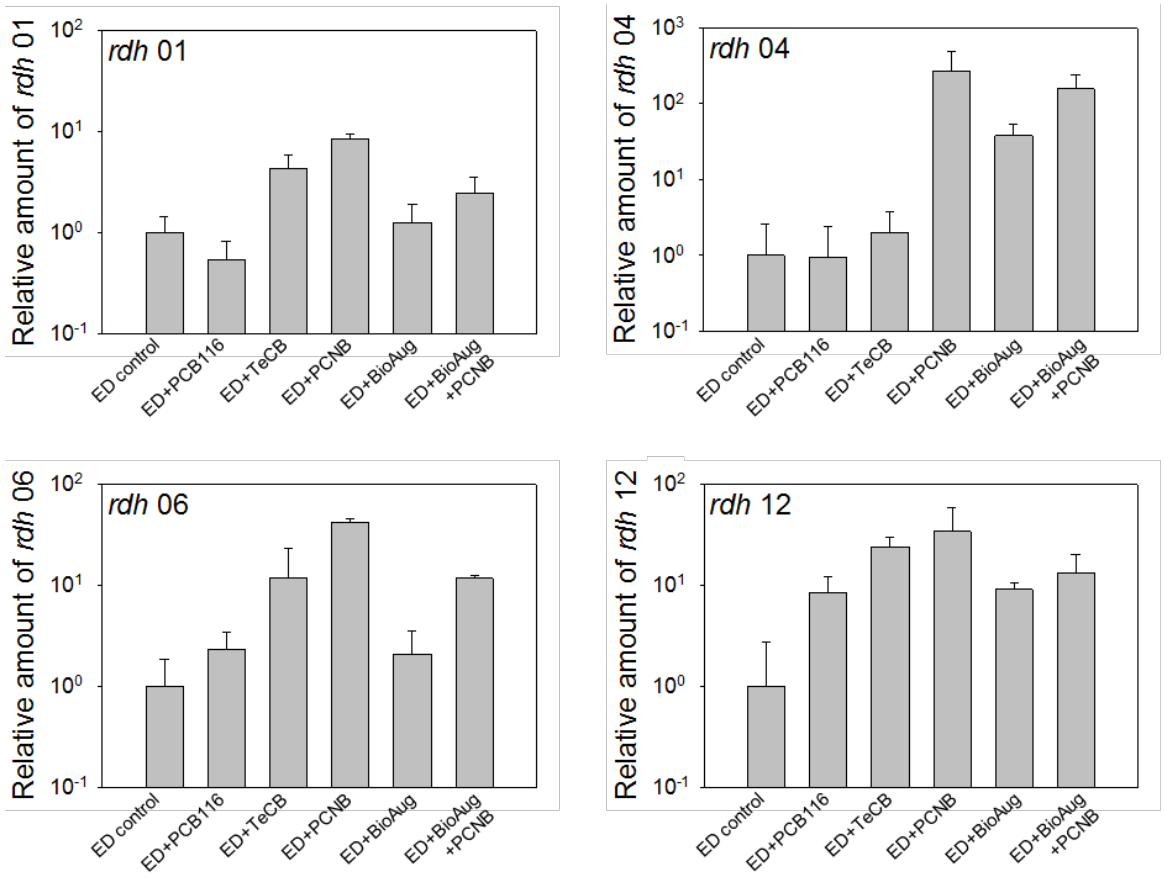


Figure 2-8. qPCR assay of 4 putative *rdh* genes in Anacostia River microcosms after 135 days of incubation. Amount of *rdh* gene is calculated relative to the average amount of *rdh* gene in ED control set as 1. Putative *rdh* gene amplicons correspond to the following *D. ethenogenes* strain 195 and *Dehalococcoides* sp. CBDB1 genes: *rdh* 01 (DET0180 and cbdb_A187), *rdh* 04 (DET0306 and cbdb_A1495), *rdh* 06 (DET0318 and cbdb_A1588), and *rdh* 12 (DET1545 and cbdb_A1638). ED: electron donor, TeCB: tetrachlorobenzene, PCNB: pentachloronitrobenzene, BioAug: *D. ethenogenes* strain 195 mixed culture.

Quantification of *rdh* genes by qPCR. The relative amount of four *rdh* genes in Anacostia River microcosms was measured (**Fig. 2-8**) to verify the semi-quantitative data based on the PCR band intensity assay (**Fig. 2-6**). Since *rdh* 05 primers designed for conventional PCR assay (**Table 2-1**) generated high background noise, we could not measure the amount of *rdh* 05 by using SYBR green-based qPCR (data not shown). The patterns of four *rdh* genes based on different treatment were the same or similar to those based on PCR band intensity analysis. In particular, the relative amount of *rdh* 04 gene in PCNB amended microcosms was approximately two orders of magnitude higher than that in TeCB amended microcosms (**Fig. 2-8**).

***rdh* gene-based phylogenetic analysis.** The two putative *rdh* genes 04 and 05 amplified from haloprimer amended and/or bioaugmented microcosms were sequenced and compared with relevant *rdh* genes in *D. ethenogenes* strain 195, *Dehalococcoides* sp. strain CBDB1, and *Dehalococcoides* sp. strain BAV1. Since the amplified regions of the two different *rdh* genes 04 and 05 partially overlapped in the highly homologous iron-sulfur cluster binding motif region,

we were able to generate a phylogenetic tree of these two *rdh* gene PCR products. Excluding primer regions, 61 bp of DNA sequences were aligned and used to generate a phylogenetic tree (data not shown). The *rdh* gene DNA sequences split into two groups, *rdh* 04 and 05, and each branch was further split into two subgroups, *rdh* genes from Pinellas subgroup and from Cornell subgroup with high bootstrap support. The same branching patterns were observed when 193 bp of *rdh* 04 and 105 bp of *rdh* 05 DNA sequences were analyzed separately by neighbor joining analysis (data not shown). All *rdh* genes of haloprimer amended microcosms grouped with *rdh* genes from the Pinellas subgroup. The *rdh* 04 gene fragment (1993 bp) amplified from PCNB amended microcosms was 100% identical to cbdb_A1495 in strain CBDB1, excluding two ambiguous base pairs. The *rdh* 05 gene fragments (105 bp) from PCNB or TeCB amended microcosms were 100% identical to cbdb_A88 in strain CBDB1 or DehaBAV1_0104 in strain BAV1. In the bioaugmented microcosms we could only detect *rdh* genes of the Cornell subgroup, although 16S rRNA analysis showed the presence of both bioaugmented Cornell subgroup and indigenous Pinellas subgroup *Dehalococcoides* sp. in these cultures. The *rdh* 04 gene fragments (193 bp) from bioaugmented or bioaugmented plus PCNB-treated microcosms were 100% identical to DET0306 in strain 195, while the 105 bp *rdh* 05 gene fragments from bioaugmented or bioaugmented plus PCNB-treated microcosms were 100% identical to DET0311 in strain 195.

Discussion

We monitored the bacterial communities mediating dechlorination of weathered PCBs in Anacostia River sediment microcosms in order to understand how different bioremediation treatments affected the rates of dechlorination and the dechlorinating community. Both PCNB amendment and bioaugmentation increased PCB dechlorination rates, while more limited PCB dechlorination was observed in other treatments, including microcosms amended with the TeCB haloprimer (**Fig. 2-1**, Krumins et al., 2009). Furthermore, enhanced PCB dechlorination activity stimulated by PCNB treatment, bioaugmentation, or PCNB plus bioaugmentation was observed in these Anacostia River sediment microcosms even after 415 days of incubation (Krumins et al., 2009). The different treatments thus resulted in sustained shifts in the dehalogenation activity of the sediment microcosms.

To complement the phylogenetic approach used previously (Krumins et al., 2009), we used DNA sequences of major DGGE bands to generate a phylogenetic tree (**Fig. 2-2**) and developed TRFLP and nested PCR-DHPLC methods (**Fig. 2-3**) to identify the key members of the bacterial community believed to be responsible for reductive dechlorination. DGGE analyses based on nested PCR approach provided high resolution of the *Chloroflexi* community and nested PCR with group-specific primers was able to increase the detection limit of low population density *Dehalococcoides* species, representing less than 1% of the total TRFLP signal intensity (**Fig. 2-5**). Nested PCR-DGGE analysis effectively distinguished between haloprimer-activated indigenous species and *D. ethenogenes* strain 195 used for bioaugmentation (**Fig. 2-2A**). The haloprimer-activated indigenous species belonged to the Pinellas subgroup of the *Dehalococcoides*, which includes known dechlorinating *Dehalococcoides* strains, such as FL2, CBDB1, and BAV1 (Bedard, 2008). Interestingly, previous work on tetrachloroethene dechlorination monitored by reductive dehalogenase genes *tceA*, *vcrA* and *bvcA* also suggested the presence of the Pinellas subgroup in PCE-enriched Anacostia River sediments (Himmelheber

et al., 2007). The close relationship between this indigenous Pinellas subgroup and PCB dechlorination activity can be inferred by the greater extent of PCB dechlorination observed in the PCNB amended microcosms (Krumins et al., 2009).

Phylogenetic analyses targeting 16S rRNA genes (**Figs. 2-2 and 2-4**), however, could not explain the differences in the PCB dechlorination activity observed in the two different haloprimer treatments. The 16S rRNA genes of *Chloroflexi* species are highly conserved and do not correspond with different dechlorination substrate specificities (Hendrickson et al., 2002; Duhamel et al., 2004; He et al., 2005; Kube et al., 2005; Sung et al., 2006). For this reason, Band 1 (**Fig. 2-2A**) could include multiple strains that have different dehalogenation activities, but carry identical lengths and GC contents in the V3 region of their 16S rRNA genes. This may be the same reason why *Chloroflexi*-specific TRFLP data only showed one peak, which includes multiple strains that carry an identical *MnlI* restriction enzyme site in their 16S rRNA gene sequences (**Fig. 2-4A**). It should be noted that reductive dehalogenase (*rdh*) genes are frequently associated with mobile genetic elements (Rhee et al., 2003; Kube et al., 2005; Seshadri et al., 2005). Therefore, a focus on only *Chloroflexi* spp. may thus miss other species involved in PCB dechlorination.

The 16S rRNA based community analysis suggests that the two different haloprimers enriched the same Pinellas-type *Dehalococcoides* community even though their PCB dechlorination activities were different. This apparent discrepancy can be explained by analyzing putative *rdh* genes, which effectively differentiated *rdh* gene patterns between the TeCB and PCNB treated microcosms; two of the monitored *rdh* genes (*rdh* 04 and *rdh* 05) were abundant in PCNB treated microcosms, but not in TeCB treated microcosms (**Fig. 2-6**). This semi-quantitative data was confirmed by qPCR (**Fig. 2-8**), which clearly distinguished the difference in the amount of the *rdh* 04 gene in microcosms amended with PCNB vs. TeCB (**Fig. 2-8**).

The *rdh* 04 and *rdh* 05 genes were detected in PCNB amended microcosms with or without bioaugmentation with *D. ethenogenes* strain 195, while strain 195 was not detected in PCNB amended microcosms without bioaugmentation (**Fig. 2-2A**). Therefore, we can assume that the *rdh* 04 and *rdh* 05 genes detected in PCNB amended microcosms without bioaugmentation are most likely from the enriched indigenous *Dehalococcoides* spp. The phylogenetic analysis based on the DNA sequences of *rdh* 04 and *rdh* 05 genes also supports that these two *rdh* genes amplified from PCNB amended microcosms without bioaugmentation derived from indigenous *Dehalococcoides* spp., since their sequences group with *rdh* genes of Pinellas subgroup. In contrast, the two *rdh* gene homologs detected in the bioaugmented microcosms grouped with *rdh* genes of the Cornell subgroup, supporting that these two *rdh* genes originated from bioaugmented *D. ethenogenes* strain 195.

Previously, it was found that multiple *rdh* genes were simultaneously transcribed during dechlorination (Waller et al., 2005) and protein segments of cbdb_A80 and cbdb_A84 were detected in a culture of *Dehalococcoides* sp. strain CBDB1 spiked with 1,2,3- and 1,2,4-trichlorobenzene (Adrian et al., 2007). Cbdb_A88 (*rdh* 05) was also one of the three reductive dehalogenase protein fragments detected in a culture of *Dehalococcoides* sp. strain CBDB1 growing on 2,3-dichlorophenol (Morris et al., 2007). However, the transcription of all 32 putative *rdh* genes, including cbdb_A1495 (*rdh* 04) and cbdb_A88 (*rdh* 05), was observed in a

culture of *Dehalococcoides* sp. strain CBDB1 spiked with 1,2,3- or 1,2,4-trichlorobenzene, suggesting a complex transcriptional response (Wagner et al., 2009).

The presence and amount of genes in environmental samples have been used as molecular markers to assess their related activity, such as dechlorinating genes for monitoring dehalogenation of chlorinated ethenes (Fung et al., 2007; Futamata et al., 2007; Behrens et al., 2008; Rahm and Richardson, 2008; Carreon-Diazconti et al., 2009). Since no known *rdh* gene involved in PCB dechlorination has been identified to date, further study on these two *rdh* genes (*rdh* 04 and *rdh* 05) may help in developing methods to monitor PCB dechlorinating bacteria. It is also necessary to clarify the relationship between these two *rdh* genes and PCB dechlorination, whether they are directly involved or merely present in the genome of the PCB dechlorinating species and thus co-enriched.

With the high resolution molecular tools applied here, we demonstrated the presence of two or more *Dehalococcoides* strains (strain 195 and an indigenous *Dehalococcoides* Pinellas subgroup species) and two putative *rdh* genes (*rdh* 04 and *rdh* 05) in PCB dechlorinating microcosms. We expect our results to support the future development of *in situ* bioremediation approaches for the treatment and detoxification PCB contaminated sediments.

3. PCB Dechlorination Pathways in Biostimulated/Bioaugmented Anacostia River Sediment Microcosms

(Du, S, Park J-W, Zhen, H, Rodenburg LA, Krumins V, Kerkhof LJ, Häggblom MM and Fennell DE. 2011. PCB Dechlorination Pathways in Biostimulated/Bioaugmented Anacostia River Sediment Microcosms, unpublished)

Abstract

The predominant dechlorination pathways for weathered polychlorinated biphenyls in microcosms of Anacostia River, Washington, DC sediment were deduced using a tandem quadrupole Gas Chromatography Mass Spectrometry (GC-MS/MS) system that identified 209 PCB congeners in approximately170 chromatographic peaks. PCB dechlorination pathways were also determined for freshly spiked Aroclor 1254 in activity enrichments from these microcosms, and in a mixed culture containing *Dehalococcoides ethenogenes* strain 195 which had been used as a bioaugmentation agent. The dechlorination pathways for two predominant sediment PCB congeners found in Anacostia sediments and in Aroclor 1254— PCBs 118 (245-34) and 170 (2345-234) were also assessed in activity enrichments from the microcosms. Dechlorination of weathered PCBs occurred mainly at the *meta*- and *para*- positions. The preferential substrate for dechlorination of PCBs in Anacostia microcosms were PCBs with substitution patterns 34, 234-, 2345- and 2346-. Congeners containing the chlorine substitution groups 236- and 2356- showed no detected dechlorination in weathered sediments, but were dechlorinated by sub-cultures of the microcosms amended with fresh Aroclor 1254. The mixed culture containing *D. ethenogenes* strain 195 exclusively dechlorinated PCBs containing doubly-flanked chlorines at the *meta*- and *para*- positions. The pathways of PCB congener dechlorination were variable between replicates, this variability was mirrored in the predominant phylotypes of the dechlorinating bacterial community as detected by denaturing gradient gel electrophoresis, and the heterogeneity appeared more pronounced when strain 195 had been bioaugmented to the sediment.

Introduction

Polychlorinated biphenyls (PCBs) include 209 individual congeners each with a different chlorination status. PCBs were manufactured for industrial use primarily as technical mixtures of congeners (including Aroclors). Despite the prohibition of PCB production in the 1970s, PCBs are still widely present in the environment because of their persistence and relative resistance to degradation. PCB accumulation in aquatic sediments has caused extensive public health concerns and the remediation of PCB-contaminated sediment has become a regulatory priority over the years. In contrast to conventional dredging, *in situ* bioremediation is an attractive alternative for treating PCB-impacted sediments because of potential for cost savings and minimization of the disturbance to the surrounding environmental system.

We previously examined biostimulation and bioaugmentation for enhancing dechlorination of low concentration historical PCBs in microcosms developed using sediments from the Anacostia River, Washington DC (Krumins et al., 2009). The Anacostia River is a freshwater tidal river in the Potomac River Drainage Basin and is classified as a warm-water stream with mean temperatures ranging from 3°C in January to 26°C in August, and summer temperatures of 18°C

to 32°C (SRC and NOAA, 2000). The lower Anacostia River, near the Washington Naval Yard, has been used as a site for a validation study of active sediment capping technologies (Reible et al., 2006). PCB concentrations in Anacostia sediment reported from previous engineering studies ranged from 0.4 to 9.1 mg/kg, and the congener profiles suggest mixtures of Aroclors (Horne Engineering Services, Inc., 2003). We previously reported average total PCB concentrations for all initial time point samples in sediment microcosms developed from Anacostia River sediments of 2.1 ± 1.4 mg/kg dry weight (mean ± 1standard deviation). Distinct from other well-studied river/estuarine systems which often have a single documented predominant Aroclor contaminant, the complex array of congeners in Anacostia River sediments which are present at relatively low concentrations arise from a weathered mixture of urban and industrial sources. The congener profile of PCBs in Anacostia River sediment is closest to the profile of Aroclor 1254.

It is expected that the mixed array of congeners present in Anacostia River sediment originating from multiple sources may have selected for diverse dechlorinating microbial populations. We previously reported that reductive dechlorination of weathered, low-level PCBs was differentially enhanced in microcosms of Anacostia River sediments under different treatment conditions (Krumins et al., 2009; see Accomplishments Section 1) including biostimulation with alternate halogenated compounds and bioaugmentation with a mixed culture containing *Dehalococcoides ethenogenes* strain 195. Further, different native dechlorinating bacteria and dehalogenase genes were enhanced in response to different treatments (Park et al., 2011; see Results and Discussion Section 2).

The dechlorination pathway resulting from microbial dehalogenation could have an impact on sediment toxicity and on subsequent treatment needed to fully restore a site. Therefore, the delineation of the effect of differing treatments and, thus, dechlorinator population shifts on the dechlorination pathway is desired. PCB dechlorination pathways for different treatments was not reported by Krumins et al. (2009), since that study utilized gas chromatography-electron capture detection (GC-ECD) for the analysis of PCB congeners. GC-ECD is incapable extensive detection of less chlorinated congeners and co-eluting congeners can often not be resolved. Krumins et al. (2009) reported results from 72 chromatographic peaks, representing 159 PCB congeners and determined mol % increases or decreases in homolog groups. To fully describe the effect of biostimulation and bioaugmentation on PCB dechlorination pathways in Anacostia River sediments, we developed a congener specific PCB analytical method resolving 170 peaks representing 209 PCB congeners using a tandem gas chromatography-mass spectrometry (GC-MS-MS) system. This technique allowed enhanced identification and quantification of individual PCB congeners that could be used to explore the pattern of dechlorination in Anacostia sediment microcosms.

Successful development of *in situ* bioremediation for PCB-contaminated sediment requires site-specific knowledge of the diversity, abundance and selective activities of the *in situ* dechlorinating microbial communities. In theory, maximal PCB dechlorination could be achieved by combining the bacterial strains capable of accomplishing specific dechlorination pathways. Therefore, species-specific and treatment-specific dechlorination pathway studies are desired for the successful design of bioremediation of PCB impacted site. Here we demonstrate a combined approach of assessing PCB dechlorination pathways via a congener specific PCB

analysis and monitoring of the dechlorinating bacteria that are stimulated or have been added under different conditions. Therefore in the current study we determined the ability of the Anacostia River bacterial community to dechlorinate commercial PCB mixtures; delineated the treatment-specific dechlorination pathways; and characterized dechlorination specificity related to known PCB dechlorination processes.

Experimental Methods

Anacostia River sediment microcosms. The details for the construction of the Anacostia River sediment microcosms were previously published (Krumins et al 2009). Briefly, microcosms were constructed using 200 mL sediment recovered from the Anacostia River capping site control plot (Reible et al., 2006). The following treatments were run in triplicate: unamended live and killed (autoclaved for 40 min at 121ºC on three successive days) controls; electron donor only control; stimulation with alternate halogenated electron acceptors (haloprimers) tetrachlorobenzene (TeCB), pentachloronitrobenzene (PCNB), or PCB 116 (2,3,4,5,6-pentachlorobiphenyl); bioaugmentation with a mixed culture containing *Dehalococcoides ethenogenes* strain 195; and biostimulation with PCNB plus bioaugmentation. An electron donor mixture containing lactate, propionate, acetate, and butyrate was added to all microcosm treatments (exclusive the live and killed controls) to a concentration of 1 mM each.

Microcosms were sampled as described by Krumins et al. (2009). For the current study preserved, unextracted Anacostia microcosm samples collected at Day 0 and Day 135 together with new samples (~ 6 g wet weight) collected at Day 1000 using the same sampling technique (Krumins et al. 2009), were air dried and extracted for PCBs. The samples were analyzed for the dechlorination pathway study using a GC-MS-MS congener-specific analytic method. The dry weight of the sediment was noted and the mass of each congener was normalized to the sediment dry weight. An additional 1 mL sample was also collected from each microcosm on Day 1000 for molecular analysis.

Aroclor 1254 dechlorination activity tests of Anacostia River sediment microcosms. **A**roclor 1254 was loaded on 0.25 g of a dried, previously autoclaved reference sediment (Fennell et al., 2004) to achieve a target concentration of 5 ppm (wet weight basis). Specifically, 150 µL of an Aroclor 1254 stock solution of 1000 ppm in toluene was added onto the reference sediment which had been placed into a 60 mL serum bottle. To remove the toluene, the solvent soaked sediment was allowed to air dry for four days. Before autoclaving (40 min at 121ºC), 0.5 mL Milli-Q water was added into the 60 mL serum bottle to moisturize the sediment, and the bottles were sealed with a Teflon®-backed septum and crimped with an aluminum crimp cap. The autoclaved serum bottles were then purged with sterile anoxic nitrogen for 30 min and placed in a disposable gloved bag under a stream of anoxic nitrogen gas.

To initiate the activity test, the original microcosms selected for the activity test (i.e., live control; electron donor only; tetrachlorobenzene; PCB 116; pentachloronitrobenzene (PCNB); bioaugmented with mixed culture containing *Dehalococcoides ethenogenes* strain 195; and PCNB plus bioaugmentation) were opened under an anoxic nitrogen headspace in a disposable glovebag and stirred thoroughly. 10 mL of sediment slurry was removed from each replicate microcosm using a sterile wide mouth glass pipette and this inoculum was transferred to the sterile 60 mL serum bottle containing the Aroclor 1254 amended sediment. Next, 20 mL of

anaerobic minimal medium (Zinder, 1998) was added via a sterile glass syringe. The constructed microcosms were stirred, re-capped and then amended according to the protocol of Krumins et al. (2009).The activity tests were and incubated at 26ºC in the dark.

Activity test samples were collected on days 0, 30, 60, 90, 120, 150, 180, 310 and 470 with a 1 mL sterile syringe were analyzed for the PCB dechlorination pathway study using the GC-MS-MS congener-specific analytic method.

PCB congener pathway sub-cultures. To confirm the dechlorination pathway of individual PCB congeners in Aroclor 1254, activity sub-cultures were established using inocula from individual activity tests from selected treatments. A 2 mL aliquot of sediment slurry from each selected Aroclor 1254 activity test was transferred to a sterile, anoxic 20 mL serum bottle containing the reference sediment which had been amended with one of the PCB congeners found in abundance in Aroclor 1254—i.e., either PCB 118 (245-34) or PCB 170 (2345-234) in the same manner as described for Aroclor 1254. Seven mL of anaerobic minimal medium (Zinder, 1998) was then added, and the serum vial was capped with a sterile Teflon®-backed septum under a sterile anoxic nitrogen gas headspace and crimped with an aluminum crimp cap. The target concentration of each individual congener in the pathway sub-culture was 3 ppm. The selected treatments from which activity sub-cultures were constructed included: live control; the TeCB-amended treatment; the PCNB-amended treatment; and the PCNB plus mixed culture containing *D. ethenogenes* strain 195 amended treatment. The sub-cultures were prepared in triplicate for each treatment and incubated at 26ºC in the dark. Samples from Days 0, 90 and 220 were collected using a 1 mL sterile syringe. Chemical analysis of the spiked individual congeners as well as their potential products utilized the GC-MS-MS PCB congener-specific analytical method.

Aroclor 1254 activity test with a mixed culture containing *Dehalococcoides ethenogenes* strain 195. A mixed culture containing *Dehalococcoides ethenogenes* strain 195 was grown at 25°C on PCE and butyric acid as described previously (Liu and Fennell, 2008). 1 g dry sterile sediment (Fennell et al., 2004) was added to a sterile 160 mL bottle. The sediment was wetted by adding 0.1 mL of 1000 mg/L Aroclor 1254-acetone stock solution. The organic solvent was evaporated overnight and the bottles were sealed with a sterile Teflon®-coated gray butyl rubber stopper and crimped with an aluminum crimp cap. Next, the bottles were purged for 20 min with sterile, anoxic 80% nitrogen/20% carbon dioxide gas and autoclaved (40 min at 121ºC). 100 mL of mixed culture was transferred to each bottle under anoxic and sterile conditions. The final concentration of Aroclor 1254 was 1 ppm. Butyric acid was added as an electron donor to a final concentration of 100 µM on days 0, 48, 87 and 143. 50 µL of a 50 g/L of fermented yeast extract solution was provided as a nutrient source and spiked on days 0 and 48. A vitamin stock solution was added at set up for all treatment groups (Fennell et al., 2004).Three sets of triplicate bottles were established at 25°C. One set of bottles received Aroclor 1254 as the sole halogenated substrate, the second set of bottles received 1,2,3,4-TeCB (as a known growth substrate for strain 195) at a final concentration of 25 µM on days 0 and 48 and the third set of bottles was autoclaved at 121ºC for 1 hr on each of three consecutive days to serve as a killed control. All bottles were inverted and shaken in the dark at 120 rpm and sampled periodically over more than 200 days.

Consequently, the deduced pathways from these various sub-culture studies established from Anacostia sediment microcosms with different amendments could be compared with the study of dechlorination of Aroclor 1254 with the bioaugmented *Dehalococcoides ethenogenes* strain 195 alone.

Extraction of PCBs. The microcosm samples from days 0 and 135 analyzed in the present study were the replicates of samples collected previously, but which had been stored at -20ºC (Krumins et al., 2009). Additional samples were collected on Day 1000. Sediment slurries removed from the microcosms were allowed to air dry for 24-48 h before being extracted in a Dionex ASE 200 Accelerated Solvent Extractor (Dionex, Bannockburn, IL, USA) following EPA Method 3545. PCB surrogate standards consisting of PCB 14, 23, 65, 166 were added into the dried sediment immediately before the extraction. The ASE extraction method was as previously described (Krumins et al., 2009) except that the extraction solvents were changed to a mixture of methylene chloride:hexane (25:75 volume:volume (v:v)) instead of using hexane alone. The extracts from the ASE were solvent exchanged to hexane and then reduced to 1 mL under a gentle nitrogen gas purge. The extract was fractionated using a 2.5% water-deactivated Florisil ® column, with 35 mL of petroleum ether eluting the PCB fraction. The extract was reduced to ~ 3 mL using a rotary evaporator and was further concentrated to ~ 0.5 mL under a nitrogen gas purge. Internal standards PCBs 30 and 204 were injected into the samples prior to instrumental analysis.

The activity test and sub-culture samples were extracted using the method described previously (Krumins et al., 2009). Briefly, 4 mL diethyl ether was added to 7 mL screw-cap vials where the samples (1 mL) were collected, and then amended with 100 µL of PCB surrogate standards (deuterated PCB 65 and PCB159). The samples were shaken overnight and then centrifuged before the solvent was decanted to clean 7 mL vials. The extraction was repeated twice and all the extracts were combined. Sodium sulfate was added to remove water and the solvent volume was reduced under gentle nitrogen flow. Solvent exchange was made by adding 3 mL of hexane after the extract volume had been reduced to about 3 mL. The solvent-exchanged extracts were further concentrated to 1 mL under nitrogen and a Florisil® clean-up was performed as described above.

GC/MS/MS analysis of PCBs. Congener specific analysis of all 209 PCB congeners was conducted using a tandem quadrupole mass spectrometry (GC-MS-MS) system (Waters Quattro Micro GC) and following a method modified slightly from EPA Method 1668A. A 30 m Supelco SP-Octyl, fused silica capillary column (30 m x 250 µm i.d. x 0.25 µm film thickness), with helium as the carrier gas at a constant flow rate of 0.8 mL min^{-1}, was used for the congener separation. The MS-MS operating parameters for the determination of PCBs are presented in **Table 3-1**. To achieve maximum sensitivity, the two most abundant isotopes M^+ and $[M + 2]^+$, were monitored and the total ion current (TIC) was used for quantification with the aim of achieving maximum sensitivity. The instrument provides detection limits similar to an Electron Capture Detector, while also allowing the unequivocal identification of more PCB congeners. PCB dechlorination and dechlorination pathways were investigated through identification and quantification of historical PCB congeners and potential dechlorination products eluting from the GC-MS-MS system. This method theoretically could separate the 209 PCB congeners into approximately 170 peaks. Approximately 117 chromatographic peaks representing

Table 3-1. GC-MS-MS operating parameters for the determination of PCBs. The MS operating conditions were the following: the temperature of the transfer line was held at 250°C during the chromatographic run. EI source is operated at 200°C with an electron energy of 70 eV and a trap current of 100 A. The MRM mode was operated at an argon collision gas pressure of 3.0×10^{-3} mBar.

Functions	PCBs	Start time (min)	End time (min)	Parent Ion (m/z)	Daughter Ion (m/z)	Dwell (secs)	Collision Energy (ev)
1	Mono-PCB	15	32	188+190	153	0.05	15
2	Di-PCB	15	32	222+224	152	0.05	15
3	Tri-PCB	23	42	256+258	186	0.05	15
4	Tetra-PCB	32	55	289.9+291.9	220	0.05	23
5	Penta-PCB	34	65	323.9+325.9	254	0.05	25
6	Hex-PCB	49	75	359.8+361.8	289.90	0.05	25
7	Hepta-PCB	58	75	393.8+395.8	323.90	0.05	22
8	Octa-PCB	64	76	429.8+431.8	360.0	0.05	22
9	None-PCB	69	77	463.7+465.7	394.0	0.05	22
10	Deca-PCB	77	90	497.7+499.7	428	0.05	22

approximately 153 PCB congeners were consistently detected in our samples from Anacostia River.

A calibration standard solution with a full suite of 209 PCB congeners was prepared from five PCB congener solutions purchased from AccuStandard (New Haven, CT). Deuterated PCB congeners purchased from C D N Isotopes (Quebec, Canada) were used as surrogate (PCBs 65 and 159) and internal (PCBs 30 and 116) standards. Average percent recoveries (±SD) determined by GC/MS/MS for PCBs d65 and d159 were 81 ± 16 and 73 ± 15% respectively. The mole percent (mol %) of the resolved PCB congeners was computed based on the molar concentration of each congener (normalized to sediment dry weight) divided by the sum of the molar concentrations of all detected congeners. Lab blanks were run with each batch of extractions to check for contamination from the laboratory or equipment. PCB masses in the three trip blanks and in the laboratory blanks were less than 5% of the masses in samples. Thus, the data were not blank corrected.For replicate samples, an average ± one standard deviation mol % was reported. The extent of dechlorination of a specific congener was expressed as the change in mol % at any time with respect to the initial mol % on day 0.

Molecular Analyses. For microcosms, DNA was extracted from 0.3 grams of sediment and for activity tests and subcultures DNA was extracted from the solids (collected by centrifugation at 16,000 g) from 1 mL of sediment slurry using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Nested PCR was performed to amplify the DNA of the putative dechlorinating community, and resulting DNA products were analyzed by denaturing gradient gel electrophoresis (DGGE) (see section 1.1). First, DNA samples extracted from microcosms were amplified with primers 338F (Lane, 1991) and Chl1101R targeting a 790 bp region of the *Chloroflexi* 16S rRNA gene (Park et al., 2011; see Section 2). The resulting PCR

products were purified using the UltraClean™ PCR Clean-Up Kit (MoBio Laboratories Inc., Carlsbad, CA), and these PCR products were then re-amplified using general bacterial PCR primers 341F-GC and 534R (Muyzer et al., 1993). All PCR reaction mixtures contained 10 mM Tris–HCl (pH 8.3 at 25 °C), 2.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphates, 10 picomole of each primer, and 1 U of Taq DNA polymerase. The temperature profile for nested PCR with 338F and Chl1101R was 94 °C for 5 min followed by 35 cycles of 94 °C for 20 sec, 55 °C for 45 sec, and 72 °C for 45 sec. A final extension step was carried out for 7 min at 72 °C, after which the DNA was stored at 4 °C. General bacterial PCR conditions were as described previously (Park and Crowley, 2006).

Samples of PCR product (20 µL) were mixed with 2x DGGE dye and loaded onto 8% polyacrylamide gels in 1× Tris-acetate EDTA (TAE) buffer using a DCode™ universal mutation detection system (Bio-Rad Laboratories, Hercules, CA). The polyacrylamide gels were made with a linear denaturing gradient from 40% at the top to 60% denaturant at the bottom. The electrophoresis was run for 14 h at 60°C and 60 V. After electrophoresis, the gels were stained with ethidium bromide and photographed on a UV transilluminator. Image analyses of the DNA profiles and band intensities were conducted using Quantity One® (version 4.5.0; Bio-Rad Laboratories, Hercules, CA) and Scion Image (Beta version 4.02, US National Institutes of Health, Frederick, MD). The smiling of bands near the edges of the DGGE gels was corrected using Adobe® Photoshop® (Adobe Systems Inc., San Jose, CA).

DGGE bands of interest were excised and DNA was eluted overnight in 50 µL of MilliQ H_2O at 4 °C. After centrifuging, DNA in the supernatant was amplified using general bacterial primers as described above. The PCR products were cloned into pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Corp. Carlsbad, CA), according to the manufacturer's instructions. The cloned PCR products were sequenced using a 16-capillary ABI PRISM® 3100 Genetic Analyzer. Sequence similarity searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The programs CLUSTAL X (1.64b) and GeneDoc (2.0.004) were used to align the sequences and determine their homologies to known bacterial 16S rRNA gene sequences.

Results and Discussion

PCB congener profiles in Anacostia sediment microcosms. The analysis of PCBs in Anacostia microcosms confirmed ongoing dechlorination after 1000 days (4 years) in effective treatments, although at modest rates. The results indicated that amendment with pentachloronitrobenzene (PCNB) or PCNB plus bioaugmentation were the most effective in enhancing dechlorination. **Figure 3-1** shows the mol % distribution time course trend of 12 selected congeners in selected Anacostia River sediment microcosms. These selected congeners that are displayed are generally present in high abundance in Anacostia sediments and are involved in the dechlorination process, i.e. either serve as substrates or are produced as dechlorination products. Most of the observed dechlorination substrates in Anacostia microcosms were penta- to hepta-CBs including PCBs 118 (245-34), 128 (234-234), 138 (234-245), 131(2346-23), 141 (2345-25), 170 (2345-234), 180 (2345-245), and 183 (2346-245) with determined dechlorination extent varying from 20-40 mol %. Substantial dechlorination of one tetra-CB, PCB 66 (24-34) also occurred with 60 mol% reduction over 1000 days.

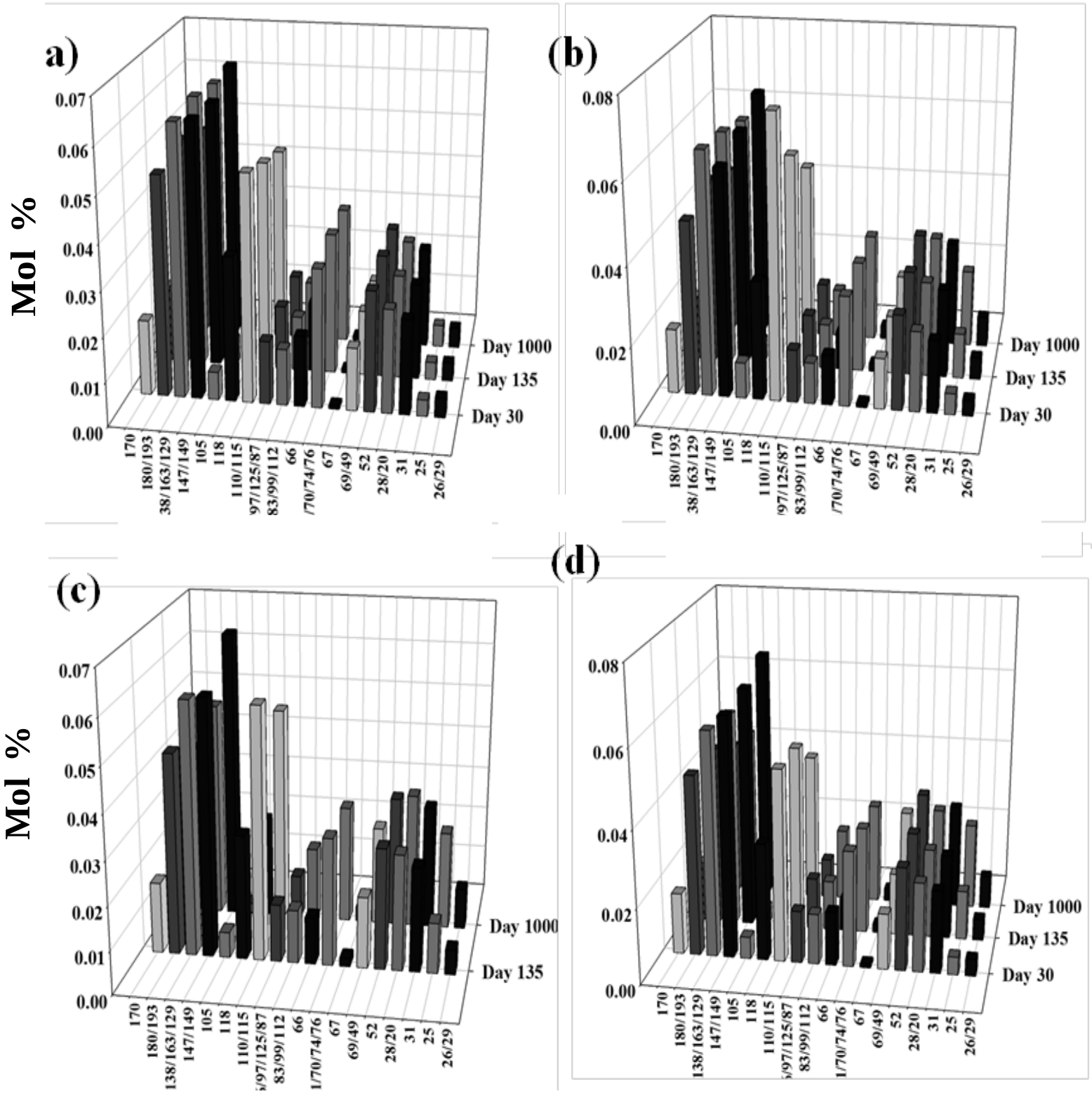


Figure 3-1. Time course of specific congeners during dechlorination in the original Anacostia River sediment microcosms. Treatments include: a) live control; b) Tetrachlorobenzene; c) PCNB; and d) PCNB + bioaugmentation.

Among the PCB congeners showing an increasing trend over the time course were the tri- and tetra-CBs, PCB 25 (24-3) and PCB 49 (24-25). These two congeners were the most highly accumulated dechlorination products, increasing by 400 mol % and 55-75 mol % in PCNB and PCNB plus strain 195 treatments, respectively. In addition, there was also detected accumulation of other tri-CBs including PCB 26 (25-3), PCB 28 (24-4) and PCB 31 (25-4), and of a penta-CB, PCB 99 (245- 24).

Analysis of microcosm sample PCB distributions using the congener-specific analytical method demonstrated dechlorination at the *meta*- and *para*- positions, as commonly reported in previous studies of PCB dechlorination. The preferential substrate for dechlorination of PCBs in Anacostia microcosms were chlorophenyl rings with substitution patterns 34, 234-, 2345-, and 2346-. Congeners containing the chlorine substitution groups 236- and 2356- showed no detected dechlorination. Congeners falling into this group included PCB110 (236-34), 132 (236-234), 149 (236-245), 151 (2356-25), 174 (2345-236), and 187 (2356-245). This is consistent with previous reports that congeners chlorinated in both *ortho* positions of either ring, i.e., at carbons 2 and 6, were not readily dechlorinated in some systems (Bedard et al., 1996). It has been proposed that this is possibly caused by steric hindrance; however, other evidence indicated that the dechlorination of congeners with three or four *ortho* chlorines were not limited under certain circumstances (Bedard et al., 2006).

The dechlorination pathways of some of these selected congeners have been reported either for individual congeners or from studies reporting Aroclor dechlorination by specific *Dehalococcoides* spp. (Bedard et al., 2006; Fagervold et al., 2007; Van Dort et al., 1997). For example, in earlier studies flanked *meta* chlorines were preferentially removed, e.g. PCB138 (234-245) was dechlorinated to PCB 99 (24-245) and then to PCB 47 (24-24). In addition to dechlorination of *meta* chlorines, *para* dechlorination also occurred in the Anacostia microcosms, e.g., PCB 170 (2345-234) to PCB 130 (2345-23) and PCB 66 (24-34) to PCB 25 (24-3), and PCB118 (245-34) to 70 (25-34). Heterogeneity of dechlorination activity among replicates in microcosm treatments was also observed. This was most pronounced in the PCNB+strain 195 treatment. Among the three replicates, one was nearly inactive one was active and one was highly active.

Dechlorination of Aroclor 1254 in activity test. Dechlorination of freshly spiked Aroclor 1254 occurred in biostimulated and bioaugmented activity tests established from individual Anacostia River microcosms 4 years after the initial stimulation. Effective treatments including biostimulation and bioaugmentation with pentachloronitrobenzene (PCNB) and strain 195, demonstrated dechlorination activity throughout a 470 day incubation period. However, in these two treatments, some replicates were substantially more active than others, refecting a heterogenous response to the stimulation of dechlorination. Dechlorination in other treatments including bioaugmentation with strain 195 alone, PCB116 amendment and the tetrachlorobenzene amendment was insubstantial, consistent with the results determined in previous study (Krumins et al., 2009). No dechlorination was detected in the live control replicates.

Substantial heterogeneity among replicates was observed for the activity test for the treatment augmented with PCNB and the mixed culture containing *D. ethenogenes* strain 195. This

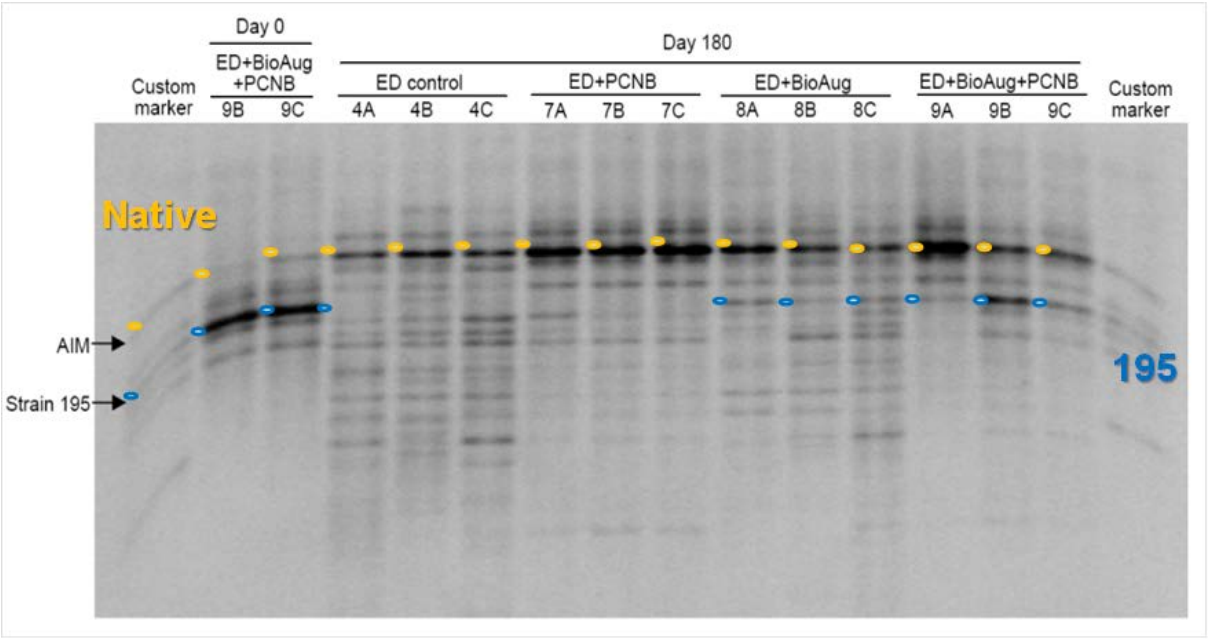


Figure 3-2. Image of DGGE results for putative dechlorinating *Chloroflexi* in activity tests with Aroclor 1254. "Native" indicates a *Dehalococcoides* phylotype native to Anacostia sediments and "195" indicates the added strain 195. [ED=electron donors; BioAug = addition of mixed culture containing strain 195; PCNB = pentachloronitrobenzene; and A, B, C represent tests prepared from individual original microcosms.]

heterogeneity was also reflected in the *Chloroflexi* bacterial community on Day 180 as monitored by DGGE (**Fig. 3-2**). Bands denoted "strain 195" and "native" represent phylotypes previously observed to have the greatest band intensities in the Anacostia River sediment microcosms (Krumins et al., 2009). The 16S rRNA gene sequence from the band "strain 195" was identical to that of *D. ethenogenes* strain 195 used in the bioaugmentation treatments and eluted at the same distance on the gel as an amplicon produced from strain 195 and used as a positive control. The band "native" was consistent with a previously observed phylotype in Anacostia River sediment microcosms and its sequence belongs to the Pinellas subgroup of *Dehalococcoides* spp., which contains strains CBDB1, BAV1, FL2 and GT among others (Adrian et al., 2000; Sung et al., 2006; He et al., 2003, 2005). The intensity of band strain 195 was initially strong after bioaugmentation on day 0, however, by Day 180 was still detectable in the system (consistent with results reported by Krumins et al., 2009) though its intensity was lessened. PCNB appeared to stimulate the native *Dehalococcoides* spp. (native), leading to an increase in the DGGE band intensity in many replicates by day 180 over what had been observed on Day 0.

In the PCNB plus bioaugmentation treatment, it is notable that a single, highly active replicate (A) was apparent, while the remaining two replicates were either slightly active (C) or inactive (B) (i.e., similar to the control) in dechlorinating Aroclor 1254. This heterogeneity among replicates has also been reported in other dechlorination studies of PCBs (Bedard et al., 2006). In the present study, the heterogeneity reflects the fact that each replicate activity test was prepared from a separate microcosm. Since the primary goal of this study was to explore the treatment-specific dechlorination pathway, we selected the most effective dechlorinating

Table 3-2. Change in PCB homolog distribution in selected effective replicate and treatments of the activity test with Aroclor 1254.

PCB homolog	Mole %(Avg ± stdev) at day 470					
		The most active replicates used in the pathway assessment				
	Control	PCNB +strain 195 (A)	PCNB +strain 195 (C)	PCNB (C)	[a]Avg. PCNB +strain 195	Avg. PCNB
Penta-deca- CB	79.69±1.26%	17.75%	35.20%	42.07%	39.72±24.55%	51.27±8.11%
tetra-CB	18.76±1.23%	59.26%	48.73%	45.62%	45.43±15.73%	40.43±4.66%
tri-CB	1.31±0.08%	22.55%	15.59%	11.91%	14.44±8.74%	7.96±3.44%
di-CB	0.22±0.03%	0.44%	0.48%	0.40%	0.40±0.10%	0.34±0.05%

[a] Note that PCNB + strain 195 (B) was not substantially different than the control

replicates showing more complete dechlorination profile for further examination of the parent and daughter product congeners.

Thus, the results from three representative replicates were chosen to illustrate the treatment specificity in this study: PCNB+bioaugmentation (A) (denoted PCNB+strain 195 A) (the most active replicate) and PCNB+ bioaugmentation (C) (denoted PCNB+strain 195 C) (the next most active replicate), representing the dechlorinating activity from the treatment augmented with PCNB and strain 195; and PCNB (C), representing the dechlorination activity from the treatment augmented with PCNB alone. The replicates for PCNB alone had a more homogenous response.

Dechlorination in activity tests incubated with 5 ppm fresh Aroclor 1254 was detected within 60 days after the incubation was initiated and proceeded to a much greater extent by day 470, than was observed in the original microcosms as discussed in Section 1. The substantially greater extent of dechlorination likely reflects the greater bioavailability of the spiked fresh Aroclor compared to the weathered historical PCBs. It is not suggested here that the effect with spiked Aroclor could reflect dechlorination that could be achieved against weathered PCBs in the biostimulated environment. Rather, it was expected that the pathways of dechlorination of the different PCB congeners present in the Aroclor would reflect pathways of dechlorination of the same congeners in the weathered mixture.

After day 470, dechlorination of the spiked Aroclor produced a shift in the homologue distribution from primarily hexa- and penta- to predominately tetra- and trichloro-biphenyls, indicative of dechlorination of specific congeners that occurred during the incubation period. The mol % of different homolog groups in the most active replicates and the corresponding averages determined for the triplicates of each of the most active treatment (i.e., PCNB and PCNB+bioaugmentation) are presented in **Table 3-2**. The largest dechlorination shift was observed in the replicate PCNB+strain 195(A) **(Figure 3-3)**, where the level of penta- through deca-chlorobiphenyl homologs decreased by 80%. However, this extent of dechlorination was

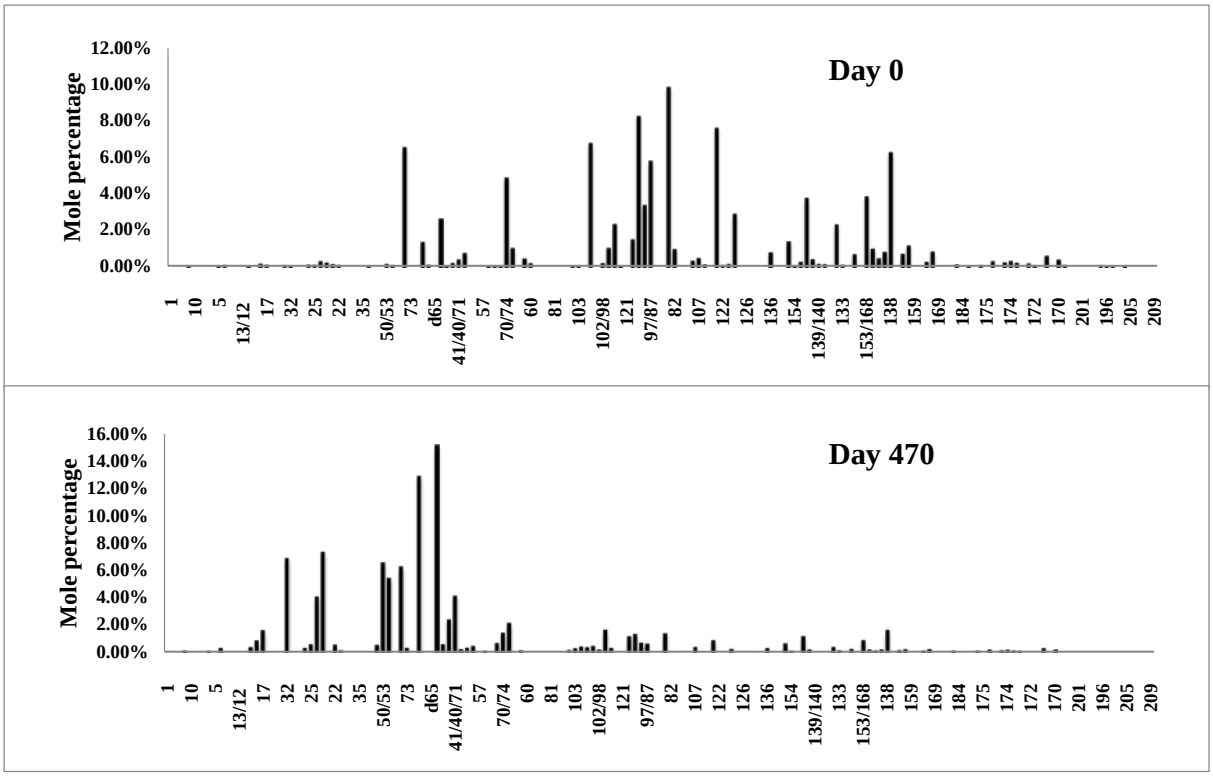


Figure 3-3. Shift in PCB congener distribution of Aroclor 1254 after 470 days of incubation in a highly active replicate of the treatment augmented with PCNB and strain 195. The concentration of spiked Aroclor 1254 was 5ppm.

not observed in all replicates. The average decline of penta- through deca-chlorobiphenyls in the triplicate treatments of PCNB+strain 195 and PCNB were lower than this most active replicate, 45 mol % and 32 mol %, respectively (**Table 3-2)**.

Tetrachlorobiphenyls formed the major dechlorination products of Aroclor 1254 at the end of the incubation. For example, tetrachlorobiphenyl accounts for up to 62 mol % of total PCBs in replicate PCNB+strain 195(A), with the overall average across all three replicates of 44 ± 17 mol % (average ± 1 standard deviation). The average mole percentage of tetrachlorobiphenyl was 40 ± 5 mol % in the PCNB-amended treatment (**Table 3-2)**. Furthermore, there was also a substantial amount of trichlorobiphenyl formed in all replicates. In general, the average accumulation of trichlorobiphenyl in the treatment PCNB + strain 195, 11 ± 7 mol % was higher than that in the PCNB only treatment, 6 ± 3 mol % (**Table 3-2)**. [In contrast, in weathered sediments, Section 1, the most successful treatment (PCNB) showed a mole fraction increase in less chlorinated congeners (sum of *all* di- to tetra-CB congeners) of 20 ± 1.9 percent (average ± standard deviation) over 415 days.] Rates of dechlorination were not calculated for the spiked Aroclor test. The heterogenous nature of the extent of dechlorination observed on each of the separate microcosms (and thus the separate activity tests described here which came from these individual microcosms) indicate heterogeneity in the microbial populations. Thus, the activities and pathways deduced in the following subsections are indicative of the most active populations in the Anacostia microcosms.

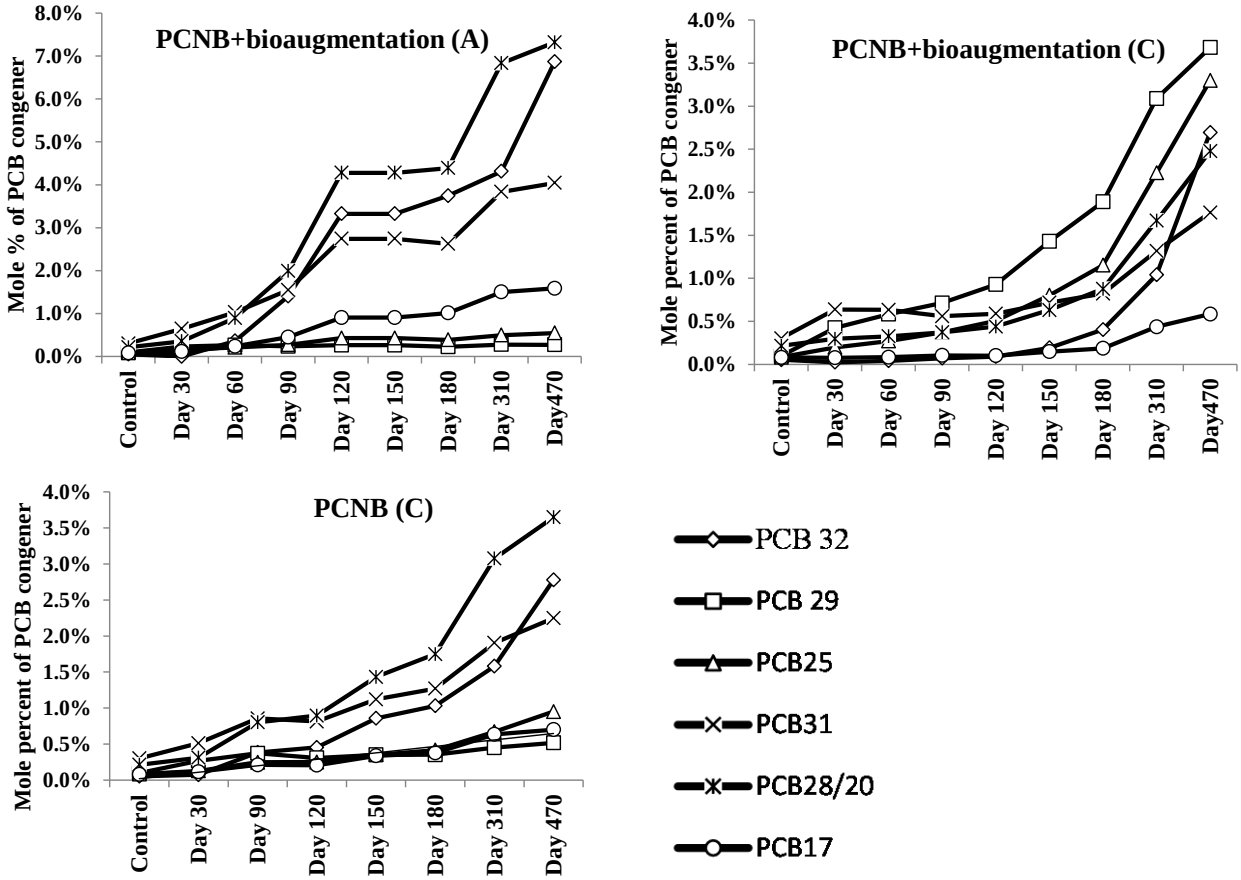


Figure 3-4. Mol % change in trichlorobiphenyl congeners during dechlorination of Aroclor 1254 in selected activity test replicates: (A) replicate A augmented with PCNB and strain 195; (B) replicate C augmented with PCNB and strain 195; and (C) replicate C augmented with PCNB.

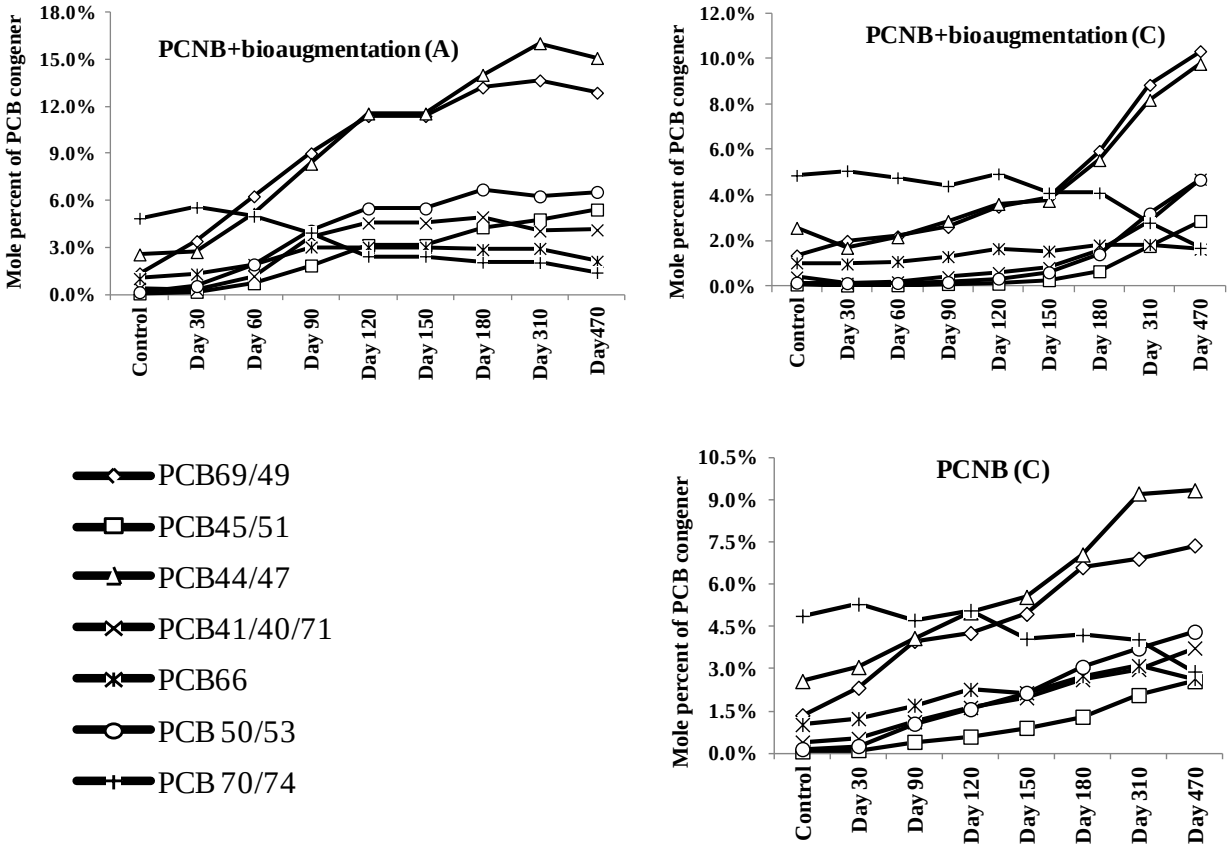


Figure 3-5. Mol % change in tetrachlorobiphenyl congeners during dechlorination of Aroclor 1254 in selected replicates: (A) replicate A augmented with PCNB and strain 195; (B) replicate C augmented with PCNB and strain 195; and (C) replicate C augmented with PCNB.

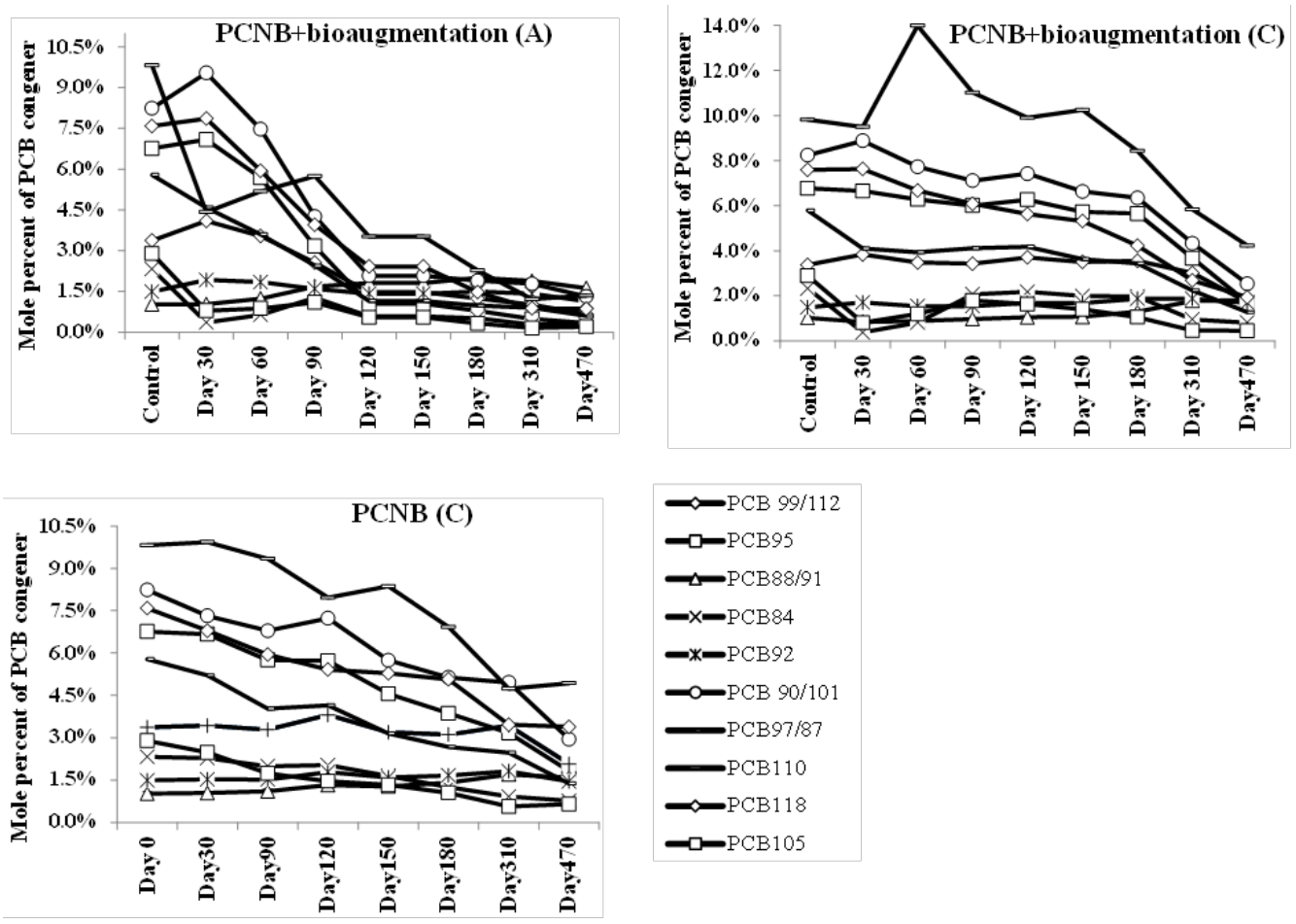


Figure 3-6. Mol % change in pentachlorobiphenyl congeners during dechlorination of Aroclor 1254 in selected replicates: (A) replicate A augmented with PCNB and strain 195; (B) replicate C augmented with PCNB and strain 195; and (C) replicate C augmented with PCNB.

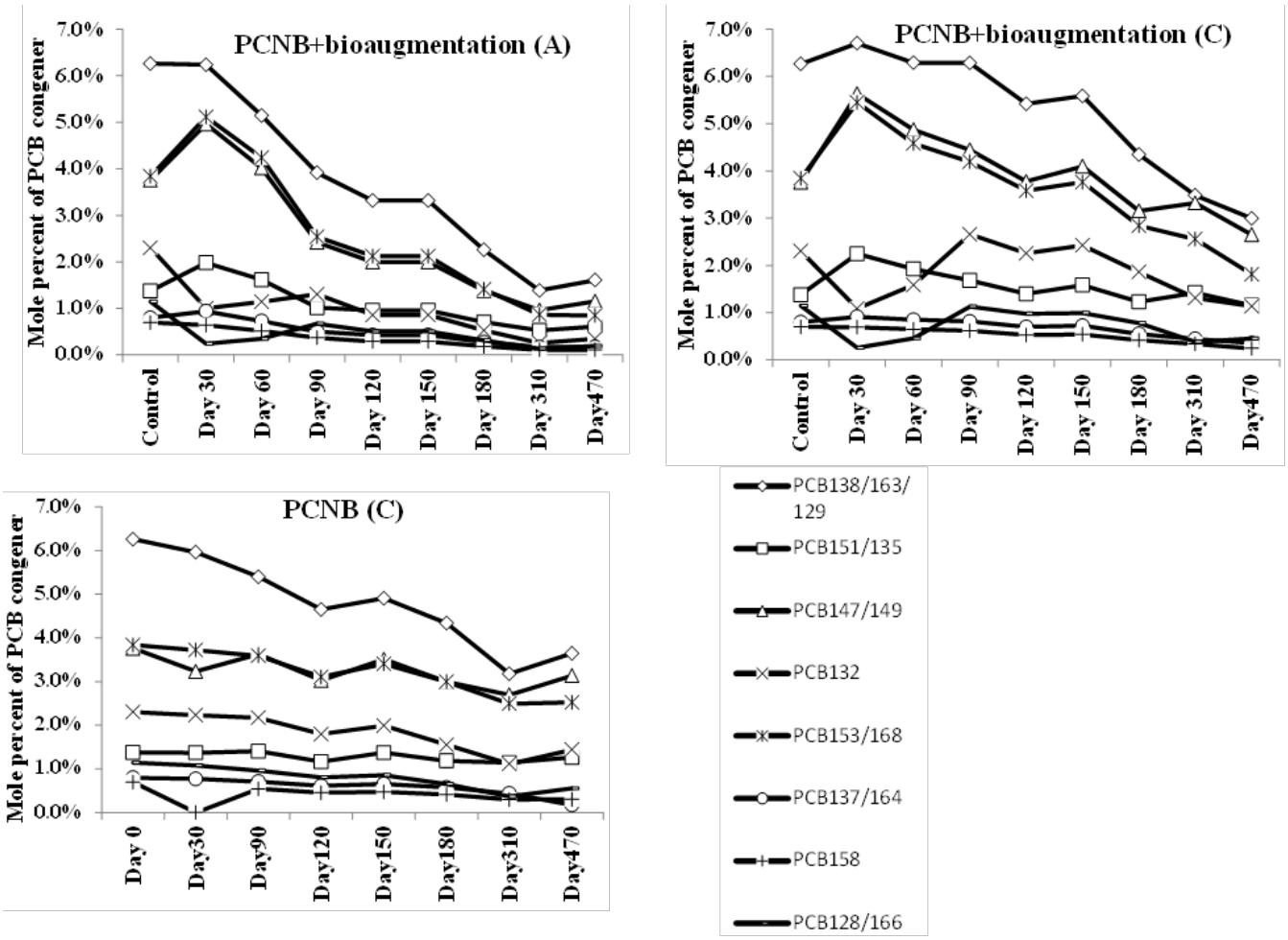


Figure 3-7. Mol % change in hexachlorobiphenyl congeners during dechlorination of Aroclor 1254 in selected replicates: (A) replicate A augmented with PCNB and strain 195; (B) replicate C augmented with PCNB and strain 195; and (C) replicate C augmented with PCNB.

Congener-specific analysis of dechlorination of Aroclor 1254. The time course of individual PCB congeners from different homologue groups including tri-, tetra-, penta- and hexa-CB over the 470 day incubation time was examined and results from selected replicates are shown in **Figures 3-4 to 3-7**. As mentioned previously, several of the tetrachlorobiphenyl congeners constituted the predominant dechlorination products of Aroclor 1254 in all the dechlorinating replicates after 310 days incubation. The major dechlorination products included PCB 47 (24-24), PCB 49 (24-25), PCB 53 (25-26) and PCB51 (24-26). In replicate PCNB +strain 195 (A), the summation of the mol % contribution of these four dechlorination products accounted for over 40% of the total PCBs by day 310. Overall, these four major congeners constituted 24 ± 16% and 19 ± 5% of all PCBs in treatments PCNB+strain 195 and PCNB, respectively. As the major terminal dechlorination products, PCB 47, 49, 51, and 53 showed increasing trends over the entire incubation time though at different rates (**Fig. 3-4**). In general, the accumulation rates of PCBs 47 and 49 were substantially higher than that of PCBs 51 and 53. Some of the tetra-CBs such as PCB 70/74, PCB 66 and PCB 41/71 were intermediates, i.e. accumulating in the beginning of the study and declining or reaching plateaus, after prolonged incubation. The time course data of the trichlorobiphenyls indicated a slower formation rate compared with that of tetrachlorobiphenyls (**Fig. 3-5**). The major trichlorobiphenyls accumulating during the dechlorination process included PCBs 26/29 (25-3/245), 25 (25-4), 31 (25-4), 32 (26-4), 28(24-4), and 17 (24-2). The distributions of these trichlorobiphenyl dechlorination products varied among these selected replicates (**Fig. 3-5**). Specifically, PCBs 31 (25-4) and 28 (24-4) appeared as the most abundant trichlorobiphenyls in PCNB +strain 195 (A) (**Fig 3-5A**). Nevertheless, in PCNB+strain 195(C), PCBs 26/29 (25-3/245) and 25 (25-4) were the major trichlorobiphenyls, with the presence of lesser amounts of PCBs 28 (24-4) and 31 (25-4) (**Fig. 3-5B**). Similar to what we observed in replicate PCNB+strain 195(A), PCBs 31 (25-4) and 28 (24-4) were present as the most abundant trichlorobiphenyls in replicate PCNB(C). This observed pattern is consistent with the findings from the individual congener activity test with the spiked PCBs 118 (34-245) and 170 (2345-234), which is discussed in a later section. Time course measurement of pentachlorobiphenyls (**Fig. 3-6**) and hexachlorobiphenyls (**Fig. 3-7**) indicated that most of the congeners were parent compounds for dechlorination, decreasing over time. Only a few PCBs, i.e., PCBs 99/112 and 128, show a slight initial increase and a later decline. This observation is likely related to the fact that they were also the immediate dechlorination products of the less abundant heptachlorobiphenyls. This trend is not reproduced in all the replicates, which may be partially attributed to the fact that the abundance of precursor congeners was lower, therefore the initial accumulation of these congeners as dechlorination products might be masked by their subsequent decline caused by their further dechlorination.

Specificity of dechlorination in activity tests. Chlorine distribution of the spiked Aroclor 1254 before and after dechlorination in different treatments revealed that in addition to the major *meta*-chlorine removal mechanism, dechlorination also occurred in *para* positions though to lesser extent (**Table 3-3**). The dechlorination pathways were inferred based upon the presence of the product congeners and their relative abundance.

We propose the dechlorination pathways of the major substrates in Aroclor 1254 based on the observed major dechlorination products in the three selected replicates and we performed mass balances as a check of their validities for day 470 values (**Tables 3-4 to 3-6**). As an attempt to test the proposed pathways, we conducted a mass balance to determine if good agreement could

Table 3-4. Mass balance on major highly chlorinated PCBs serving as parent compounds and their likely predominant dechlorination products in replicate PCNB+strain 195.

	Substrates	decrease (mol%)	total decrease (mol%)	Intermediate	decrease (mol%)	Product	Increase (mol%)	total increase (mol%)	difference (mol%)
95	236-25	6.43%	6.43%			26-25	6.41%	6.41%	-0.02%
110	236-34	8.47%	8.47%	26-34	3.74%	26-4	6.81%	10.55%	2.07%
90/101	235-24/245-25	6.93%							
97/**87**	234-25	5.19%							
141	2345-25	0.81%	13.79%			25-24	11.55%	11.55%	-2.24%
137	2345-24	0.62%							
170	2345-234	0.23%							
118	245-34	6.73%	12.89%	66(24-34)	1.10%	24-4	7.10%	11.95%	
105	234-34	2.69%				25-4	3.74%		-0.94%
70/74	25-34/245-4	3.47%							
99/112	245-24	2.71%							
138	234-245	4.65%							
128	234-234	1%	11.93%			24-24	12.56%	12.56%	0.62%
153	245-245	2.99%							
137	2345-24	0.62%							
149	236-245	2.61%		91(24-236)	0.59%	26-24	5.36%	5.36%	
84	236-23	2.05%	6.61%			236-2			-0.66%
132	234-236	1.95%		95(25-236)					

Table 3-5. Mass balance on major highly chlorinated PCBs serving as parent compounds and their likely predominant dechlorination products in replicate PCNB+strain 195.

	Substrates	decrease (mol%)	total decrease (mol%)	Intermediate	decrease (mol%)	Product	Increase (mol%)	total increase (mol%)	difference (mol%)
95	236-25	5.07%	5.07%			26-25	4.53%	4.53%	-0.54%
110	236-34	5.59%	5.59%	26-34	4.66%	26-4	2.64%	7.30%	1.70%
90/101	235-24/245-25	5.71%							
97/87	234-25	4.51%							
141*	2345-25	0.67%	11.46%			25-24	8.99%	8.99%	-2.46%
137 *	2345-24	0.44%							
170*	2345-234	0.12%							
118	245-34	5.65%	5.65%			24-3	3.21%	6.80%	1.15%
						25-3	3.58%		
70/74	25-34/245-4	3.19%	3.19%			24-4	2.26%	3.72%	0.53%
						25-4	1.46%		
105	234-34	0.36%							
99/112	245-24	1.66%							
138	234-245	3.27%							
128	234-234	0.70%	8.21%			24-24	7.22%	7.22%	-0.99%
153	245-245	2.03%							
137*	2345-24	0.44%							
170*	2345-234	0.12%							
149	236-245	1.11%		91(24-236)	0.79%	26-24	2.80%	2.80%	-0.20%
84	236-23	1.51%	3.79%			236-2			
132	234-236	1%							

* we assumed that the dechlorination of those congeners containing 2345-chlorophenyl ring was through two pathways: 40% of two successive meta chlorine dechlorination to 25-chlorophenyl ring and 60% of first para then meta chlorine dechlorination to 24-chlorophenyl ring.

Table 3-6. Mass balance on major highly chlorinated PCBs serving as parent compounds and their likely predominant dechlorination products in replicate PCNB.

	Substrates	decrease (mol%)	total decrease (mol%)	Intermediate	decrease (mol%)	Product	Increase (mol%)	total increase (mol%)	difference (mol%)
95	236-25	4.94%	4.94%			26-25	4.16%	4.16%	-0.78%
110	236-34	4.88%	4.88%	26-34	2.58%	26-4	2.73%	5.31%	0.42%
90/101	235-24/245-25	5.29%							
97/**87**	234-25	4.41%							
141*	2345-25	0.57%	10.92%			25-24	8.26%	8.26%	-2.66%
137*	2345-24	0.62%							
170*	2345-234	0.02%							
118	245-34	4.20%	4.20%	24-34		24-4 25-4 25-3 24-3	3.43% 1.95% 0.42% 0.87%	6.67%	0.86%
70/74	25-34/245-4	0.97%	0.97%						
105	234-34	2.25%	2.36%						
99/112	245-24	1.30%							
138	234-245	2.61%							
128	234-234	1%	6.47%			24-24	6.77%	6.77%	0.30%
153	245-245	1.32%							
137*	2345-24	0.62%							
170*	2345-234	0.02%							
149	236-245	0.62%	2.46%	91(24-236)	0.57%	26-24	2.49%	2.49%	0.60%
132	234-236	0.86%				236-2			
84	236-23	0.97%							

*we assumed that the dechlorination of those congeners containing 2345-chlorophenyl ring was through two pathways: 40% of two successive *meta* chlorine dechlorination to 25-chlorophenyl ring and 60% of first *para* then *meta* chlorine dechlorination to 24-chlorophenyl ring.

intermediates and the corresponding decrease of the proposed parent substrates. The mass balance is nearly perfect in the replicate PCNB+strain 195 (C) (**Table 3-4**) and the replicate PCNB(C) (**Table 3-5**). The mass balance is not closed for the observed increase of PCB 47 (24-24) in replicate PCNB+strain 195 (C) (**Table 3-6**), which may either be caused by the overestimation of the product or the underestimation of some of the substrates and perhaps because of an interfering chromatographic peak observed only in this replicate.

The PCB congeners in Aroclor 1254 contain an abundant amount of chlorobiphenyl rings with the chlorine substitution pattern of 2345-, 234-, and 245-, which constitute about 50% of the total PCBs. Complete *meta*-dechlorination of these chlorophenyl rings would cause the accumulation of 24- substituted chlorophenyl rings, which is reflected by the presence of PCB congeners such as PCBs 47 (24-24) and 49 (25-24) as the congeners with the largest mol % increase in the present study. Congeners with 2345-substitution such as PCBs 137 (2345-24), 141 (2345-25) and170 (2345-234) were reported to be dechlorinated in both *meta*- and *para*- positions previously (Bedard et al., 2006; Fagervold et al., 2007). It has been further proposed that approximately 60% of their loss was by serial removal of double flanked *para*- and *meta*-chlorines, i.e. generating a 25-substituted chlorobiphenyl ring, while 40% of the loss was through removal of two *meta*-chlorines which generated 24-substituted chlorobiphenyl rings. In the present study, we considered both of these two dechlorination pathways in replicates

PCNB+strain195 (C) and PCNB (C), and split the observed decrease of these congeners into two different products with the relative proportions apportioned as reported previously (Fagervold et al., 2007) (**Tables 3-5 and 3-6**).

Dechlorination of spiked individual PCB congeners in sub-cultures. Dechlorination was monitored in sub-cultures of the activity tests amended with PCB 118 (234-34) and PCB 170 (2345-345), predominant congeners in Anacostia sediment. Dechlorination of these two PCB congeners was detected in all replicates, however, the extent of dechlorination varied. PCB 118 was dechlorinated by approximately 80 mol % from day 0 to day 90 in the replicates transferred from PCNB+strain 195 (A) and PCNB+strain 195(C), while moderate dechlorination was observed in replicates prepared from PCNB(C). The dechlorination of PCB 170 proceeded at a slower and relative consistent rate among the sub-cultures prepared from these three replicates, showing approximately 30 mol % dechlorination by day 90. Based on the appearance of different dechlorination products in different replicates, and their relative proportions, we inferred the dechlorination pathways of PCB 118 (**Fig. 3-8**) and 170 (**Fig. 3-9**). PCB170 has been reported to be dechlorinated to either PCB 47 or PCB 49 (Fagervold et al., 2007). This study showed the

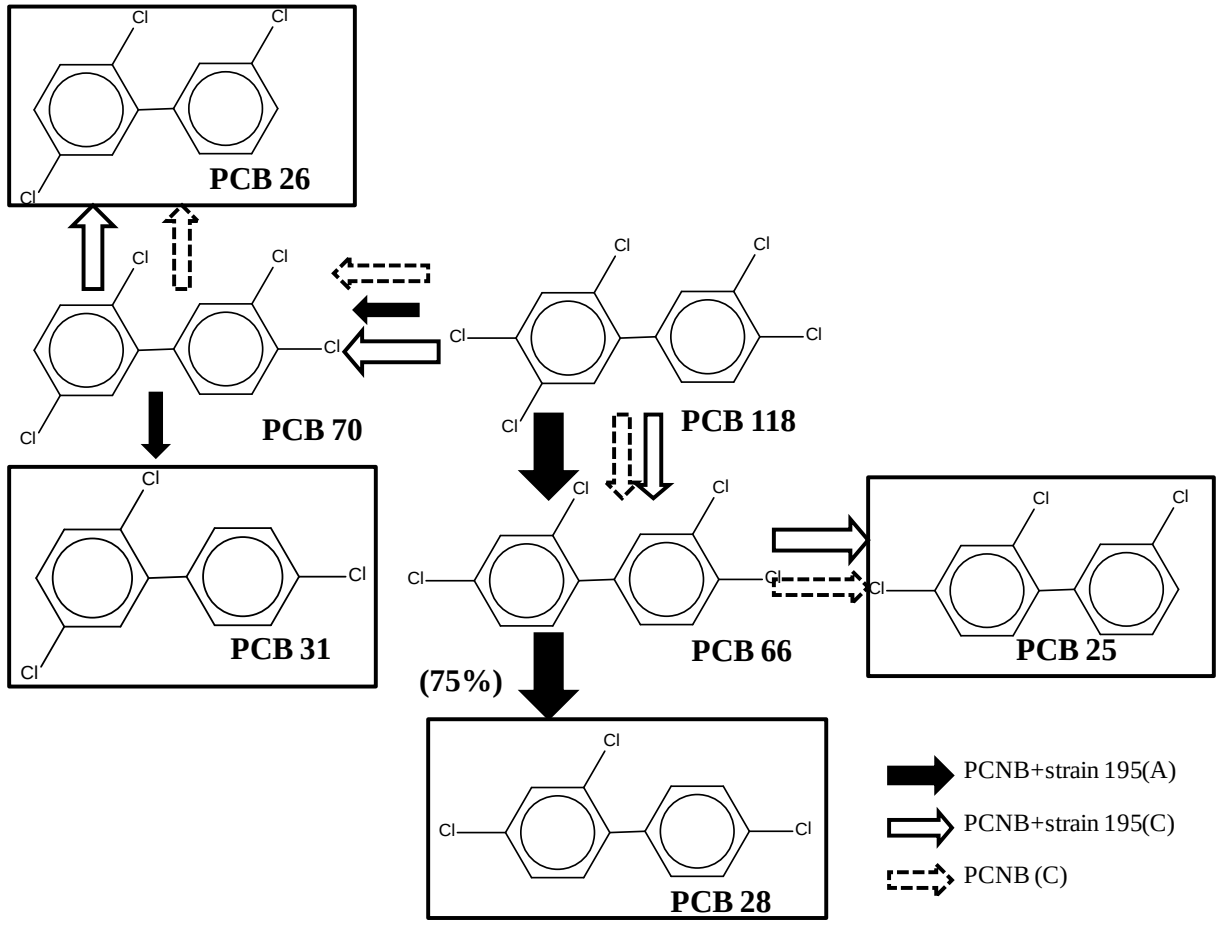


Figure 3-8. Proposed dechlorination pathway for PCB 118 in spiked individual congener subculture. The pathways are shown for subcultures of different replicates: black solid arrows, PCNB+strain 195(A); open arrows, PCNB+strain 195(C); dashed arrows, PCNB (C). The predominant end products are boxed. The size of the arrow is proportional to their dominance.

same terminal dechlorination product, with PCB 49 accounting for about 60% of the total products. The results also indicated that dechlorination only occurred in the flanked *meta* positions in sub-cultures prepared from replicate PCNB + strain 195 (C). In the sub-cultures from the other two replicates, dechlorination occurred in both the flanked *meta*- and *para*-positions. In addition, the dechlorination of flanked *meta*-chlorines was favored over that of *para*-chlorines, as deduced from the relative abundance of the dechlorination products from these two different pathways. Furthermore, congeners containing 236- and 2356-substituted chlorophenyl rings also served as substrates in the sub-cultures. Removal of *meta*-chlorines from these chlorophenyl rings generated the 26-substituted chlorophenyl rings, as reflected in the accumulation of PCBs 51 (24-26) and 53 (25-26). This observation was entirely distinct from activity observed for Anacostia River sediment microcosms, in which the dechlorination of these 236- and 2356-substituted chlorophenyl rings was not observed.

Comparison of dechlorination pathway deduced from sub-culture test with that from strain 195 test. As an attempt to examine the species-specific dechlorination activity, we also examined dechlorination of Aroclor 1254 by a mixed culture containing *D. ethenogenes* strain 195 to determine its specific effect in contaminated sediments to which it was bioaugmented. Dechlorination of Aroclor 1254 by the culture was slow (**Fig. 3-10**), but enhanced by the

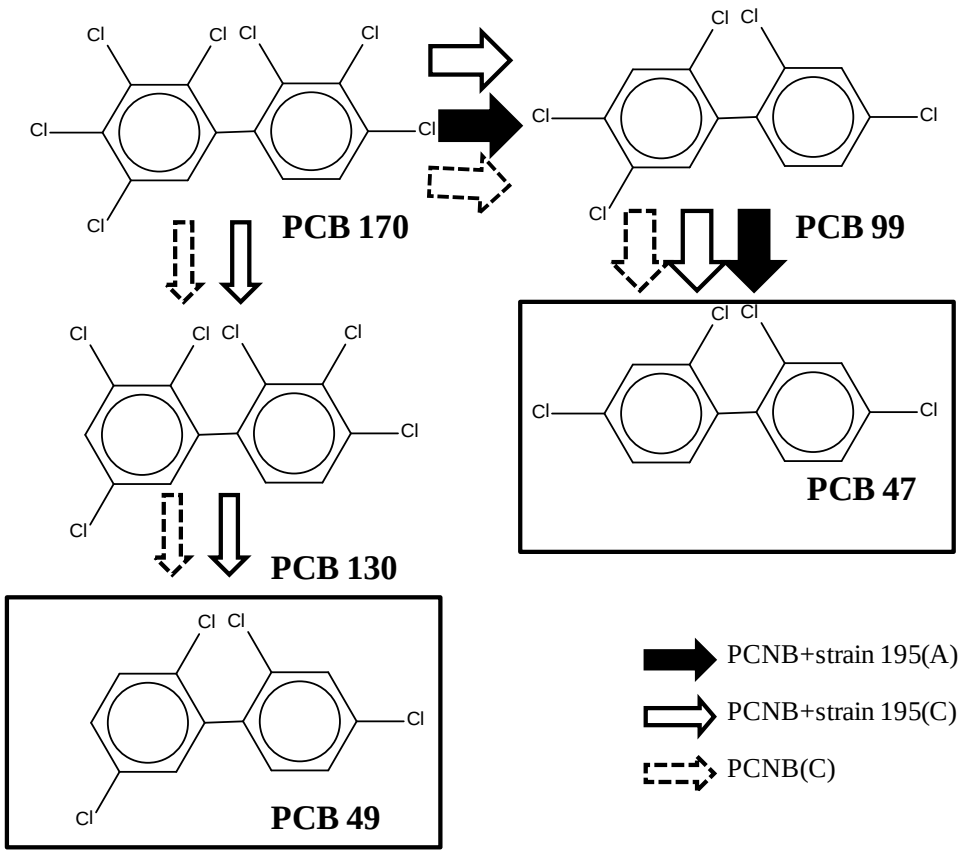


Figure 3-9. Proposed dechlorination pathway for PCB 170 in spiked individual congener subculture. The pathways are shown for subsultures from different replicates: black solid arrows, PCNB+strain 195(A); open arrows, PCNB+strain 195(C); dashed arrows, PCNB (C). The predominant end products are boxed. The size of the arrow is proportional to their dominance.

addition of tetrachlorobenzene (a known growth substrate). The congener specific results indicated that the mixed culture exclusively dechlorinated PCBs containing doubly-flanked chlorines at the *meta*- and *para*- positions. The deduced dechlorination pathways based on the observations from tests with Anacostia River sediments and the strain 195 mixed culture are compared in **Table 3-7**. Dechlorination of doubly-flanked chlorines was reported previously for bacterium DF-1 (Wu et al., 2000; Wu et al., 2002) and *D. ethenogenes* strain 195 (Fennell et al., 2004). In comparison, the dechlorination processes mediated by the Anacostia microbial community in the activity test included dechlorination of flanked chlorines (including both doubly and singly flanked) at the *meta*- and *para*-positions. The accumulated terminal dechlorination products in Anacostia sediment microcosms were not typical of any known dechlorination process, but rather a combination of several processes. The key reductive dechlorination products for process N are PCBs 47, 51,100, while the key dechlorination products in Anacostia sediment were tetra-CBs including PCBs 47, 49 and 53 and tri-CBs including PCBs 28 or 29 and 25 (varying according to the specific replicate). Furthermore, the fact that addition of PCB 116 as an alternate halogenated electron acceptor was not effective in stimulating the dehalogenating population in Anacostia sediment to dechlorinate weathered PCBs, but did stimulated extensive process N dechlorination of the Aroclor 1260 residue in Housatonic River sediment (Van Dort et al, 1997), provide another line of evidence that the observed dechlorination in Anacostia sediment is different from the previously described process N.

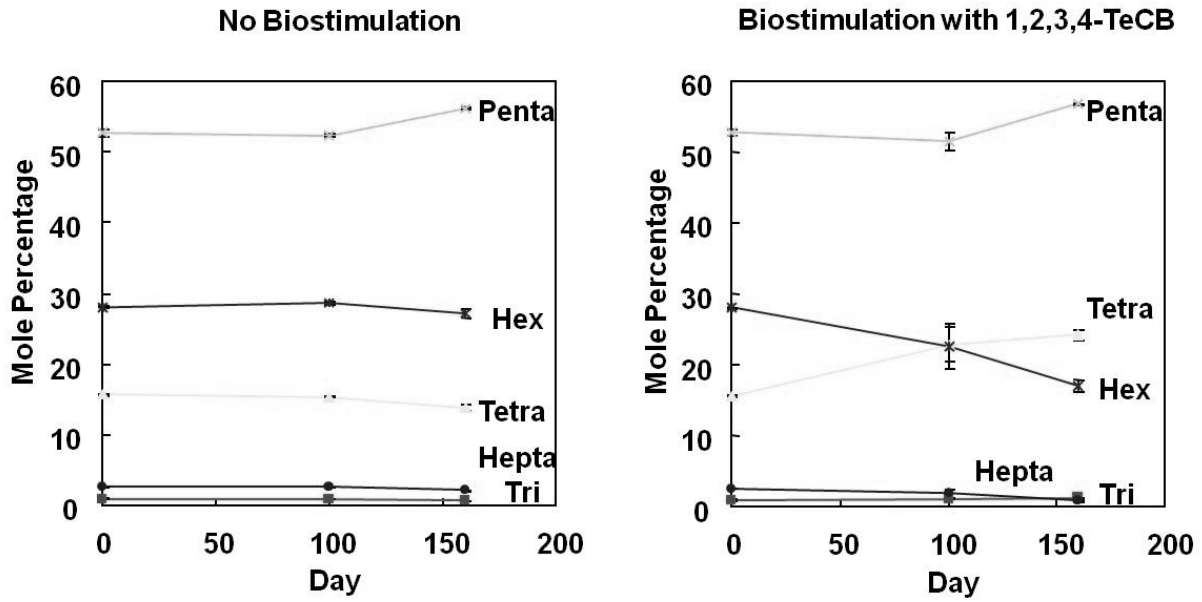


Figure 3-10. Time course change in mole % of different PCB homologs (hepta-, hexa, penta-, tetra- and tri-chlorinated biphenyls) during dechlorination of Aroclor 1254 by a mixed culture containing Dehalococcoides ethenogenes strain 195 with and without additions of the known growth substrate tetrachlorobenzene.

Comparison of the dechlorination pathways in activity tests and sub-cultures with that from Anacostia sediment microcosms. In general, dechlorination pathways observed in Anacostia sediment microcosms with weathered PCBs were in agreement with that observed in the activity tests amended with fresh Aroclor 1254. However, some discrepancies were also

observed. First, a broader range of substrates for dechlorination was observed in the activity test and subculture samples. For example, PCB congeners containing 236- and 2356-substituted rings were dechlorinated in activity test samples but not in weathered PCBs in Anacostia sediment microcosms. As a result, a broader suite of daughter products were detected in the activity tests compared with the Anacostia sediment microcosms. Specifically, some of the predominant terminal dechlorination products such as PCBs 32 (26-4), 51 (24-26) and 53 (26-25) were absent in Anacostia sediment microcosms after prolonged incubation.

Table 3-7. Deduced PCB dechlorination pathways based on observations from Anacostia activity tests and strain 195 tests with Aroclor 1254.

Congener dechlorination pathway			**Dechlorination activity**
Anacostia microcosm activity culture		**Strain 195 mix culture**	
95 (236-25)	**→ 53 (25-26)**		***ortho*-flanked *meta***
101 (245-25)	**→ 49 (24-25)**		***para*-flanked *meta***
101 (245-25)	**→ 52 (25-25)**		***meta*-flanked *para***
90 (235-24)	**→ 49 (25-24)**		***ortho*-flanked *meta***
87 (234-25)	**→ 49 (24-25)**	**87 (234-25) → 49 (24-25)**	**double flanked *meta***
110 (236-34)	**→ 71 (26-34)**		***ortho*-flanked *meta***
105(234-34)	**→ 66 (24-34)**	**105(234-34) → 66 (24-34)**	**double flanked *meta***
118 (245-34)	**→ 66 (24-34)**		***para*-flanked *meta***
66 (24-34)	**→ 28 (24-4)**		***para*-flanked *meta***
66 (24-34)	**→ 25(24-3)**		***meta*-flanked *para***
118 (245-34)	**→ 70 (25-34)**		***meta*-flanked *para***
70 (25-34)	**→ 26 (25-3)**		***meta*-flanked *para***
70 (25-34)	**→ 31 (25-4)**		***para*-flanked *meta***
170 (2345-234)	**→ 130(234-235)**		**double flanked *para***
130(234-245)	**→ 99 (245-24)**		**double flanked *meta***
138 (234-245)	**→ 99 (245-24)**	**138 (234-245) → 99 (245-24)**	**double flanked *meta***
153 (245-245)	**→ 99 (245-24)**		***para*-flanked *meta***
99 (245-24)	**→ 47 (24-24)**		***para*-flanked *meta***
99 (245-24)	**→ 49 (25-24)**		***meta*-flanked *para***
132 (234-236)	**→ 91 (236-24)**	**132 (234-236) → 91 (236-24)**	**double flanked *meta***
149 (236-245)	**→ 91 (236-24)**		***para*-flanked *meta***
91 (236-24)	**→ 51(26-24)**		***ortho*-flanked *meta***
128 (234-234)	**→ 47 (24-24)**	**128 (234-234) → 47 (24-24)**	**double flanked *meta***
137(2345-24)	**→ 47 (24-24)**	**137(2345-24) → 47 (24-24)**	**flanked *meta***
41(234-2)	**→ 17 (24-2)**		**double flanked *meta***

4. Mesocosm-Scale Investigation of PCB Dechlorination and Examination of PCB Dechlorination Pathways

Abstract

We investigated potential enhancements to reductive dechlorination of polychlorinated biphenyls (PCBs) in sediments from two contaminated sites with relatively low historic PCB concentrations: the Anacostia River, a tidal freshwater waterway in Washington, DC and Kearny Marsh, a freshwater impoundment in the New Jersey Meadowlands. Sediment mesocosms were amended with electron donors, haloprimers (tetrachlorobenzene or pentachloronitrobenzene), indigenous microbial communities pre-activated on haloprimers, and/or a mixed culture containing *Dehalococcoides ethenogenes* strain 195. Mesocosm treatments were also compared between capped (Aquablok™) and uncapped systems. These treatments were evaluated as a means to increase dechlorinator populations and dechlorination activity for *in situ* bioremediation.

Treatment effectiveness was evaluated by examining changes in the chlorination level of the pre-existing PCBs from the collected mesocosm samples over time. A congener specific PCB analytical method resolving 117 peaks with 153 PCB congeners was developed using a tandem gas chromatography-mass spectrometry system for enhanced identification and quantification of individual PCB congeners when compared to gas chromatography-electron capture detection. Dechlorination results obtained with the congener-specific method averaged across triplicate mesocosm treatments have to date been compared only with the background chlorination level in uncapped controls. Analytical results for capped controls are in progress. In comparison with the uncapped controls, dechlorination was observed in some individual mesocosm replicates from the treatments bioaugmented with activated indigenous microorganisms (AIM) or from treatments bioaugmented with a combination of AIM and a mixed culture containing *D. ethenogenes* strain 195 and haloprimed with pentachloronitrobenzene.

The specific PCB dechlorination pathways were also investigated using the new congener-specific analytical method through the analysis of saved and re-sampled sediment microcosm samples collected at operational day 1000. The results of this analysis for Anacostia River to date demonstrated dechlorination of PCBs at the *meta*- and *para*-positions as reported in previous studies of PCB dechlorination.

Experimental methods

Mesocosm preparation. Mesocosms were constructed for Anacostia River and Kearny Marsh using sediment and site water (**Fig. 4-1**), and a matrix of treatments and amendment combinations were utilized (**Table 4-1**). Electron donors, halogenated co-amendments and bioaugmented bacteria (both laboratory strains and activated indigenous bacteria) were added through a variety of processes. All these studies have included microcosms run in parallel to provide additional treatment variables and sufficient replication for statistical analyses.

The Anacostia and Kearny Marsh Sediment mesocosms were constructed in 200 mm diameter glass vessels. Each vessel contained approximately 200 mm (final settled thickness) of

sediment, with the Kearny Marsh mesocosms also including 25 mm of bentonite clay beneath the sediment layer to aid in core sample recovery. Depending on the treatment, the sediment was either capped with 150 mm of Aquablok, or uncapped, with an additional 200 mm of site water on top. The total sediment volume was approximately 6 L.

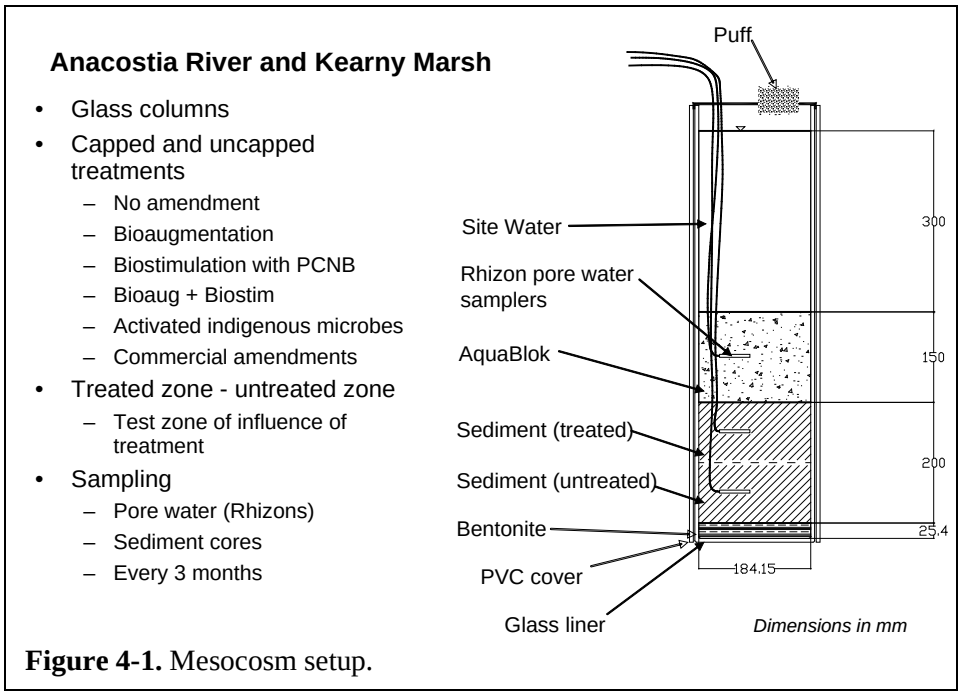


Figure 4-1. Mesocosm setup.

Sediment mesocosms were amended with electron acceptors, halogenated co-substrates (tetrachlorobenzene or pentachloronitrobenzene) as "primers", indigenous microbial communities pre-activated on haloprimers, and/or a mixed culture containing *Dehalococcoides ethenogenes* strain 195. The complete protocol is shown in **Table 4-1**.

Table 4-1. Experimental protocol for mesocosms tests of PCB dechlorination in Anacostia River and Kearny Marsh sediments.

TREATMENT	KM	Ana
Control	X	X
Pentachloronitrobenzene (PCNB) (20 µM) + ED	X	X
Bioaugmentation (*D. ethenogenes* strain 195 mixed culture, 2% vol:vol) + ED	X	X
PCNB + Bioaugmentation	X	X
Activated Indigenous Microorganisms (AIM)	X	X
PCNB + Bioaugmentation + AIM	X	X

The treatments were mixed into the top 100 mm of sediment, and the sediment was allowed to settle for two days before capping. Polyoxymethylene (POM) coupons were installed into the water column above the sediment or cap to provide an analysis of PCB equilibration into the overlying water. Rhizon pore water samplers were installed in the upper (treated) 100 mm of sediment as well as the lower (untreated) sediment. The glass vessels were covered, with air

exchange moderated by a polyurethane foam (PUF) plug. The mesocosms were incubated in the dark in a temperature-controlled greenhouse. The mesocosms were sampled four days after treatment addition (to allow for settling of the sediment and swelling of the Aquablok), at Day 30 and Day 90, 180, 270. Sediment cores (11 mm diameter) were collected and stored for molecular and PCB analysis. The temperature, pH, and dissolved oxygen concentration of the surface water was measured. Surface water samples and sediment pore water samples (collected *via* rhizons) were frozen for future anion and cation analyses.

Mesocoms have now been monitored for over 300 days. Pore water, overlying water, solid phase polyoxymethylene (POM) coupons, sediment cores, and the polyurethane foam gas samplers (PUFs) were collected at sampling events. The PUFs will be analyzed for PCBs to assess outgassing from the mesocosms. The mesocosms were first sampled four days after treatment addition (to allow for settling of the sediment and swelling of the Aquablok). Sediment cores (11 mm diameter) were collected and stored at -20°C for molecular and PCB analysis. The temperature, pH, and dissolved oxygen concentration of the surface water was measured using a filed probe. Surface water samples and sediment pore water samples (collected *via* rhizons) were frozen for future anion and cation analyses. Specific analyses for the various media sampled are shown in **Table 4-2**.

Table 4-2. Mescosm sampling and analyses.

Medium	Sampling method	Samples/ mesocosm	Analysis
Air	Polyurethane foam plug	1[a]	PCBs
Overlying water	Grab	1	pH, DO, ORP, anions, cations (IC)
	Polyoxymethylene (POM) coupon	1	PCBs PCNB and daughter products
Pore water	Rhizon samplers (5 mL total volume)	3	Anions, cations
Sediment	1 cm ID core	2[b]	PCBs PCNB and daughter products Molecular

[a] – an additional 10% of PUFs will be analyzed per sampling event as field blanks
[b] – 2 or 3 samples per core – treated (top), untreated (bottom) sediment and cap

Molecular/Microbial Analyses. The molecular/microbial measurements (i.e., population structure, number of dehalogenating species and population density, diversity of dehalogenase genes) will be correlated to the extent of biotransformation observed under different treatment conditions in the micro- and mesocosms. This work is in progress and will be reported at a later date.

Chemical analysis. Mesocosms were sampled for PCB dechlorination at Day 0, 30, 90, 180, 270 by employing an automatic pipette. Sediments were allowed to air dry for 24-48 h before being extracted in a Dionex ASE 200 Accelerated Solvent Extractor (Dionex, Bannockburn, IL, USA)

following EPA method 3545. PCB surrogate standards consisting of PCB 14, 23, 65, 166 were added into the dried sediment right before the extraction. The average weight of the mesocosm samples was approximately 8.3 (±2.4, n=39) wet weight and 4.4 (±1.5, n=39) after air drying. The ASE extraction method was as previously described (Krumins et al., 2009) except that the extraction solvents were changed to a mixture of methylene chloride:hexane (25:75 volume:volume (v:v)) instead of using hexane alone. The underlying reason for this solvent change was to achieve more effective extraction of the organochlorine pesticides (OPCs) which we will also track in these mesocosms. The extracts were solvent exchanged to hexane and then reduced to 1 ml under gentle nitrogen gas flow. The extract was fractionated using a 2.5% water-deactivated Florisil column, with 35 ml of petroleum ether eluting the PCB fraction. The second fraction containing some organochlorine pesticides was eluted with 50 ml of a 1:1 (v:v) petroleum ether:methylene chloride mixture. The extract was reduced to around 3 ml with a rotary evaporator and was further concentrated to ~ 0.5 ml under a nitrogen gas stream. Internal standards PCBs 30 and 204 were injected into the samples prior to instrumental analysis.

Quantitative congener-specific PCB analysis was performed by using a tandem quadrupole GC/MS/MS system (Waters Quattro Micro GC). All samples were measured for a suite of 209 PCB congeners using a method similar to EPA method 1668A. The MS-MS operating parameters for the determination of PCBs are presented in **Table 4-3**. In order to achieve maximum sensitivity, the two most abundant isotopes M^+ and $[M + 2]^+$, were monitored and the total ion current (TIC) was used for quantification with the aim of achieving maximum sensitivity. This instrument provides detection limits similar to an Electron Capture Detector, while also allowing the unequivocal identification of more PCB congeners. The extent and pathways of PCB dechlorination were investigated through identification and quantification of historical PCB congeners and potential dechlorination products eluting from the GC-MS-MS

Table 4-3. GC-MS-MS operating parameters for the determination of PCBs. The MS operating conditions were the following: the temperature of the transfer line was held at 250°C during the chromatographic run. EI source is operated at 200°C with an electron energy of 70 eV and a trap current of 100 A. The MRM mode was operated at an argon collision gas pressure of 3.0 x 10-3 mBar.

Functions	PCBs	Start time (min)	End time (min)	Parent Ion (m/z)	Daughter Ion (m/z)	Dwell (secs)	Collision Energy (ev)
1	Mono-PCB	15	32	188+190	153	0.05	15
2	Di-PCB	15	32	222+224	152	0.05	15
3	Tri-PCB	23	42	256+258	186	0.05	15
4	Tetra-PCB	32	55	289.9+291.9	220	0.05	23
5	Penta-PCB	34	65	323.9+325.9	254	0.05	25
6	Hex-PCB	49	75	359.8+361.8	289.90	0.05	25
7	Hepta-PCB	58	75	393.8+395.8	323.90	0.05	22
8	Octa-PCB	64	76	429.8+431.8	360.0	0.05	22
9	None-PCB	69	77	463.7+465.7	394.0	0.05	22
10	Deca-PCB	77	90	497.7+499.7	428	0.05	22

system. This method separated the 209 PCB congeners into around 170 peaks. Approximately 117 chromatographic peaks representing approximately 153 PCB congeners were consistently detected in our samples.

The OCPs were analyzed using gas chromatography/mass spectrometry in negative chemical ionization mode (GC/MS-NCI) operating in selective ion monitoring mode (SIM) with a Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer. The details for this method have been reported elsewhere (Gioia et al., 2005).

The mole percent of the resolved PCB congeners was calculated based on the molar concentration of each congener versus the sum of the molar concentrations of all detected congeners. We examined the mole percent distribution of some of the major parent congeners present in the sediment and the expected dechlorination products as proposed by other anaerobic dechlorination studies reported in the literature.

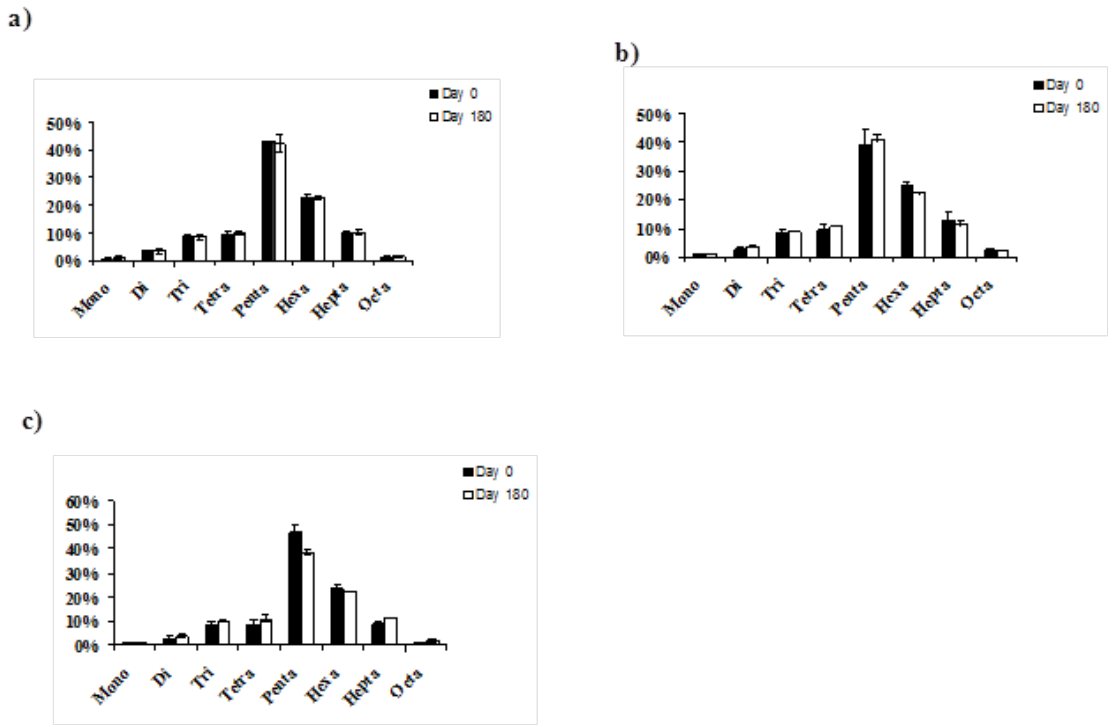


Figure 4-2. Time course of homologue distribution of PCBs at day 0 and day 180 in mesocosm treatments: a) PCNB; b) activated indigenous microorganism (AIM); c) PCNB + Bioaugmentation + AIM. Error bars are standard deviation of measurements from triplicate samples.

Results

Dechlorination in Anacostia mesocosms. The mesocosm samples were collected at Day 0, 30, 90, 180 and 270 and while analyses are still on-going, results from some mesocosms are reported here. **Figure 4-2** shows the averaged results from three replicates of the mole percent distribution of the PCB homologues at Day 0 and Day 180 for selected treatments. In general, dechlorination results averaged across triplicate mesocosms indicated only slight changes in less

chlorinated congeners at Day 180 when compared to Day 0 (**Fig. 4-2**). For the PCNB treatment (**Fig. 4-2a**), the mole fraction of all PCB homologs were relatively similar at Day 0 and Day 180. For the treatments bioaugmented with an enrichment of indigenous dehalogenators (AIM) (**Fig. 4-2b**) or a combination of PCNB, strain 195 and an enrichment of indigenous dehalogenators (AIM) (**Fig. 4-2c**), the mole fraction of less- chlorinated congeners (from mono- to tetra- PCB) were increased slightly while slight decreases were observed for the more-chlorinated congeners (hexa- and/or hepta- PCB). Analysis of additional mesocosm samples including the capped control is on-going and a full statistical analysis of the significance of the dechlorination in the bioaugmented and biostimulated treatments as compared to controls, will be performed when the complete data set has been analyzed. Variations in dechlorination extent among triplicates of specific treatments were observed. Dechlorination clearly occurred in individual replicates of Anacostia mesocosms receiving bioaugmentation with an enrichment of indigenous dehalogenators (AIM) (**Fig. 4-3b**) or those treated with PCNB, AIM and a mixed culture containing *Dehalococcoides ethenogenes* strain 195 (**Fig. 4-3c**). For instance, the relative percentage increases for tetra-PCBs were 13 and 38 % with a concomitant decrease in hexa-PCBs of 13 and 12 % for two replicates, respectively, of the treatment bioaugmented with an enrichment of indigenous dehalogenators (AIM). However, the dechlorination effect was ambiguous in other similarly treated replicates (**Fig. 4-3c**). It is possible that sampling artifacts could mask the dechlorination signal.

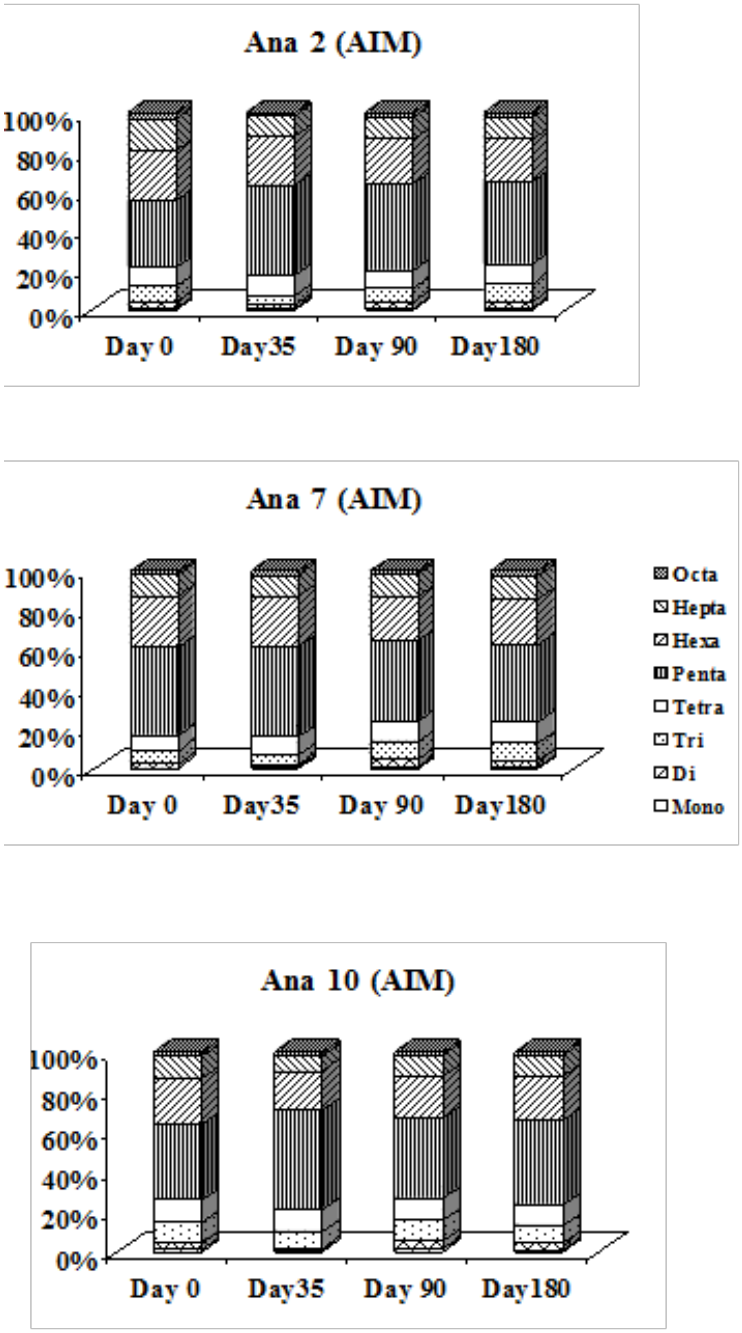


Figure 4-3. Time course of changes of PCB homologue distribution in individual replicates from two different treatments: a) and b): activated indigenous microorganisms (AIM); c) AIM + PCNB + bioaugmentation.

PCB dechlorination pathways in Anacostia microcosms. Previously preserved, unextracted Anacostia microcosm samples collected at Day 0 and Day 135 together with new samples collected at Day 1000 applying the same sampling technique as described previously (Krumins et al., 2009), were analyzed for a dechlorination pathway study using the GC-MS-MS congener-specific analytic method. Changes in specific congeners over time and concurrent changes in paired likely parent-daughter congener sets are reported here. An addition 1 ml sample was collected from each microcosm for molecular analysis.

Analysis of PCB dechlorination at day 1000. Results for selected likely parent-daughter congener sets are shown in **Figure 4-4**. This analysis of Anacostia microcosms at Day 1000 confirmed ongoing and prolonged dechlorination over 1000 days (4 years) in effective treatments. The results indicated that amendment with pentachloronitrobenzene (PCNB) or PCNB plus bioaugmentation were most effective in enhancing dechlorination. The dechlorination pathway of these selected congeners have been have been studied either with individual spiked congener studies or from dechlorination activities observed with Aroclors (Bedard et al., 2006; Fagervold et al., 2007). The decrease in the relative abundance of potential parent congeners and the corresponding accumulation of their potential dechlorination daughter products matched the pathways previously reported for *Dehalococcoides* spp. in other studies.

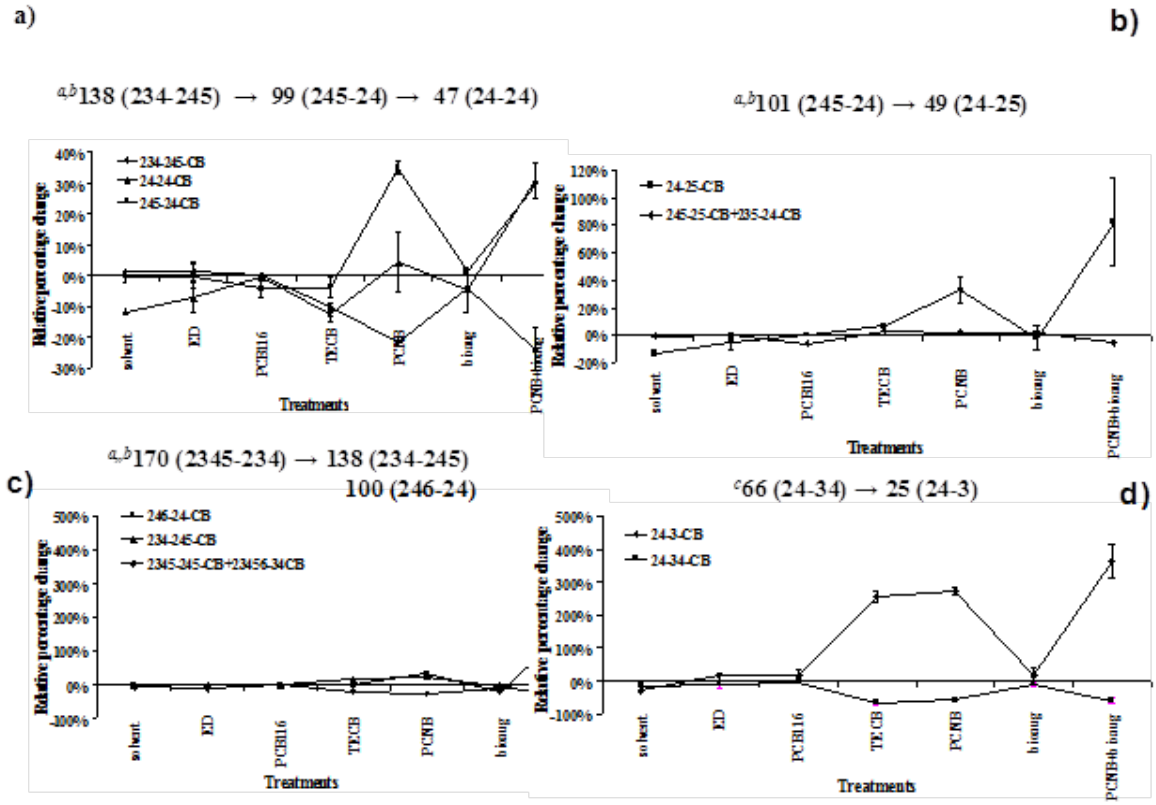


Figure 4-4. Percent change in abundance of specific PCB congener relative to controls in Anacostia sediment microcosms at Day 1000. Treatments: Solvent (Solvent); electron donor (ED); PCB 116 (PCB116); tetrachlorobenzene (TeCB); pentachloronitrobenzene (PCNB); mixed culture with *Dehalococcoides ethenogenes* strain 195 (Bioaug); PCNB plus mixed culture with *D. ethenogenes strain* 195 (PCNB + Bioaug). [Note pathways reported previously: a Bedard et al., 2006; b Fagervold et al., 2007; c Bzdusek et al., 2006.]

For example, 2345-substituted PCBs were preferentially dechlorinated in the *meta* position (Bedard et al., 2006; Fagervold et al., 2007). In addition, *para* dechlorination also occurred in the microcosms, e.g., PCB 170 (2345-234) to PCB 130 (2345-23) and PCB 66 (24-34) to PCB 25 (24-3).

Time course analysis of PCB dechlorination. Analysis of the individual congener over time could indicate if these congeners are substrates, intermediates or terminal products. A preliminary analysis of congener changes over time from one replicate microcosm of three different treatments is shown in **Figure 4-5**. More data from triplicate samples at multiple time points are in the process of being analyzed.

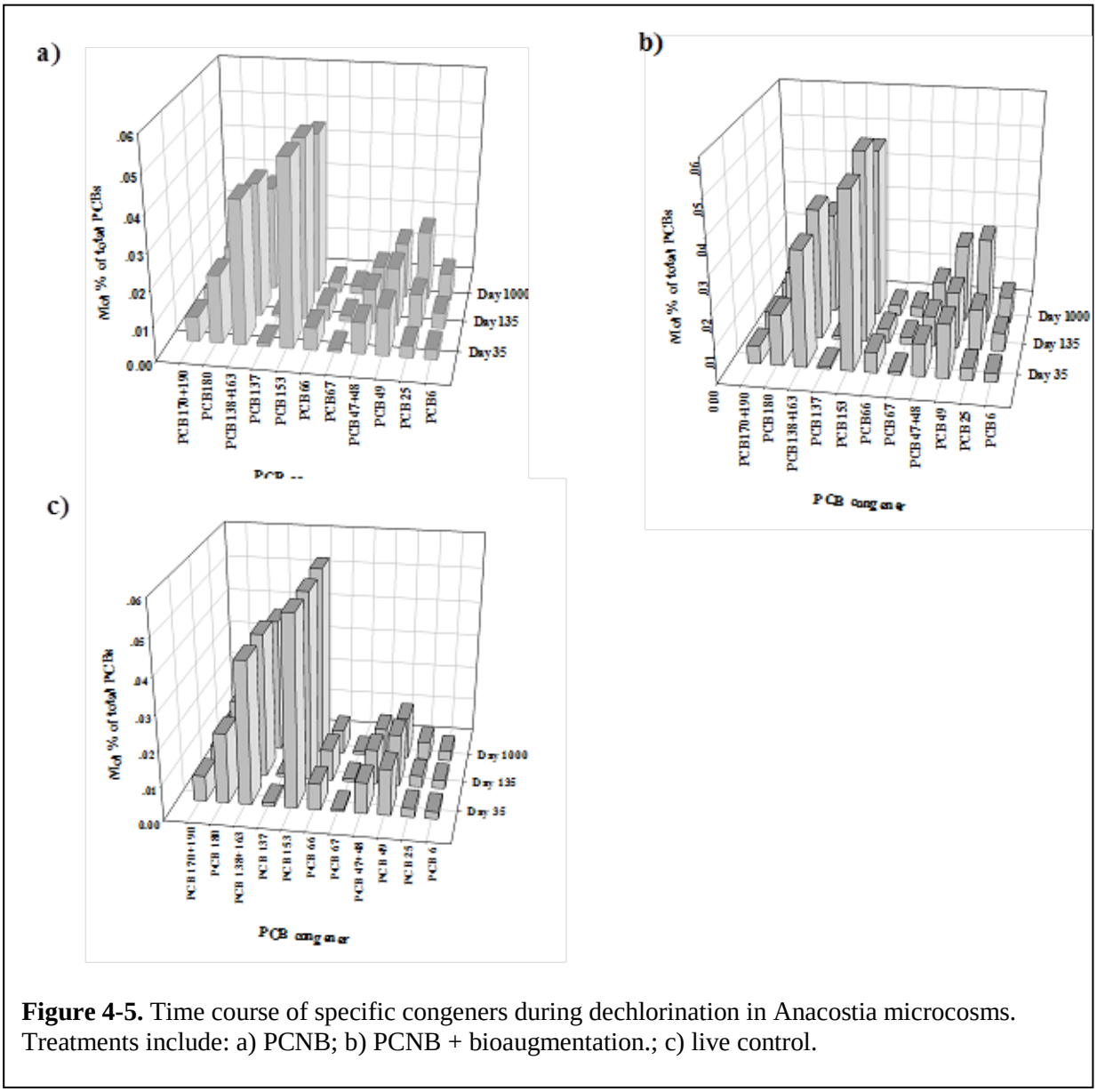


Figure 4-5. Time course of specific congeners during dechlorination in Anacostia microcosms. Treatments include: a) PCNB; b) PCNB + bioaugmentation.; c) live control.

5. Effects of Bioaugmentation on Indigenous PCB Dechlorinating Activity in Sediment Microcosms.
(Fagervold SK, Joy E. M. Watts JEM, May HD Sowers KR. 2010. Effects of bioaugmentation on indigenous PCB dechlorinating activity in sediment microcosms. Water Research 45:3899-3907.)

Abstract

Bioaugmentation is an attractive mechanism for reducing recalcitrant pollutants in sediments, especially if this technology could be applied *in situ.* To examine the potential effectiveness of a bioaugmentation strategy for PCB contamination, PCB dehalorespiring populations were inoculated into Baltimore Harbor sediment microcosms. A culture containing the two most predominant indigenous PCB dehalorespiring microorganisms and a culture containing a strain with a rare *ortho*-dechlorination activity and a non-indigenous strain that attacks double-flanked chlorines, were inoculated into sediment microcosms amended with 2,2',3,5,5',6-hexachlorobiphenyl (PCB 151) and Aroclor 1260. Although we observed a similar reduction in the concentration of PCB 151 in all microcosms at day 300, a reduced lag time for dechlorination activity was observed only in the bioaugmented microcosms and the pattern of dechlorination was altered depending on the initial combination of microorganisms added. Dechlorination of Aroclor 1260 was most extensive when all four dehalorespiring microorganisms were added to sediment. Overall numbers of dehalorespiring microorganisms in both bioaugmented and non-bioaugmented microcosms increased 100- and 1000-fold with PCB 151 and Aroclor 1260, respectively, and they were sustained for the full 300 days of the experiments. The ability of bioaugmentation to redirect dechlorination reactions in the sediment microcosms indicates that the inoculated PCB dehalorespiring microorganisms effectively competed with the indigenous microbial populations and cooperatively enhanced or altered the specific pathways of PCB dechlorination. These observations indicate that bioaugmentation with PCB dehalorespiring microorganisms is a potentially tractable approach for *in situ* treatment of PCB impacted sites.

Introduction

PCBs are persistent organic pollutants that are ubiquitously dispersed throughout the ecosystem as a result of cycling between air, water, and soil. Once released into the environment, PCBs can bioaccumulate throughout the food chain as a result of absorption in the fatty tissue of animals, such as fish and marine mammals; within humans, PCBs have been detected in human adipose tissue, milk and serum (Johansen et al., 1993; Muir et al., 1992; Neff, 1984; Safe, 1993; Schecter et al., 1994). Among their toxic effects, PCBs have been reported to act as endocrine disrupters (Crisp et al., 1998) and possible carcinogens (Safe, 1993). The commercial PCB mixture Aroclor 1260, which was widely used as dielectric fluid in liquid-filled transformers and capacitors between 1930 and 1971, is especially recalcitrant to degradation, primarily due to its high chlorine content and hydrophobicity (Alder et al., 1993; Quensen et al., 1990; Versar, 1976). However, microbial reductive dechlorination, a process in which microorganisms use PCBs as terminal electron acceptors for respiration, can transform highly chlorinated congeners in commercial mixtures such as Aroclor 1260 (Brown et al., 1987).

In addition to the potential for lower cost, *in situ* bioaugmentation with PCB dechlorinating microorganisms would provide several advantages over traditional methods such as dredging and

capping, including minimal disruption to benthic habitats in sensitive rivers and wetlands and the ability to treat shallow locations or those with restricted accessibility. Although anaerobic bioaugmentation studies have been successful for *in situ* treatment of chlorinated ethenes (Lendvay et al., 2003; Major et al., 2002), another class of organochlorines most commonly found in contaminated groundwater, effective bioaugmentation of PCB contaminated sites has not, as yet, been demonstrated. There have been reports on biostimulation of PCB dechlorination using other chlorinated compounds (halopriming) to enhance the numbers of dechlorinators and increase rates of contaminant breakdown (Bedard et al., 1996; Deweerd and Bedard, 1999; Klasson et al., 1996; Van Dort et al., 1997) though, unfortunately, the biostimulants are often themselves persistent organic pollutants. Other studies investigated the effects of enrichment cultures on bioaugmentation (Bedard et al., 1997; Natarajan et al., 1997), but the fates of the dechlorinating microorganisms were not determined in these earlier studies because their identity was unknown.

The patterns of PCB dechlorination in anaerobic sediments varies between sites and has been attributed to the activities of different indigenous dehalorespiring populations (for reviews see Bedard and Quensen (1995) and Smidt and de Vos (2004)). Since the discovery that some "*Dehalococcoides"* spp. and related species within the *Chloroflexi* are capable of dehalorespiring PCBs, there have been reports on the enrichment and isolation of strains with specific dechlorinating capabilities (Bedard et al., 2007; Cutter et al., 2001; Fagervold et al., 2005; Wu et al., 2002). Fagervold et al. (2005) showed that PCB 132 was dechlorinated in two discreet steps by 2 phylotypes enriched from Baltimore Harbor (BH) sediments, demonstrating that PCBs could be cooperatively dechlorinated by a consortium of dehalorespiring bacteria. Furthermore, Aroclor 1260 was later shown to be dechlorinated by a consortium consisting of three phylotypes that were reproducibly enriched from BH sediments (Fagervold et al., 2007). The dechlorination pattern resulting from the activity of the microbial consortia predominantly attacks flanked *meta* chlorines most characteristic of process N, which has been reported in sediments from Woods Pond and Silver Lake (Bedard and Quensen, 1995). In contrast, Adrian et al. (2009) reported the reductive dechlorination of Aroclor 1260 by "*Dehalococcoides*" sp. CBDB1 as more predominant attack of *para* chlorines most characteristic of Process H, reported in Hudson River sediments (Bedard and Quensen, 1995). These combined observations support the hypothesis that the variation of microbial dechlorination patterns observed in different sites is due to the presence and activity of specific species and consortia of dehalorespiring bacteria. However, it is unclear if the combined interactions of the indigenous population of dehalorespiring bacteria and resulting products can be altered by changing the total number and ratio of individual species in the consortium.

In this study sediment microcosms, spiked with PCB 151 (2,2',3,5,5',6-hexachlorobiphenyl) or Aroclor 1260, were bioaugmented with the two most predominant indigenous PCB dehalorespiring microorganisms from BH sediments, and a culture containing a species with a rare *ortho*-dechlorinating activity and a non-indigenous species selective for dechlorination of double-flanked chlorines. The study examines the effects of bioaugmentation on both the dehalogenating activities and fate of the indigenous dehalorespiring population in BH sediment microcosms.

Materials and Methods

Bacterial cultures used for bioaugmentation. The specific activities of the microorganisms used to bioaugment the sediments have been characterized previously (Fagervold et al., 2007; May et al., 2006; Wu et al., 2000). Bacterium *o*-17 reductively dechlorinates flanked *meta* chlorines and flanked *ortho* chlorines in congeners containing up to 3 *ortho* chlorines (Fagervold et al., 2007; May et al., 2006). *"Dehalobium chlorocoercia"* strain DF-1 dechlorinates double-flanked in the *para* or *meta* positions, but does not dechlorinate single flanked chlorines (Wu et al., 2000). Phylotype DEH10 dechlorinates the double-flanked *meta* chlorines in 2,3,4-substituted chlorobiphenyl rings and *para*-flanked *meta* chlorines when no double-flanked chlorines are available. Phylotype SF1 dechlorinates all 2,3,4,5-substituted chlorobiphenyl rings, preferentially in the *meta* position, although some *para* dechlorination has been observed (Fagervold et al., 2007).

The inocula used for bioaugmentation were maintained by sequential transfer of 10 % v/v into estuarine salts medium (E-Cl) as described below. *D. chlorocoercia* strain DF-1, originally enriched from Charleston Harbor sediments, was maintained in co-culture with a *Desulfovibrio* spp. with 10 mM sodium formate and 2,3,4,5-CB (PCB 61) (Wu et al., 2000). A co-culture containing Bacterium *o*-17 originally enriched from BH sediment was maintained with a *Desulfovibrio* spp. with 20 mM sodium acetate and 2,3,5,6-CB (PCB 65) (Cutter et al., 2001). Phylotypes DEH10 and SF1 were initially enriched from BH sediment (Fagervold et al., 2007) and maintained with a fatty acid mixture (acetate, propionate and butyrate, 2.5 mM each) and 2,2'3,5',6-CB (PCB 95) or 2,2',3,4',6-CB (PCB 91), respectively, and 0.1% (w/v), sterile, dry BH sediment. Both phylotypes were maintained as highly enriched cultures.

Microcosm experiments. Defined E-Cl medium containing a low sulfate concentration (<0.3 mM) was dispensed anaerobically (10 ml) into 20 ml anaerobe tubes and sealed under an atmosphere of N_2-CO_2 (4:1) (Berkaw et al., 1996) with modifications described previously (Fagervold et al., 2005). A fatty acid mixture (acetate, propionate and butyrate, 2.5 mM each) was added as an electron donor and carbon source (Berkaw et al., 1996). Aroclor 1260 or PCB 151 was added in 10 µl acetone to final concentrations of 100 and 50 ppm, respectively. All treatments contained 1.5 g v/v freshly collected BH sediment that was contaminated with less than 2 ppm weathered PCB. Treatment 1 contained 1.5 g (wet) BH sediment as a source of indigenous microorganisms at a pre-enriched background level of approximately 1.5 x 10^5 total dehalorespiring cells per 10 ml culture. Treatment 2 contained 1.5 g fresh BH sediment with the addition of 0.4 ml each of SF1 and DEH 10 cultures, which is equivalent to approximately 3 x 10^5 cells of each phylotype per 10 ml. Treatment 3 contained 1.5 g fresh BH sediment with the addition of 1 ml of a co-culture containing *o*-17 and 1 ml of a co-culture containing DF-1, 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. Sterile controls were prepared as in treatment 1, followed by sterilization (autoclaved for 20 minutes, 121°C). All cultures were prepared in triplicate and incubated at 30 ^oC in the dark.

Analytical techniques. Microcosms were sampled on day 0 immediately after inoculation and subsequently every 50 days in an anaerobic glove box (Coy Laboratory Products, Grass Lake,

MI). Each culture sample (0.5 ml) was extracted with 3 ml of hexane for 12 h 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 ^{63}Ni electron capture detector as described previously (Fagervold et al., 2005). Unpaired two sample Student's t-tests, assuming equal variance, were used to determine the significance between the mean concentrations of 151 at different time points, in augmented versus non-augmented microcosms, as well as the total, *meta*, *para* and *ortho* chlorines at day 300 in the Aroclor 1260 microcosms.

Bacterial community 16S rRNA gene analyses. DNA from pooled samples (0.5 ml from each of replicates for each treatment) was extracted every 50 days. Microcosms sampled on day 0 immediately after inoculation were extracted with the DNA® SPIN For Soil kit (MP Biochemicals, Solon, OH), followed by purification with the Promega Wizard PCR Prep Kit (Promega, Madison, WI.). All subsequent DNA extractions were extracted with UltraClean™ Soil DNA Kit (Mo Bio, Carlsbad, CA), which did not require an additional purification step. 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 as a template in all subsequent PCR reactions.

The microbial dechlorinating population within microcosms was evaluated by denaturing gradient gel electrophoresis (DGGE) of total microbial community DNA using PCR amplification with primers specific for 16S rRNA genes of a monophyletic group within the *Chloroflexi*, Chl348FGC and Dehal884R (Fagervold et al., 2005). DGGE was performed using the D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA.) (Watts et al., 2001). To identify strains from DGGE profiles, bands were excised and incubated in 30 μl TE overnight at 4 °C, subjected to PCR and checked for purity using DGGE and subsequently sequenced.

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 similarities were analyzed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

Quantitative assessment of PCB dechlorinating populations. Cell enumeration of DEH10, SF1, *o*-17 and DF-1 inocula for bioaugmentation experiments were determined by most probable number estimation of 16S rRNA gene copies from total DNA. Diluted DNA from these cultures was subjected to PCR (40 cycles) using primers Chl348F and Dehal884R (Fagervold et al., 2005), and one copy of the gene per genome was assumed based on the genomes of "*Dehalococcoides ethenogenes"* strain 195 and *Dehalococcoides* sp. strain CBDB1 (Kube et al., 2005; Seshadri et al., 2005). Numbers of putative dehalogenating *Chloroflexi* in bioaugmentation microcosms were monitored by competitive PCR, using primers Chl348F and Dehal884R (Fagervold et al., 2005; Kjellerup et al., 2008). The assays were performed on normalized DNA extracted from microcosm sub samples as described previously (Fagervold et al., 2007).

Results

Effect of bioaugmentation on the patterns of PCB 151 dechlorination. BH sediment microcosms amended with PCB 151 were bioaugmented with selected populations of dehalorespiring microorganisms to a final concentration of approximately 1 to 7 x 10^4 cells ml^{-1} and the patterns of dechlorination were monitored over 300 days. PCB 151 (2,2',3,5,5',6-CB) can be dechlorinated initially through two pathways (**Fig. 5-1**). The first pathway involves a *meta* dechlorination to PCB 95 (2,2',3,5',6-CB), which is further dechlorinated in the *meta* position to PCB 53 (2,2',5,6'-CB). The second pathway is a dechlorination in the *ortho* position to PCB 92 (2,2',3,5,5'-CB), which can be dechlorinated either in the *meta* position to PCB 52 (2,2',5,5'-CB) or in the *ortho* position to PCB 72 (2,3',5,5'-CB).

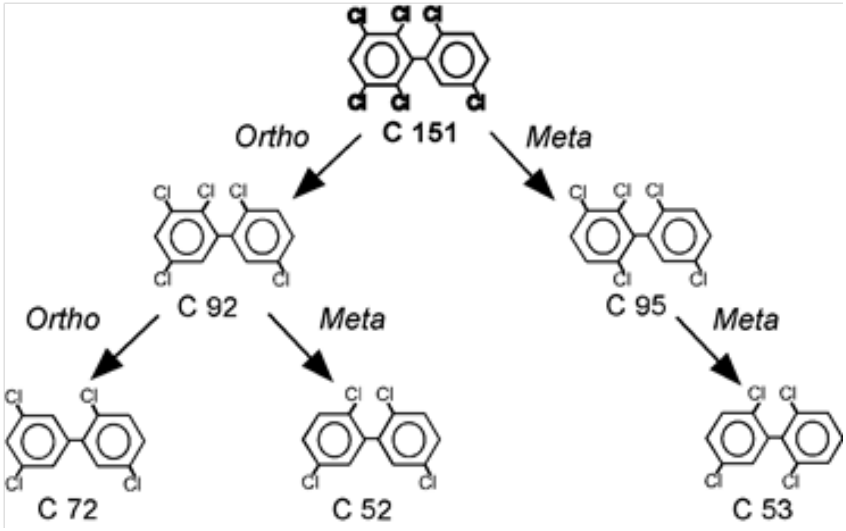


Figure 5-1. Possible pathways for dechlorination of PCB 151.

The dechlorination pathways of PCB 151 varied as a result of the different bioaugmentation treatments (**Fig. 5-2**). The PCB dehalorespiring population was monitored by DGGE analysis of the 16S rRNA gene community at time points throughout the incubation period to: 1) investigate whether the inoculated microorganisms were sustained after addition, and 2) to determine the impact of bioaugmentation on the indigenous dechlorinating community in the microcosms (**Fig. 5-3**).

All treatments were found to be significantly different from the non-bioaugmented control (1 : BH, **Fig 5-2**) as determined by unpaired Student's t-tests (p value <0.05), except for treatment 2 (BH + SF1 + DEH10) at day 200 and 300. Treatment 1, BH sediment with indigenous microorganisms only, did not dechlorinate before day 150, thereafter, both *meta* and *ortho* dechlorination was observed (**Fig. 5-2**). After 300 days of incubation, the major dechlorination product of PCB 151 for two replicates was PCB 72 (69.1 and 67.3 mol %); resulting from two sequential *ortho* dechlorinations, likely mediated by *o*-17 (**Fig. 5-3**). The major product for the remaining replicate was PCB 95 (81.2 mol %) indicating a single *meta* dechlorination, likely mediated by SF1. In a previous report, DEH10 was shown to catalyze the single *meta*-dechlorination of PCB 151 to PCB 95 in BH microcosms (Fagervold et al., 2007). However, both DEH10 and SF1 have specificity for dechlorinating flanked *meta*-chlorines. These differences observed between the two studies and between the replicate cultures in the current study are likely due to biotic factors, such as competition between the indigenous species enriched in each microcosm. An indigenous *Dehalococcoides* phylotype was initially observed

in BH sediments for all treatments at day 0 (**Fig. 5-3**), but was not subsequently detected after incubation with PCB 151. This phylotype co-migrates with DEH10 and was most similar using 16S rRNA gene similarity (472 identity over 478 bp) to another *Dehalococcoides* species, DHC ANAS (acc. number DQ855129) detected previously in trichloroethene enrichment cultures (Holmes et al., 2006).

Treatment 2, consisting of BH sediment bioaugmented with the indigenous phylotypes SF1 and DEH10, exhibited two sequential *meta* dechlorinations of PCB 151 to PCB 53 (46 ± 0.1 mol % at day 300) in all three replicates in only 50 days, with less than 5 mol % accumulation of PCB 95 (**Fig. 5-2**). Both SF1 and DEH10 were detected at day 0 in these microcosms, but only DEH10 was subsequently detected throughout the experiment, to day 300 (**Fig. 5-3**). A second less intense band was detected in treatment 2, with 97 % (471/478 bp) sequence identity to an uncultured *Chloroflexi* (clone VHS-B3-87). This phylotype was also detected weakly in treatments 3 and 4 during active dechlorination of PCB 151, but its role is unknown.

In contrast, dechlorination was observed at 100 days in treatment 3 (BH sediment with *o*-17 and DF-1) and the major product after 300 days was PCB 72 at 44 ± 7 mol %. This pathway is probably the result of two sequential *ortho* dechlorination steps, with PCB 92 as an intermediate. Some *meta* dechlorination (18 ± 5 mol %) of the intermediate PCB 92 to PCB 52 was observed on day 300. DF-1 does not dechlorinate PCB 151 because this congener does not contain double-flanked chlorines.

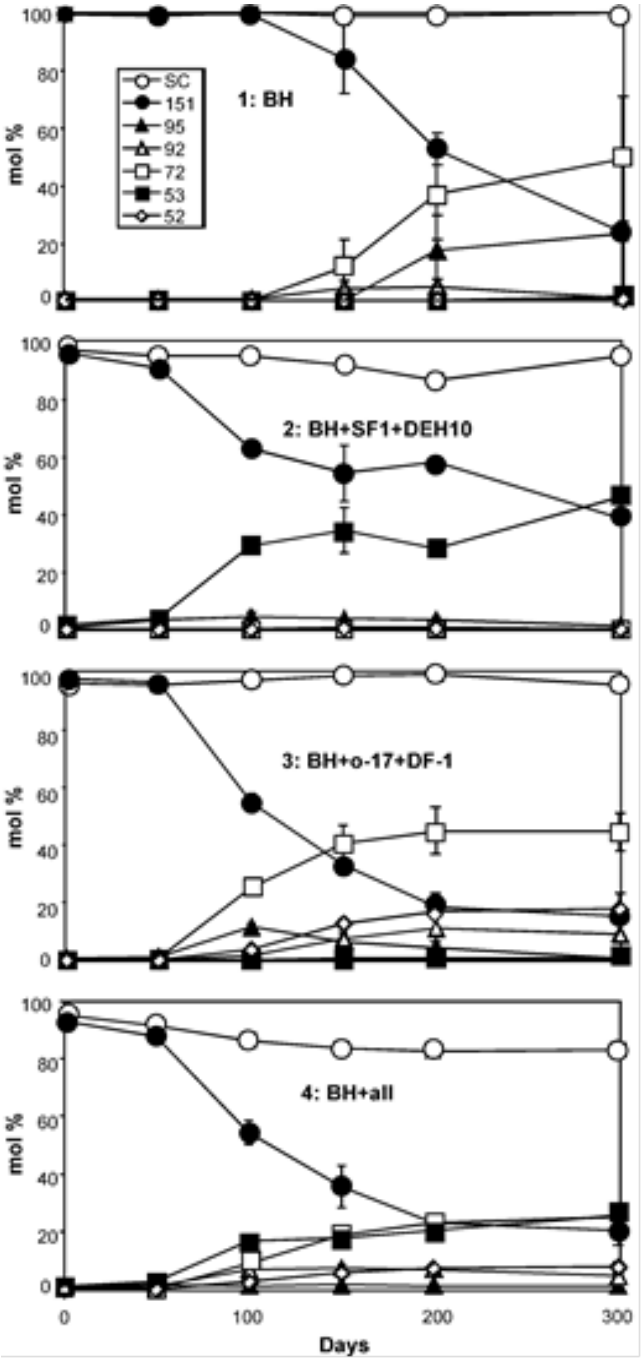


Figure 5-2. Dechlorination of PCB 151 in BH sediment after the following treatments: BH sediment with no bioaugmentation (1); BH sediment bioaugmented with SF-1 and DEH10 (2); *o*-17 and DF-1 (3); SF-1, DEH10, *o*-17 and DF-1. Congeners detected include PCB 151 (●), PCB 95 (▲), PCB 92 (△), PCB 72 (□), PCB 53 (■), PCB 52 (◇) and a sterile control (○). Each datum point is the mean and standard deviation for three replicate cultures.

The greatest range of dechlorination activities was observed in treatment 4, BH sediment with all four phylotypes. *Meta* dechlorination of PCB 151 to PCB 53 (26 ± 3 mol %) was detected after 300 days with little accumulation of the intermediate congener PCB 95 (< 2 mol %). *Ortho* dechlorination of PCB 151 to PCB 72 (25 ± 3 mol %) was detected with < 5 mol % accumulation of PCB 92. However, some *meta* dechlorination to PCB 52 (8 ± 2 mol %) was also detected at day 300. The activity corresponds to a more diverse microbial community of putative dechlorinators with all four different phylotypes detected at day 300. DEH 10 and *o*-17 appeared as the predominant DGGE bands (**Fig. 5-3**) and the uncultured *Chloroflexi* and SF1 as minor bands, which although not quantitative, is consistent with the dechlorination pathways observed.

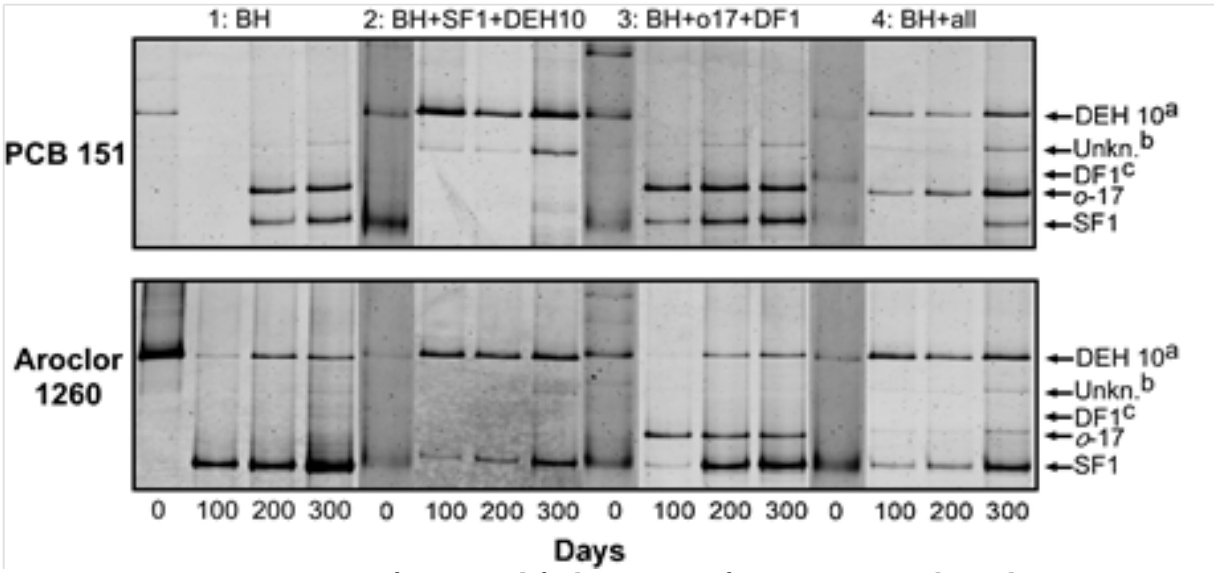


Figure 5-3. Composite DGGE of PCR amplified 16S rRNA from PCB 151 and Aroclor 1260 microcosms after different treatments. Day 0 samples for treatments 2, 3 and 4 was subjected to 40 PCR cycles rather than the normal 26 cycles (Fagervold et al., 2005) and run on a separate gel. [a] Phylotype detected in day 0 samples co-migrated with DEH10 but was a different phylotype with sequence identity (99 %) to uncultured *Chloroflexi* clone DHC ANAS (DQ855129).

[b] Unkn = unknown, sequence identity (97 %) to uncultured *Chloroflexi* clone VHS-B3-87 (DQ294968). [c] Expected position of DF-1.

Effect of bioaugmentation on the patterns of Aroclor 1260 dechlorination. BH sediment microcosms amended with Aroclor 1260 exhibited a decrease in the total chlorines per biphenyl for all four treatments (**Fig. 5-4**), in comparison to sterile controls. All treatments were found to be significantly different (p value <0.05) from the non-biaugmented control and treatment 4, BH sediment bioaugmented with all four dechlorinators, exhibited the most extensive dechlorination, with the reduction of 1.77 chlorines per biphenyl over 300 days; while treatment 2, BH sediment bioaugmented with SF1 and DEH10, was similar with an average reduction of 1.70 chlorines per biphenyl. The remaining treatments exhibited a total reductive dechlorination of approximately 1.40 chlorines per biphenyl in 300 days.

The total reduction of *meta* chlorines was similar to the reduction of total chlorines (**Fig. 5-4**, panels A and B), however the unpaired t-test showed that treatment 3, BH sediment bioaugmented with *o*-17 and DF-1 was not statistically different from the non-bioaugmented microcosm. Also some differences in the reduction of *ortho* chorines were observed between the different treatments. Treatment 3, BH sediment bioaugmented with *o*-17 and DF-1 dechlorinated PCB congeners in the *ortho* position to a greater extent than other treatments, with an average reduction of 0.24 and 0.21 *ortho* chlorines per biphenyl over 300 days, respectively. In contrast, treatment 4, BH sediment bioaugmented with all four microorganisms, dechlorinated in the *para* position to a greater extent than other treatments, however, this might be a reflection of overall higher dechlorination by this treatment. All treatments were significantly different (p value <0.05) from non-bioaugmented microcosms, regarding *ortho* and *para* chlorines, at day 300.

Effect of bioaugmentation on the indigenous population of PCB dehalorespiring bacteria. To determine whether microorganisms used for bioaugmentation were capable of growth and sustainable dechlorination in the presence of the indigenous dahalorespiring population, and the general microbial community within the sediment; dehalorespiring microorganisms were enumerated during growth in the number of 16S rRNA gene copies, per µl normalized DNA. In all four treatments the numbers of dechlorinators increased approximately 100- and 1000-fold with PCB 151 and Aroclor 1260, respectively, regardless of whether the culture was bioaugmented. The average numbers of 16S rRNA gene copies per µl normalized DNA in most microcosms with PCB 151 increased from $1.65 \times 10^3 \pm 1.68 \times 10^3$ at day 0 to $2.40 \times 10^5 \pm 9.48 \times 10^4$ at day

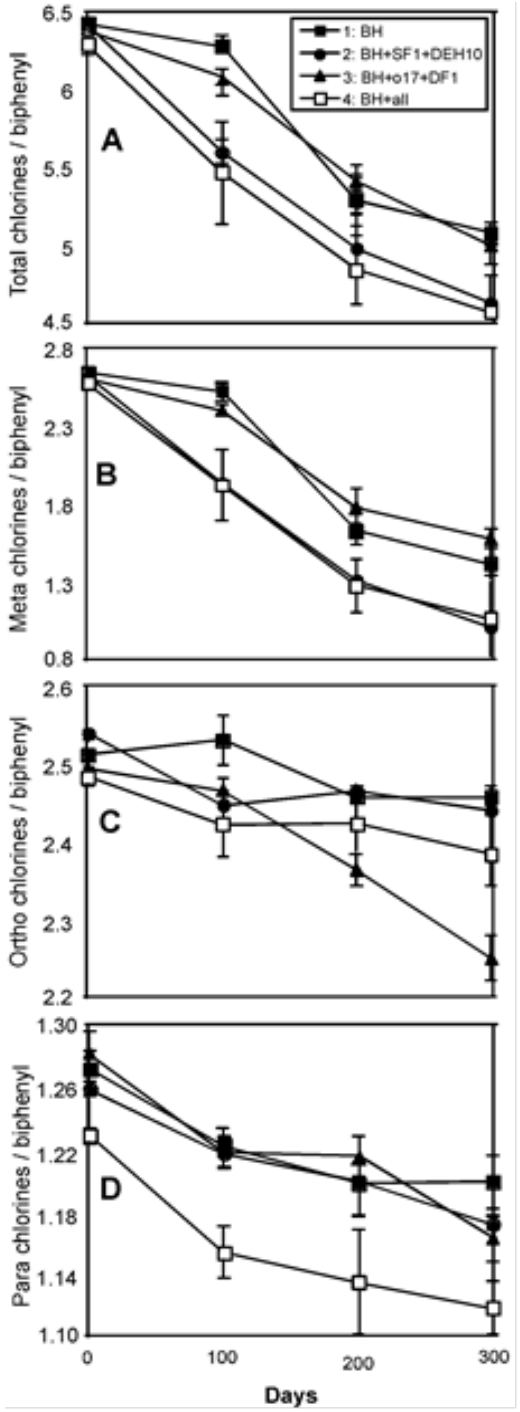


Figure 5-4. Dechlorination of Aroclor 1260 in microcosms with different treatments (see legend) calculated as total chlorines per biphenyl (A), and *meta* (B), *ortho* (C) and *para* (D) chlorines per biphenyl. BH = Baltimore Harbor sediments. Each datum point is the mean and standard deviation for three replicate cultures.

100, then remained relatively constant today 300. Slower initial growth was observed only for treatment 1 with PCB 151 (indigenous population without bioaugmentation), but similar numbers were observed after 200 days. The average numbers of 16S rRNA gene copies per µl normalized DNA in microcosm dechlorinating Aroclor 1260 increased from $1.06 \times 10^3 \pm 380$ at day 0 to $1.32 \times 10^6 \pm 6.94 \times 10^5$ at day 100 then remained relatively constant today 300. The results indicate that total number of dehalorespiring microorganisms in the microcosms, both indigenous and/or inoculated microorganisms, increased to a finite total or climax community within the first 100 days of active dechlorination and this steady state was maintained for the remainder of the incubation experiments.

The numbers of dechlorinating microorganisms in bioaugmented microcosms was approximately $2x10^4$ cells per ml sediment or $1x10^5$ cells per g dry wt. of sediment at day 0. By comparison, Krumins et al. (2009) used approximately 3×10^6 cells per ml sediment or $7x10^6$ cells per g dry wt. of sediment of *D. ethenogenes* strain 195 per ml in microcosm experiment to test stimulation of PCB dechlorination activity. Although lower numbers of microbes were used for bioaugmentation in the current study, they still had a considerable effect on the composition of the microbial community and on the pathways of PCB dechlorination.

Although indigenous DEH10, SF1 and *o*-17 were enriched from BH sediments in treatment 1, the effect of bioaugmentation is evident from differences in both the dechlorination pattern and changes in the microbial community in treatments 2 through 4 (**Fig. 5-3**). In treatment 1 with PCB 151 *o*-17 and SF1 are enriched, which is consistent with two sequential *ortho* dechlorinations of PCB 72 and a single *meta* dechlorination to PCB 95. In treatment 2, the predominance of DEH10 concurrent with a two-step *meta* dechlorination to PCB 53 is consistent with results reported previously (Fagervold et al., 2007). In treatment 3, the predominance of *o*-17 is consistent with a two-step *ortho* dechlorination to PCB 72 and the presence of SF1 is consistent with smaller amounts of single step *meta* dechlorinations to PCB 95 and 52. Finally, adding all four microorganisms resulted in predominance of DEH10 and *o*-17 and concurrent two step *meta* and *ortho* dechlorinations, respectively, as the dominant dechlorination pathways. SF1 was weakly detected, which is consistent with smaller amounts of single step *meta* dechlorination products observed.

Likewise, bioaugmentation with different microorganisms had a substantial effect on the dehalorespiring population in cultures amended with Aroclor 1260. Treatment 1 without bioaugmentation is consistent with a prior study showing that DEH10 and SF1 are the predominant microorganisms observed in BH sediments enriched with Aroclor 1260 (Fagervold et al., 2007). Bioaugmentation with DEH10 and SF1 in treatment 2 resulted in detection of both phylotypes as expected, a reduction in the lag time and more extensive dechlorination of *para* and *meta* chlorines at 300 days. Treatment 3 resulted in sustained detection of o-17 throughout the 300-day incubation period and significant increase in *ortho* dechlorination compared with treatments 1 and 2. DF-1 was not detected in the microcosm and there was no major change in the *meta* and *para* dechlorination patterns, which indicates that DF-1 did not compete successfully with the indigenous dehalorespirers DEH10 and SF1 for utilizing Aroclor 1260. Bioaugmenting with all four dehalorespiring bacteria resulted in the same lag time and total dechlorination pattern as observed after bioaugmentation with only DEH10 and SF1. However, a less intense *o*-17 band was detected throughout the 300 days, concurrent with some *ortho*

dechlorination. Interestingly, there was an increase in the extent of *para* dechlorination than with other treatments. One possible explanation is the increased removal of *ortho* chlorines generated more congeners with double flanked *para* chlorines, which are generally more susceptible to dechlorination than single flanked *meta* chlorines (Bedard and Quensen, 1995).

Discussion

Bioaugmentation with PCB dehalorespiring bacteria had a considerable effect on the dehalogenating activity of PCB 151 and Aroclor 1260, in sediment microcosms. The addition of PCB dechlorinators changed the specific dechlorination activities and increased the extent of dechlorination after 300 days, compared with enrichments containing only indigenous dechlorinating populations. A one-way ANOVA analysis showed no significant difference between dechlorination rates between treatments (p-value >0.05), which indicates that the more extensive dechlorination observed in bioaugmented microcosms after 300 days was the result of the reduced lag time. This is consistent with the enumeration data indicating that total numbers of dehalorespiring microorganisms in both enriched and bioaugmented cultures increased to the same steady state maximum levels during the incubation period. This observation is not surprising as nutrients, including nitrogen, phosphate, sulfur, trace metals, vitamins, PCBs and fatty acids, were not limiting in these experiments, although it is not clear why bioaugmentation in some cases repressed growth of an indigenous dechlorinating species in the presence of excess PCB. Furthermore, we observed heterogeneity of activity between non-bioaugmented replicate samples both in this study and previously (Fagervold *et al*., 2007), but not in the bioaugemented samples. This may be an artificial microcosm effect, and a number of factors could be involved; for example, bioaugmentation of microcosms may reduce the spatial patchiness of dechlorinating microorganisms within the microcosm thereby reducing heterogeneity (Jessup *et al*., 2004). In addition, some indigenous species detected in enrichments with PCB151 were no longer detectable after bioaugmentation (**Fig. 5-3**). The results indicate that bioaugmentation was successful, because bioaugmentation with greater initial cell numbers enabled the inoculum to successfully outcompete the indigenous dechlorinating population (e.g., PCB 151, treatment 2). However, this does not explain why selected activities of the indigenous population could be completely repressed in the presence of excess PCB. The free energy yield is similar for products of the alternative pathways (Holmes et al., 1993), so this selection is unlikely due to thermodynamic differences. Likewise, the similarity between rates for dechlorination of parent compound PCB 151 via alternate pathways rules out kinetic differences as a reason for selection. One possible explanation is that PCBs adsorb to sediment particles due to their hydrophobic nature and as the more abundant species colonize the particles as biofilms, the PCBs are no longer available to species that are less abundant during the initial inoculation. The observation that bioaugmented microorganisms were sustainable as long as activity continued, supports the latter hypothesis that PCBs were no longer bioavailable to other species despite the fact that high levels of parent PCB151 (>10 ppm) were detected after 300 days for all treatments.

Although these results show that bioaugmentation is a potential treatment strategy for PCB dechlorination in sediments, several questions must be addressed to determine if bioremediation of weathered PCBs is feasible. One unknown is the effectiveness of bioaugmentation for dechlorinating low levels of PCBs most commonly associated with weathered PCB contaminated sites. In the presence of weathered PCBs where the concentration is more limiting both kinetic

and availability issues might affect the effectiveness of *in situ* PCB transformation. A recent study by May et al. (2008) showed that bioaugmentation with DF-1 stimulated the reductive dechlorination of Aroclor 1260 (>5 ppm) in contaminated soil, which suggests that using bioaugmentation for treatment of low levels of weathered PCBs is feasible. Furthermore, Krumins et al., (2009) found that the addition of *D. ethenogenes* strain 195 and pentachloronitrobenzene to microcosms stimulated the dechlorination of weathered PCB in contaminated sediments from Anacostia River, Washington DC. Interestingly, in microcosms bioaugmented with *D. ethenogenes* strain 195, this strain could not be detected after 281 days, although its addition did have an initial stimulatory effect on dechlorination. The results of the current study demonstrate clearly that the dehalorespiring microorganisms added to sediment microcosms can successfully compete with indigenous populations reducing the lag time and diverting the pathways of dehalogenation. The ability to alter the synergistic activities of the indigenous dehalogenating community suggests that bioaugmentation is a potentially viable approach for enhancing reductive dehalogenation of PCB impacted sediments. Future studies will focus on the effects of bioaugmentation on indigenous populations under limiting conditions to identify the kinetic and availability limitations in weathered sediments subject to *in situ* treatment.

Conclusions

- A reduced lag time for dechlorination activity was observed in all bioaugmented microcosms and the pattern of dechlorination was altered depending on the initial combination of microorganisms added
- Dechlorination pattern heterogeneity observed in sediment enrichments was not observed in bioaugmented cultures, indicating that bioaugmentation effectively redirected the dechlorination pathways
- The ability of bioaugmentation to redirect dechlorination reactions in the sediment microcosms and sustainability of bioaugmented microorganisms after 300 days indicates that the inoculated PCB dehalorespiring microorganisms effectively competed with the indigenous microbial populations and cooperatively enhanced or altered the specific pathways of PCB dechlorination
- Although the mechanism by which bioaugmented microorganisms could outcompete the indigenous population in the presence of excess substrate in not known, the results of this study support the feasibility of using *in situ* bioaugmentation with dehalorespiring bacteria as an environmentally less evasive and lower cost alternative to dredging for treatment of PCB impacted sediments.

6. Aroclor 1260 Contaminated Soils Show Spatial Variability of Dechlorinating Bacteria and PCB Reducing Activities

(Kjellerup BV, Paul P, Ghosh U, May HD, Sowers KR. Manuscript submitted)

Abstract

Soil from a storm water runoff ditch contaminated with weathered Aroclor 1260 was analyzed for historical and current PCB dechlorination potential. Analysis of PCB transforming microbial communities and dechlorinating potential in microcosms using 2,3,4,5,6-CB as a surrogate target for activity in microcosms in the PCB impacted soil revealed spatial heterogeneity of both the microbial populations and specific dechlorination pathways. Rates of dechlorination activity also varied throughout the site, but no relationships were observed between dechlorination activities and microbial communities indicating that inhibition of the activity by metals, pesticides or other contaminants detected at the site might occur. Dechlorinating bacterial phylotypes were for the first time identified in soil samples, but no relationships between the types of dechlorinating activity and the identified phylotypes were observed. Several of the phylotypes found in soil were closely related to PCB dechlorinating phylotypes previously found in sediment. Therefore, bioaugmentation with the pure culture DF-1 isolated from sediment was tested in microcosms with soil from three locations with varying PCB concentrations. Significant dechlorination (reduction of 0.4 chlorines per biphenyl in 145 days) of weathered Aroclor 1260 contaminated soil occurred in the sample containing 4.6 ppm PCB, but not in the other tested samples with higher PCB concentrations emphasizing the spatial heterogeneity at the site. This is the first time that successful bioaugmentation of PCB contaminated soil has been shown with anaerobic bacteria and shows the potential for testing the approach in full scale.

Introduction

Polychlorinated biphenyls (PCBs) are persistent organic pollutants that are still present in the environment despite a U.S. production ban in 1976 (Kimbrough, 1995). Prior to this, commercial mixtures of PCBs (trade name Aroclor in the U.S.) were applied for a range of industrial applications such a high voltage transformers, insulating materials and hydraulic liquids (Fischbein et al. 1982; Ouw et al., 1976). PCBs are hydrophobic due to their chemical composition with a high affinity for adsorption to soil particles and can bioaccumulate in lipids causing hepato- and immunotoxicity, carcinogenesis, effect endocrine organs and the reproduction in humans (Carpenter, 2006; Safe et al., 1997; Vater et al., 1995) and animals (De Flora et al., 1991; Kozie & Anderson, 1991; Tanabe et al. 1994). Removal of PCBs from impacted sites has therefore been a regulatory priority for over two decades (Kannan et al., 1998). Dredging or excavation are a widely accepted remediation tool for PCB impacted sediments and soils, respectively (Wakeman & Themelis 2001), followed by disposal to a landfill or off-site treatment (De Windt et al., 2005; Hetflejs et al., 2001; Seok et al, 2005). However, the proposed risk reduction goals are often not achieved by this approach (Megasites 2007). *In situ* remediation by reduction of PCB bioavailability with absorbents such as granular activated carbon into the top layer of the sediment is an alternative approached that creates minimal disruption at significantly reduced cost (Sun & Ghosh, 2007; Zimmerman et al., 2005). However, an effective permanent approach *in situ* degradation of PCBs by bioremediation does not exist currently.

Previous reports suggested that sites contaminated with weathered PCBs would be recalcitrant to microbial dechlorination (Alder et al., 1993; Bedard et al., 1997) due to molecules being trapped in the pores of the soil particles (Hatzinger & Alexander, 1995). Desorption of PCBs depends on the partitioning of PCB molecule between the pore water and the organic matter associated with the particle as well as the diffusion of the molecules. It has been suggested that formation of strong bonds between the PCB molecule and the soil particles could cause desorption resistance (Hatzinger & Alexander 1995). Thus, analysis of the indigenous microbial populations as well as the fate of PCBs during bioaugmentation is important in order to evaluate the potential for *in situ* bioremediation of a specific PCB contaminated sites.

Complete microbial degradation of PCBs requires anaerobic reductive dechlorination of extensively chlorinated congeners followed by subsequent aerobic cleavage of the biphenyl ring and mineralization of the less extensively chlorinated congeners (Pieper & Seeger, 2008; Field, 2008). A few anaerobic bacteria within the *Chloroflexi* have been confirmed to have PCB dechlorinating activity including *Dehalococcoides ethenogenes.* (Fennell et al., 2004), bacterium DF-1 (May et al., 2008), bacterium o-17 (May et al., 2006; Cutter et al., 2001) phylotypes SF-1 and DH-10 (Fagervold et al., 2007), and others (Bedard et al., 2006). In contrast, aerobic PCB degradation can be performed by many bacterial species such as *Burkholderia xenovorans* strain LB400 (Chain et al., 2006), *Rhodococcus* sp. strain RHA1 (Masai et al., 1995) and bacteria utilizing biphenyls are ubiquitously distributed in the environment (Furukawa, 1994). Biphenyl 2,3-dioxygenases are considered the key enzymes in the oxidative pathway for PCB degradation, where bphA1 can degrade specific PCB congeners (Hoostal et al., 2002) and bphC is involved in extradiol *meta* cleavage (Erb & Wagner-Dobler, 1993). Bioaugmentation with aerobic PCB degrading bacteria has successfully been applied to soil environments (Di Toro et al., 2006; Singer et al., 2000), whereas anaerobic bioaugmentation has previously only shown success in sequential anaerobic-aerobic treatment (Adebusoye et al. 2008; Kuipers et al., 2003; Tartakovsky et al., 2001).

In this study the spatial variability of bacteria involved in PCB degradation was investigated in Aroclor 1260 (A1260) contaminated soil for the first time. The results showed that anaerobic and aerobic bacteria involved in PCB dechlorination and mineralization, respectively, were present at the site. The abundance and activity of these groups of indigenous bacteria could not be related to the localized PCB concentration, the presence of other organic or inorganic contaminants or physical/chemical parameters at the site. Thus, the obtained results provide important information about the spatial variability and heterogeneity inherent in a PCB-impacted soil site, which is crucial for designing an effective bioremediation strategy. In addition to indigenous microbial activities, the potential for on-site bioremediation tested in microcosms using the PCB dechlorinating "*Dehalobium chlorochloercia*" DF-1 had an effect at only one of three tested locations. These results show that there is a potential for bioaugmentation at the site, PCB-impacted sites must be carefully assessed for spatial variability and heterogeneity of potential dechlorination inhibitors prior to treatment.

Materials and Methods

Sample collection. Samples were collected from a storm water drainage ditch in Mechanicsburg,

PA ranging from its source at 40°13”46”N, 76 °59”37”W to approximately 730 meters downstream to 40°14”06”N, 75 °59”32”W. The open drainage ditch, which collects storm water runoff from the Naval Support Activities base and surrounding off-base properties, extends approximately 2.4 kilometers from its origin to its confluence with Trindle Spring Run. Samples contaminated with different levels of PCBs were collected in November 2005 with a 60 cm by 5 cm (OD) core sampler from the top 30 cm of soil or sediment present in PCB impacted drainage ditch located near the Mechanicsburg Navy Base, Pa. In total 20 sediment samples were collected (**Fig. 6-1**) and stored anaerobically in sealed glass jars at 4 ºC in the dark.

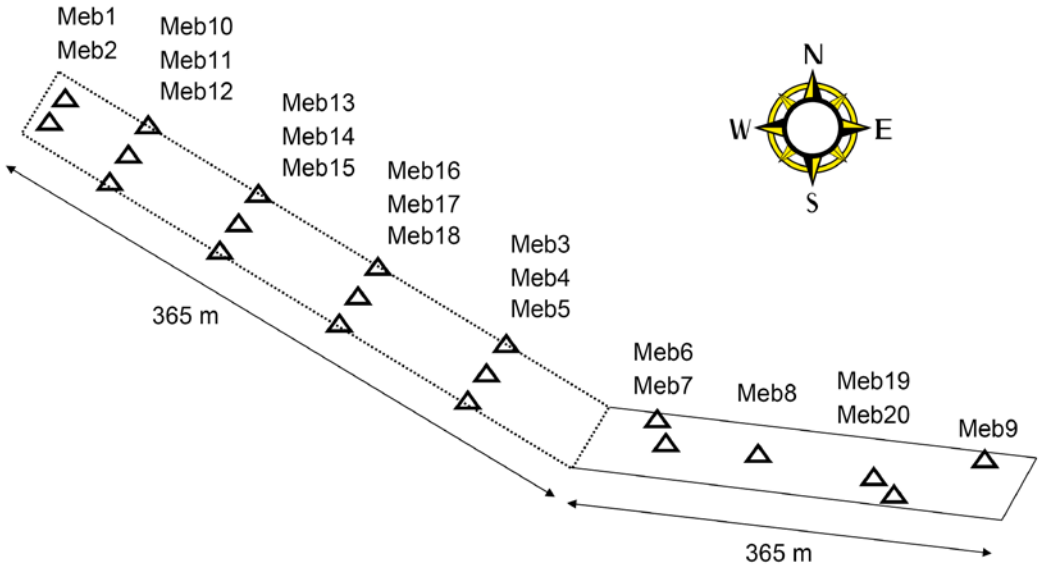


Figure 6-1. Sampling locations in the drainage ditch.

Extraction and analysis of PCBs from soil/sediment samples and bioaugmentation microcosms. The total concentration of PCBs in the samples were measured as described previously (Ghosh et al., 2000). Briefly, the samples were weighed prior to extraction, the overlying water was separated from the sediment by decanting and the remaining sample was mixed with anhydrous sodium sulfate. Following the addition of the surrogates PCB-14, PCB-65 and PCB-166 (4 ppb each) the overlying water samples were shaken in equal volume of 1:1 acetone:hexane, while the solid samples were extracted by sonication in 25 ml of 1:1 acetone:hexane for six minutes following EPA method 3550B. The extracts were then pooled and solvent exchange was performed to replace the solvents with hexane. PCB cleanup was based on EPA SW846 Methods 3660B (activated copper treatment), 3665A (sulfuric acid treatment) and 3630C (silica gel treatment). The volume obtained after clean-up was diluted according to QA protocols and the PCB congeners were analyzed using an Agilent 6890 Gas Chromatograph equipped with micro electron capture detector.

Microbial activity assays. The microbial dechlorination activity assay was used to determine the potential dechlorination activity of indigenous microbial communities in the sediment samples was described previously (Kjellerup et al. 2008). Briefly, ten ml of low-sulfate mineral F-medium (Berkaw et al. 1996b) was prepared anaerobically and the congener 2,3,4,5,6-CB (AccuStandard, CT) was added to a final concentration of 50 ppm in acetone without the

addition of endogenous electron donor. Cultures were inoculated in triplicate with 4.0 g of soil/sediment. Negative controls were prepared by autoclaving twice for 20 min at 121^{o}C one and three days after inoculation, respectively and prior to adding PCB. The cultures were incubated at 30ºC in the dark and 1 ml subsamples were collected for PCB analysis in an anaerobic glove box after 0, 52, 75, 105, 130, 163 and 200 days.

The potential inhibition of the dechlorination activity in selected sediment samples was evaluated with a modification of the activity assay described above. Sediment from Baltimore Harbor (2 g) with confirmed dechlorination activity was mixed with test sample (2 g) and inoculated into E-Cl medium. Sampling for PCB analysis was performed after 0, 50, 75, 100, 150 and 214 days.

Chemical analyses. Analyses of the following parameters were performed in the soil and sediment samples by a certified chemistry laboratory (Tetra Tech NUS 2006) using the listed EPA methods: polycyclic aromatic hydrocarbons (PAHs) including benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, dibenzo(a)anthracene and indeno(1,2,3-CD)pyrene (8310), pesticides including dieldrin, endrin aldehyde, 4-4-DDE and 4-4-DDT (8081), polychlorinated biphenyls (8082), nitrate, nitrite and sulfate (9056), total organic carbon (MSA Walker-Black method), solids content (9045), pH (9045), oxidation-reduction potential (ASTM Method D 1498-93), ammonia (E350.2), Kjeldahl-nitrogen (E351.3), phosphorous (365.2), metals (6010B, 1311/6010/70000, potassium (6010B) and oil and grease (9071A). The analytical data were validated according to United States Environmental Protection agency Region III validation procedures (Tetra Tech NUS 2006).

The dry matter content was determined in triplicate according to (American Public Health Association/American Water Works Association/Water Environment Federation 1995) and applied for calculation of the number of dechlorination bacteria by competitive PCR.

Statistical evaluations were performed using Students t-test with a statistical significance of $p<0.05$ and scatter plots, where the R^2 value was evaluated.

DNA extraction. DNA was extracted by transferring 0.75 g of sample to a sterile microcentrifuge tube containing approx. 1 g of 0.1 mm Zirconia/Silica Beads (BioSpec Products, Inc, OK) followed by addition of 200 µl of 1X TE buffer, 150 µl of phosphate buffer pH 8.0 (0.12 M) and 150 µl 1X TS-SDS buffer. The sample and buffers were mixed by hand shaking prior to 30 s of bead beating at speed "4.5" using a FastPrep120 (Q-Biogene, CA). Nucleic acid extraction and purification were performed using a phenol/chloroform based protocol described previously (Pulliam Holoman et al., 1998).

Detection and enumeration of putative PCB dechlorinating bacteria. Enumeration of putative dechlorinating bacteria in the sediment samples was performed in triplicate by a competitive PCR assay as described previously (Kjellerup et al. 2008). To confirm that potential co-extracted PCR inhibitors did not adversely affect the assay, all samples were tested in parallel with the universal primer set 341F/907R (Lane et al., 1985; Muyzer et al., 1993). All samples from the site showed positive results indicating that the inability to detect dechlorinating phylotypes was due to their abundance below the detection limit of 2×10^2 gene copies μl^{-1} (Kjellerup et al., 2008). The enumerated 16S rRNA genes copies from the cPCR assay were

normalized to the dry weight content of the soil sample. One 16S rRNA gene copy per cell was assumed based on the genome sequences of *Dehalococcoides ethenogenes* (Seshadri et al. 2005) and CBDB1 (Kube et al., 2005). DNA extraction efficiency was tested by extracting different amounts of homogenized soil and measuring the DNA concentration on a spectrophotometer at 280 nm. The results showed linear extraction efficiency in the range from 0-4 g of sediment (data not shown).

Detection of potential aerobic PCB degrading bacteria. Putative PCB degrading aerobic bacteria were detected by PCR amplification of the functional genes bphA and bphC, which encode the PCB transforming enzymes biphenyl dioxygenase and 2,3-dihydroxybiphenyl 1,2-dioxygenase, respectively. Detection of bphA was performed by the primer set bphA40-F/bphA50-R (Hoostal et al., 2002), whereas detection of bphC was performed by the primer set P42D-F/P43U-R (Erb & Wagner-Dobler, 1993). PCR was conducted in 50 µl reaction volumes using the following GeneAmp reagents (Applied Biosystems, Foster City, CA): 10 mM Tris-HCl, 75 mM KCl, 0.2 mM of each dNTP in a mix, 1.5 mM $MgCl_2$, 2.5 units of AmpliTaq DNA Polymerase, 50 pM of each primer, 1 µl of DNA template and 34.5 µl of nuclease free water. Amplification of the bphA fragment was performed as follows (40 cycles): denaturation at 45°C for 60 s, primer annealing at 31-49°C for 3 min, elongation at 72°C for 4 min and a final holding step at 4°C. For amplification of the bphC fragment (35 cycles) the following conditions were applied: denaturation at 95°C for 30 s, primer annealing at 35°C for 60 s, elongation at 72°C for 3 min, a final extension step at 72°C for 10 min and a final holding step at 4°C. PCR products of the correct length were confirmed by electrophoresis using a 1.5% agarose gel. *Burkholderia xenovorans* LB400 (Chain et al., 2006) was used for verification of the PCR protocols for bphA and bphC and as the positive control during PCR amplification.

Bioaugmentation in microcosms. In total 4 g of sediment (wet weight) was inoculated in triplicate into ten ml of anaerobically prepared low saline mineral medium (Berkaw et al. 1996a) in 25 ml anaerobe tubes sealed under N_2-CO_2 (80:20) with Teflon septa. No electron donors were added except for the components in the medium (0.0125% w/v cysteine) and residual hydrogen (≤ 5% v/v) present in the atmosphere of the anaerobic glove box used for inoculating and sampling of the microcosms. A culture of the dechlorinating bacterium DF-1 (May et al. 2008) was grown to approximately 10^7 cells per ml with PCE, which was purged with N_2/CO_2 to remove PCE and products prior to inoculating 2 ml into the sediment microcosms. Non-bioaugmented controls included medium and sediment containing indigenous microorganisms without DF-1. Controls for abiotic activity containing medium, sediment containing indigenous microorganisms and DF-1 were sterilized by sequential autoclaving for 1 hour on days 0, 2 and 4.

Microbial community analysis. Community analyses were performed by denaturing HPLC (DHPLC) using a WAVE 3500 HT system (Transgenomic, Omaha, NE) and the primers 348F/884R as described previously (Kjellerup et al., 2008). The 16S rRNA gene fragments were analyzed in an 20 µl injection volume and the fractions were collected in 96 well plates (Biorad, Hercules, CA). Fractions were dried using a Savant SpeedVac system (Thermo Electron Corporation, Waltham, MA) followed by dissolution in 15 µl nuclease free water. Each DHPLC fraction was sequenced in the 5' and 3' direction with 250 pM of primer 348F or 884R, respectively, in 5% DMSO to reduce effects from potential secondary structure using the

BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA) kit per the manufacturer's instructions and sequenced on an ABI 3130 XL automated capillary DNA sequencer (Applied Biosystems, CA) as previously described (Kjellerup et al. 2008). The 16S rRNA gene sequences and submitted gene sequences obtained from NCBI (hhtp://ncbi.nlm.nih.gov/BLAST) were compiled and aligned using the automatic nucleic acid aligner in the BioEdit sequence alignment editor. A total of 13 sequences containing from approximately 530 nucleotides were unambiguously aligned and used for calculation of trees by the neighbour joining and FITCH approaches using default settings in the PHYLIP software (hhtp://evolution.genetics.washington.edu/phylip.html). Bootstrap analyses (1000 replicates) were performed using the PHYLIP package.

Table 6-1. Analyses of the dry matter content, the total PCB concentration, the average number and percentage of chlorines less than 4 and 6, respectively and in soil samples and commercial Aroclor mixtures (Faroon et al., 2003).

Location	Dry matter (mg/g)	PCB Conc. (ppm)	Average no. of chlorines	≤6 chlorines (%)	≤4 chlorines (%)
Meb1	18.92	34.8	6.52	52.1	0.4
Meb2	17.54	264.6	6.47	53.6	0.5
Meb3	13.71	12.5	6.51	52.5	0.5
Meb4	28.25	36.6	6.54	50.7	0.4
Meb5	23.17	15.1	6.54	50.5	0.4
Meb6	26.61	3.1	6.43	54.7	1.7
Meb7	16.71	5.6	6.50	52.6	0.2
Meb8	23.71	45.3	6.54	50.5	0.5
Meb10	15.91	4.6	6.36	55.9	4.3
Meb11	26.68	2.3	6.61	45.8	1.0
Meb12	33.61	8.2	6.48	52.3	2.4
Meb13	13.55	12.1	6.47	51.8	3.2
Meb14	27.34	12.3	6.47	52.3	2.6
Meb15	29.22	35.6	6.54	47.1	2.6
Meb16	15.20	9.1	6.48	50.9	2.7
Meb17	25.10	17.6	6.52	50.9	2.0
Meb18	36.72	11.3	6.50	50.9	2.7
Meb19	20.31	20.1	6.55	48.7	1.9
Meb20	18.60	72.7	6.54	51.7	0.3
Aroclor 1016	-----	-----	3.04	100.0	100.0
Aroclor 1232	-----	-----	2.41	100.0	94.1
Aroclor 1242	-----	-----	3.31	100.0	92.3
Aroclor 1248	-----	-----	3.97	100.0	82.2
Aroclor 1254	-----	-----	5.15	96.0	18.9
Aroclor 1260	-----	-----	6.39	56.7	0.6

Results

Physical and chemical characterization of soil samples. Twenty soil samples were collected in the center of the storm water drainage ditch bed and on each of the flanking banks at incremental distances from the inlet to approximately 365 m down stream in addition to five locations between 365 and 730 m down stream from the inlet (**Fig. 6-1**). Soil samples collected from the banks were all moist, whereas both dry and moist soil samples were collected from the center of the ditch. Soil was not collected from the center of the ditch at the inlet because only 1-2 cm of sediment was present above the bedrock due to erosion from heavy storm water events, but this layer increased in depth with distance from the inlet. Soil samples collected more than 365 m downstream were submerged in water at the time of sampling. The total PCB concentration at the 20 sampling sites ranged from 2.3-264.6 ppm with an average of 32.8 ppm (± 58.9 ppm) (**Table 6-1**). The highest concentration was detected at the east bank nearest the inlet (Meb2), whereas the lowest concentration was detected at west bank sampling location Meb11. The average number of chlorines at each site ranged from 6.36-6.61, with a total average of 6.50 (± 0.053), which is similar to the average of 6.48 observed characteristically for Aroclor 1260 (**Table 6-1**). The distribution of PCB homologs was also similar to A1260 and in prior studies A1260 was reported to be the sole detected Aroclor at this site (Tetra Tech NUS 2005). In some of the samples increased concentrations of tetra chlorinated congeners were observed, but they did not co-elute with any known chlorinated pesticides (**Fig. 6-2**). The results suggest that *in situ* PCB dechlorination had occurred to some extent, although the possibility that small amounts of less extensively chlorinated Aroclor mixtures were released into the site cannot be ruled out.

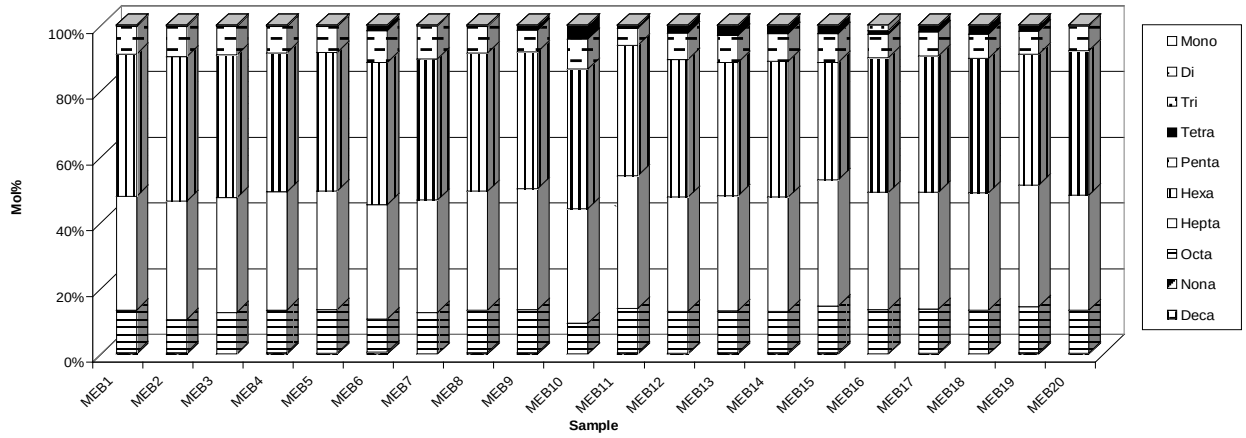


Figure 6-2. PCB homolog distribution profiles for the collected soil samples. The most typical: Meb2; Most dechlorinated: Meb10; Least dechlorinated: Meb11.

Characterization of indigenous microbial communities. Bacteria were detected in 19 of 20 samples using universal bacterial primers (**Table 6-2**). In contrast putative anaerobic dechlorinating bacteria and aerobic degrading bacteria were detected in only ten samples using primers specific for dechlorinating *Chloroflexi* and bphA/bphC genes, respectively (**Table 6-2**). At seven locations both putative aerobic and anaerobic PCB transforming bacteria were detected in the same samples. The numbers of anaerobic dechlorinating bacteria varied from $5 \cdot 10^3$ to $5 \cdot 10^6$ dechlorinating bacteria g^{-1} soil, where the numbers at several locations were below the

Table 6-2. Analyses of total bacteria, putative dechlorinating and PCB degrading bacteria and the number of dechlorinating bacteria in soil samples. *Sample Meb9 does not exist.

Location	Presence/absence of			
	Bacteria	Dechlorinating bacteria	Aerobic PCB degraders	No. of dechlorinating bacteria (16S copies·g soil^{-1})
Meb1	+	-	-	-
Meb2	+	-	-	-
Meb3	+	+	+	<D[a]
Meb4	+	+	+	$3 \cdot 10^4 \pm 2.8 \cdot 10^4$
Meb5	+	+	+	<D
Meb6	+	+	+	$3 \cdot 10^6 \pm 2.8 \cdot 10^6$
Meb7	+	+	+	$4 \cdot 10^5 \pm 2.3 \cdot 10^5$
Meb8	+	+	-	$3 \cdot 10^5 \pm 2.8 \cdot 10^5$
Meb9	-	-	-	-
Meb10	+	-	+	-
Meb11	+	-	-	-
Meb12	+	-	-	-
Meb13	+	-	+	-
Meb14	+	+	+	<D
Meb15	+	+	+	<D
Meb16	+	-	+	-
Meb17	+	+	-	<D
Meb18	+	+	-	$3 \cdot 10^3 \pm 2.8 \cdot 10^3$
Meb19	+	-	-	-
Meb20	+	-	-	-

Mean values are given and figures in brackets are ± standard deviation (n=3).
[a]DL: Detection limit = < 10^2 16S copies/g wet soil.

detection limit for enumeration. The number of aerobic degrading bacteria was not determined since an assay capable of enumerating this phylogenetically diverse group of bacteria is not available.

The identity of dechlorinating bacteria was examined by DHPLC in three samples where putative dechlorinating bacteria were most abundant (Meb6, Meb7 and Meb8) and compared to the sequences of known PCB dechlorinating bacteria within the *Chloroflexi* group (**Fig. 6-3**). Five of the 13 identified phylotypes grouped closely together with known *Dehalococcoides sp.* and the remaining eight phylotypes grouped within the broader *Chloroflexi* group. No trend was observed between the sample origin of the phylotypes and their phylogenetic relationship, since phylotypes from all three samples were located throughout the phylogenetic tree.

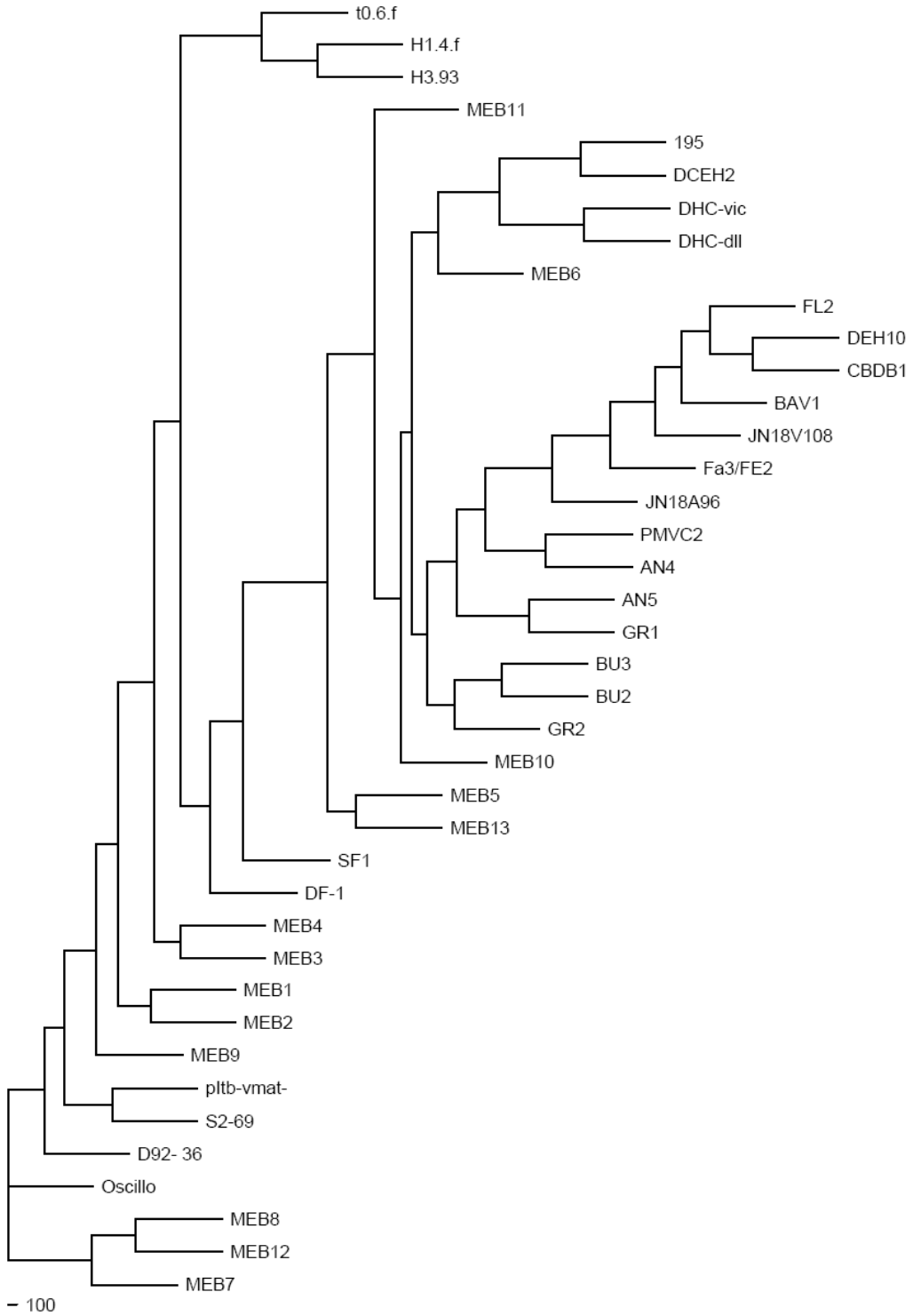


Figure 6-3. Phylogenetic tree showing the relationships between the dominant phylotypes identified in sediment samples Meb6, Meb7, and Meb8 and the closest dechlorinating species within the dechlorinating Chloroflexi group. Phylotypes from sample Meb6, Meb7 and Meb8 were located throughout the phylogenetic tree and did not show any relationship between the sample origins their phylogenetic relationship. Accession numbers are indicated in parentheses. The tree was calculated by the neighbor joining method and supported by FITCH (Ludwig et al., 2004). The scale bar indicates 10 substitutions per 100 nucleotide positions.

Dechlorination activities by indigenous communities. Dechlorination activity examined in soil samples using 2,3,4,5,6-CB, which is saturated on one biphenyl ring with chlorines (Kjellerup et al. 2008), was detected in six of the 20 samples (**Table 6-3**). The highest dechlorination rate ($6.933 \pm 5.33 \cdot 10^{-3}$ mol% 2,3,4,5,6-CB × day^{-1}) was measured in Meb16 and the lowest ($0.933 \pm 1.70 \cdot 10^{-3}$ mol% 2,3,4,5,6-CB × day^{-1}) in Meb17. No trends were observed between the dechlorination rates and the relative sampling locations (banks versus center of the ditch), since the lowest terminal plateau representing the maximum extent of dechlorination and the shortest lag phase were observed for Meb10. The observed products of the dechlorination of 2,3,4,5,6-CB were predominantly 2,3,4,5-CB/2,3,5,6-CB, but 2,3,6-CB and 2,6-CB were detected for sample Meb10. The remaining 13 samples did not show any dechlorination activity over after 200 days of incubation despite detection of putative dechlorinating bacteria by cPCR.

Table 6-3. The potential dechlorination activity of the soil samples in microcosms. The samples that were left out compared to Table 6-1 did not show any dechlorination activity.

Location	Max rate·10^{-3} [a] (mol% 2,3,4,5,6-CB × day^{-1})	Lag phase (d)	Terminal plateau after 200 days (mol% 2,3,4,5,6-CB)	Dechlorination products
Meb10	4.182 (0.73)	0-50	46.0 (7.0)	2,3,4,6-CB /2,3,5,6-CB[b] 2,3,6-CB, 2,6-CB[c]
Meb12	4.233 (4.12)	0-130	82.3 (5.8)	2,3,4,6-CB /2,3,5,6-CB
Meb13	3.400 (3.80)	0-150	78.0 (15.6)	2,3,4,6-CB /2,3,5,6-CB
Meb16	6.933 (5.33)	0-105	58.7 (27.6)	2,3,4,6-CB /2,3,5,6-CB
Meb17	0.933 (1.70)	0-200	89.7 (18.2)	2,3,4,6-CB /2,3,5,6-CB
Meb19	3.000 (5.02)	0-200	68.3 (49.7)	2,3,4,6-CB /2,3,5,6-CB
Negative control	0.067	-----	98	-----

[a]Mean values are given and figures in brackets are standard deviations (n=3).
[b]The products 2,3,4,6-CB /2,3,5,6-CB were not possible to separate during GC analysis.
[c]This product was detected in one of three replicates <5 mol%.

Effects of indigenous contaminants on activity. The absence of detectable dechlorination activity in some of the samples was further examined to determine whether high concentrations of PCBs or other contaminants inhibited activity, since putative dechlorinating phylotypes were detected in several of the active samples. Soil from inactive samples was mixed with sediment from Baltimore Harbor, MD, which transforms 2,3,4,5,6-CB to 2,4,6-CB via reductive dechlorination at the two *meta* positions. For all tested samples including sediment from Baltimore Harbor the lag phase was less than 50 days and the terminal plateau ranged between 6 and 46 mol% 2,3,4,5,6-CB (**Table 6-4**). The dechlorination rates varied from $7\text{-}12 \cdot 10^{-3}$ mol% 2,3,4,5,6-CB day^{-1}, where the highest was approximately 25% higher than obtained in the Baltimore Harbor sediment alone. This sediment sample (Meb7) was collected furthest away from the inlet of the drainage ditch, had the highest number of putative dechlorinating bacteria and had a high water content compared to the rest of the samples. The dechlorination rates for the mixed samples were all higher or at the same level as observed in the activity assays (**Table**

Table 6-4. Effect of inhibition on the dechlorination activity in microcosms using selected soil and sediment samples mixed 1:1 with actively dechlorinating sediment from Baltimore Harbor. Inhibition was determined ($p < 0.05$) for the dechlorination rate and terminal plateau.

Location	Lag phase (d)	Terminal plateau (mol% 2,3,4,5,6-CB)	Dechlorination rate $\cdot 10^{-3}$ (%2,3,4,5,6-CB day^{-1})	End product	Inhibition ($p<0.05$) Rate	Plateau
Baltimore Harbor	0-50	5.7 ± 4.5	9.1 ± 2.2[b]	2,4,6-CB[a]	-----	-----
Meb6	0-50	31.3 ± 15.9	7.3 ± 1.0	2,4,6-CB	No	Yes
Meb7	0-50	10.7 ± 3.5	12.4 ± 1.1	2,4,6-CB	No	No
Meb8	0-50	21.0 ± 19.2	8.4 ± 3.7	2,4,6-CB	No	No
Meb17	0-50	46.3 ± 6.5	7.5 ± 2.2	2,4,6-CB	Yes	Yes
Meb18	0-50	45.7 ± 10.1	10.5 ± 2.2	2,4,6-CB	No	Yes

[a] One tube showed additional terminal products such as 2,4-CB, 2,5-CB and 2,6-CB.
[b] Data are means ± standard deviation (n=3).

6-3). In all samples tested the end product of dechlorination was 2,4,6-CB, which is characteristic of BH activity rather than the tetra- chlorinated congener products detected predominantly in indigenous ditch microcosms, indicating that the indigenous BH dechlorinating activities were dominant. A statistical evaluation (Student's t-test, $p<0.05$) showed that statistically significant inhibition was found for only samples Meb17 (dechlorination rate), Meb6 and Meb18 (dechlorination plateau) and Meb17 (dechlorination rate and plateau).

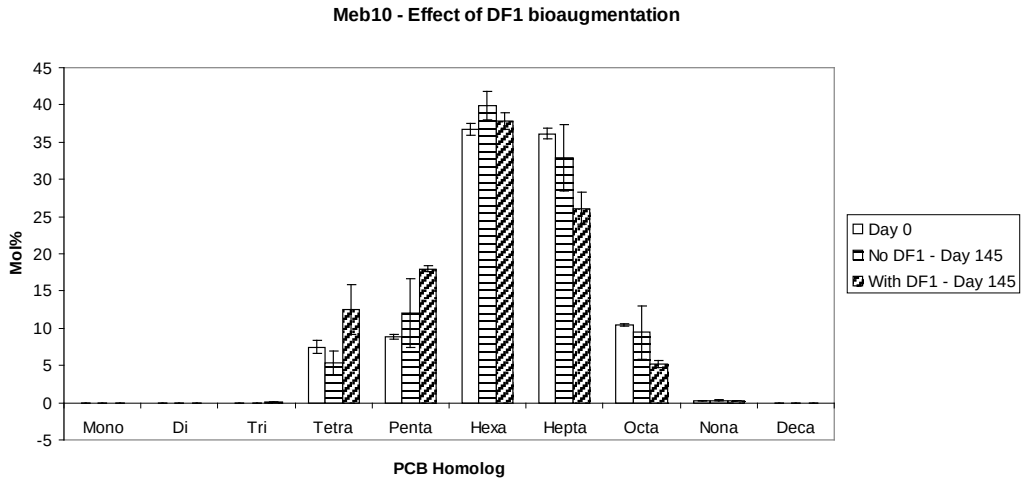
A statistical analysis was performed using Student's T-test to examine whether the presence and concentrations of other contaminants could account for the spatial variability of indigenous microbial populations and dechlorinating activities observed at the site. A correlation was observed between the presence of potential aerobic PCB degraders, the dechlorination rate ($p=0.02$) and the average number of chlorines ($p=0.05$), respectively. The presence/absence of dechlorination activity correlated with the number of chlorines<6 ($p=0.02$). In the case of dechlorination activity and the presence of dechlorinating bacteria there was a correlation with the dry matter content ($p=0.002$) and the dechlorination rate ($p=0.01$), respectively. No significant effect was apparent from the following individual or groups of contaminants: polycyclic aromatic hydrocarbons (PAHs) including benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, dibenzo(a)anthracene and indeno(1,2,3-CD)pyrene (8310); pesticides including dieldrin, endrin aldehyde, 4-4-DDE and 4-4-DDT. In addition, influence of the following nutrients and other parameters were tested and no significant influences were found: nitrate, nitrite, sulfate, total organic carbon, pH, oxidation-reduction potential, ammonia, Kjeldahl-nitrogen, phosphorous, inorganic compounds (arsenic, chromium, lead, and vanadium), potassium or oil and grease.

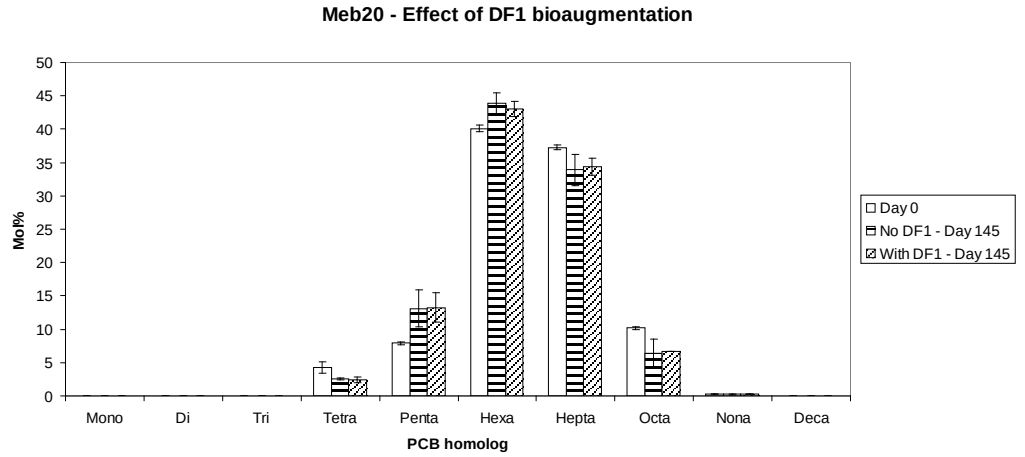
Effect of bioaugmentation on dechlorinating activity. Bioaugmentation with a pure culture of the PCB dechlorinating bacterium DF-1 was tested in microcosms with soil/sediment from three locations containing approximately 5 ppm (Meb10), 73 ppm (Meb20) and 265 ppm (Meb2) total

Table 6-5. Change in the number of chlorines per biphenyl over the five month incubation period for the bioaugmentation experiments with DF-1.

	Chlorines per biphenyl		
Location	Day 0	D145	
		No DF1	With DF1
Meb10	6.34 ± 0.02	6.30 ± 0.19	5.94 ± 0.10
Meb20	6.42 ± 0.02	6.29 ± 0.09	6.30 ± 0.04
Meb2	6.43 ± 0.01	6.42 ± 0.01	6.42 ± 0.01

weathered PCBs. These locations were selected based on the increasing concentrations of total PCB. The specific dechlorination of only double flanked chlorines by this strain could be readily distinguished from the dechlorination of single flanked and unflanked chlorines by the indigenous populations. Controls included both indigenous population without DF-1 and autoclaved controls to detect abiotic activities. The controls containing indigenous microorganisms only showed dechlorination at low levels. In the bioaugmented microcosms significant reduction was observed for Meb10 only, which contained the lowest indigenous levels of PCBs (**Table 6-5**). In addition to DF-1 a highly enriched culture of indigenous PCB dechlorinating microorganisms from MEB10 enriched with 2,3,4,5,6-PCB was also used to bioaugment Meb2, but again no significant effect on dechlorination was observed. Evaluation of the changes in homolog distributions indicated that DF-1 had a significant effect on Meb10 (**Fig. 6-4A**), with significant reductions in hepta- and octa-chlorinated congeners and significant increases for tetra- and penta-chlorinated congeners. The dechlorinated congeners in the bioaugmented Meb10 bioaugmented microcosm that were significantly reduced compared with the control were: 22'33'45'6 (PCB-75), 22'33'55'66'/22'33'44'6/233'44'5 (PCB-202/171/156), 22'33'455' (PCB-172), 22'344'55' (PCB-180), 22'33'44'5/233'44'56 (PCB-170/190), 22'344'55'6/22'33'44'56' (PCB-203/196). All congeners that were dechlorinated significantly more than the control contained both double flanked *meta* and *para* positioned congeners with the exception of PCB-175 and PCB-171 that only contained a *meta* positioned chlorine and PCB-202 that did not contain any double flanked *meta* or *para* positioned chlorines. However, the two latter congeners co-eluted with PCB-156 that contains both double flanked *meta* and *para* positioned chlorines. The products that were formed in significantly higher amounts in the presence of DF-1 were: 234'5 (PCB-63), 233'4'/2344' (PCB-56/60), 22'44'5 (PCB-99), 22'355'/22'33'6/22'346' (PCB-92/84/89). Most of these products could have been formed due to the presence of DF-1 since dechlorination of either a *meta* or a *para* positioned chlorine was occurring in the last step. The only exception was PCB-56/PCB-60 that did not contain any double flanked *meta* or *para* positioned chlorines. It remains unclear whether DF-1 catalyzed any other processes that could have resulted in the formation of these products since the intermediate dechlorination products could not be distinguished in this analysis. However, for the samples Meb20 and Meb2 containing higher concentrations of total PCB, DF-1 did not show any significant effects (**Fig. 6-4B-C**).





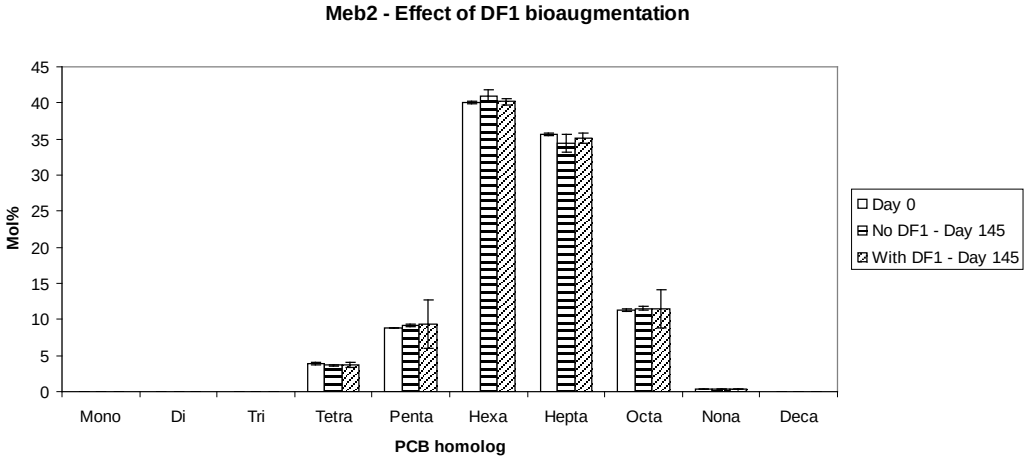


Figure 6-4A-C. Homolog distribution showing the effect of DF-1 in the bioaugmentation experiment compared to effect of the indigenous population for the three tested locations. 4A: Meb10. 4B: Meb20. 4C: Meb2.

Discussion

Bacteria capable of aerobic PCB degradation are found ubiquitously in the soil environment and several have been isolated and identified belonging to genera such as *Pseudomonas*, *Burkholderia*, *Ralstonia*, *Achromobacter*, *Comamonas*, *Bacillus* and *Rhodococcus* (Furukawa, 1994; Pieper, 2005). In contrast, anaerobic PCB-dechlorinating bacteria have previously only been detected in soil with activity based methods, but never identified. Dechlorination in soil has been detected at several Canadian military installations such as Saglek, Labrador (Master et al., 2002), Resolution Island, Nunavut (Kuipers et al., 2003), Fort Albany, Ontario (Tsuji et al. 2006). However, identification of putative anaerobic PCB dechlorinating bacteria in soil has until now not been performed and the combined methodological approach involving molecular and activity based techniques applied in this study is novel. The presence of active dechlorinating bacteria is essential for obtaining complete mineralization of PCBs since aerobic microorganisms cannot mineralize extensively chlorinated PCB congeners thus requiring dechlorination to occur as the first step of this sequential degradation process.

In the drainage ditch examined in this study, the PCB contamination originated predominantly from A1260 contamination (Tetra Tech NUS 2005). However, tetra-chlorinated congeners were observed at elevated levels in some locations together with an decreased average chlorine content compared to A1260, which could indicate presence of *in situ* dechlorination activity. However, the same effects could also be obtained from mixing with less extensively chlorinated Aroclors even though this has not been reported for this site.

Identification of the putative PCB dechlorinating phylo-types suggests that *in situ* dechlorination might take place. All 13 phylotypes from the soil samples clustered within the dechlorinating *Chloroflexi* group. Some of the soil phylotypes grouped with the *Dehalococcoides*-group (Bedard et al., 2006; He et al., 2005; Hendrickson et al., 2002; Kjellerup et al., 2008; Waller et al., 2005) and the DF-1/SF-1-group (Fagervold et al., 2005; May et al., 2008), whereas other putative dechlorinating soil phylotypes grouped within the broader *Chloroflexi* group. Two previous reports on bacterial communities in PCB contaminated soil identified bacteria closely related to *Proteobacteria*, the *Holophage-Acidobacterium* phylum, *Actinobacteria*, and *Plantomycetales* and *Cytophagales* (Nogales et al., 1999, 2001). The dominant species included the genera *Burkholderia* and *Variovorax* together with *Sphingomonas* species, *Rhodophila globiformis* group members and *Acidobacterium capsulatum* that all aerobically can degrade a variety of organic pollutants such as PCBs. However, neither anaerobic dechlorinating bacteria nor any phylotypes related to the dechlorinating *Chloroflexi* were identified in these studies.

Prior to the current study, PCB dechlorinating bacteria have only been identified in sediments. Thus, the results obtained in this study show that putative anaerobic dechlorinating bacteria are present in the examined soil samples and might play an important role in PCB dechlorination in soil. Potential aerobic PCB degrading bacteria were also detected at several locations in the drainage ditch either alone or in co-existence with dechlorinating bacteria, but the identity or abundance were not further identified due to the diverse phylogeny of aerobic PCB degraders. These observations suggest that natural attenuation of PCBs could potentially occur in soils by sequential anaerobic dechlorination followed by aerobic degradation and subsequent mineralization. However, large spatial heterogeneity was observed for aerobic and anaerobic

PCB transforming bacteria. The distribution of both aerobic and anaerobic putative PCB degrading bacteria did not correspond with any of the parameters that were evaluated in this study nor were any other relationships between the chemical parameters found. Similar observations have been done in a study where bacterial activity and community structures were examined in soil contaminated with heavy metals (Becker et al., 2006). It was found that samples collected within 1 cm distance had a 10,000 fold difference in metabolic potential and that metal concentrations did not correspond with the metabolic potential.

In previous studies of freshwater sediment the number of putative dechlorinating bacteria was approximately 10^8 bacteria per g sediment, whereas the abundance in soil in this study was 100-10,000 times lower (Kjellerup et al., 2008). Compared to other prior reports with sediments both lag phases, dechlorination rates and terminal plateaus were lower in this study (Kjellerup et al. 2008). The contrast between the number of dechlorinating bacteria and the dechlorination rates in the soil and sediment samples might have been caused by the localized environmental conditions, since most of the soil samples at the time of sampling were moist, but were not submerged in water as is the case for sediments. In a study of simulated dredged sediment that was spiked with 300 ppm A1248 it was reported that the dechlorination was reduced when the water content was reduced (Cho et al., 2001). In addition, the variation in water content caused a lag in the dechlorination of A1248, but eventually the dechlorination followed the same pathway independent of the original water content. Also, degradation studies of other contaminants in soil such as petroleum hydro carbons show that moisture content in soil influences the degradation rate (Davis et al., 2003).

A possible consequence of the reduced moisture content in the soil samples could be exposure to oxygen that could negatively influence anaerobic dechlorination. In a study of methanogenic granules capable of A1254 dechlorination, exposure to oxygen did not significantly influence the dechlorination activity (Natarajan et al., 1995). In six months 80% of the initial concentration of A1254 had been dechlorinated in samples that had been exposed to oxygen for one week. The reason for this rapid dechlorination could be the formation of biofilms in micro-niches that stay anaerobic despite the exposure to oxygen at the surface of the granule. In soil however, the presence of micro-niches has been found in several studies, where spatial and temporal heterogeneity has been observed for many parameters including oxygen, pH, redox and nutrient concentrations (Alewell et al., 2006; Davis et al., 2003; Liebner et al., 2008). Thus the spatial variation in dechlorination rates observed in the soil samples might be the result of heterogeneous moisture content affecting formation of biofilms and anaerobic micro-niches that support communities of dechlorinating bacteria.

Previously a number of studies have used 2,3,4,5,6-CB, 2,3,6-CB or 2,4,6-CB as primers for the onset of dechlorination of indigenous PCBs (Bedard et al., 1997; Klasson et al., 1996). In these studies dechlorination did not occur unless the primers were applied. In the study by Bedard et al (1997) both *meta*- and *para*-dechlorination occurred in sediment samples from the Housatonic River (Bedard et al., 1997), whereas in Klasson et al. (1996) only *meta*-dechlorination was observed in soil slurries (Klasson et al., 1996). In the current study evaluation of the end products from dechlorination of 2,3,4,5,6-CB used for determining the dechlorination potential showed that 2,3,4,6/2,3,5,6-CB (co-eluting) and for one sample (Meb10) 2,3,6-CB and 2,6-CB were formed based on *meta*- and *para*- dechlorination. In comparison only *meta*-dechlorination

was observed, when selected soil samples were mixed with Baltimore Harbor sediment in the inhibitor assay in this study. The sample showing the most diverse activity also had the highest potential dechlorination rate and showed the presence of tetra chlorinated congeners in the original soil sample despite the fact that putative dechlorinating bacteria were not detected by PCR. This shows that a combination of molecular and activity based assays is needed to obtain a sufficiently detailed evaluation of the site with regard to bioremediation and whether biostimulation or bioaugmentation would be a solution.

Successful bioaugmentation of weathered PCBs in soil has previously been observed by Klasson et al (1996), where an enrichment culture from the Hudson River was used for bioaugmentation of A1242 contaminated soil (Klasson et al., 1996). Here *meta*-dechlorination was observed after 19 weeks of incubation resulting in a reduction of the average chlorine content by 0.7 chlorines per biphenyl (Klasson et al., 1996). Also, an experiment evaluating the effect of sequential anaerobic-aerobic treatment of A1260 contaminated soil from Saglek, Labrador, Canada (Master et al., 2002) showed a decrease in the average chlorine content of 1.2 chlorines per biphenyl together with a significant homolog shift after three months of anaerobic bioaugmentation with enrichment culture BK81s (Master et al., 2002). The main products were 24-24-CB, 24-26-CB, 23-2/26-4-CB, and 235-2/24-25-CB being formed due to *meta*-dechlorination. In the current study, the effects of DF-1 on bioaugmentation of weathered A1260 contaminated soil could mainly be related to the presence of DF-1, since DF-1 dechlorinates double flanked *meta*- and *para*-positioned chlorines (May et al., 2008). One product was observed that could not have been formed due to dechlorination of a double flanked *meta* or *para* positioned chlorine (233'4'/2344'-CB). However, DF-1 might have stimulated the indigenous dechlorinating population since the activity assays had shown the possibility of both *meta*- and *para*- dechlorination. The observed processes could be compared to the effect of priming (Bedard et al., 1997; Klasson et al., 1996). In these cases 2,3,6-CB, 2,3,4,5,6-CB, or 2,4,6-CB were applied to reduce the lag phase by stimulating the dechlorinating bacteria present in the samples before dechlorination of the indigenous PCBs started (Bedard et al., 1997; Klasson et al., 1996). The reason for the lack of increased dechlorination activity at the two other bioaugmentation sites remains unclear, but might be caused by inhibition by high concentrations of PCBs, other contaminants or strong adsorption of weathered PCBs to the soil particles and thereby less bioavailability.
The results from this study suggest that a potential for PCB degradation involving both aerobic and anaerobic groups of bacteria exists at the sampling location. However, spatial heterogeneities were observed regarding presence of bacteria, contaminants and dechlorination activity making bioremediation of the drainage ditch based on one solution difficult. Bioaugmentation with an exogenous dechlorinating bacterium that was closely related to bacteria identified at the site was successful for one of three tested locations. At this location dechlorination activity had been detected prior to the start of bioaugmentation. The soil moisture content at a micro-scale level might have influenced the dechlorination activity together with the bioavailability of the weathered PCBs. Thus bioaugmentation of PCB contaminated soil sites showed to be different from sediments.

7. Dechlorination and Detoxification of 1,2,3,4,7,8-Hexachlorodibenzofuran by a Mixed Culture Containing *Dehalococcoides ethenogenes* strain 195

(Liu F, Fennell DE. 2008. Dechlorination and detoxification of 1,2,3,4,7,8-hexachlorodibenzofuran by a mixed culture containing Dehalococcoides ethenogenes strain 195. Environmental Science and Technology 42:602–607.)

Abstract

Toxic polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) with chlorines substituted at the lateral 2-,3-,7-,8- positions are of great environmental concern. We investigated the dechlorination of 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) and 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD) by a mixed culture containing *Dehalococcoides ethenogenes* strain 195. The 1,2,3,4,7,8-HxCDF was dechlorinated to 1,3,4,7,8-pentachlorodibenzofuran and 1,2,4,7,8-pentachlorodibenzofuran, and further to two tetrachlorodibenzofuran congeners, which were identified as 1,3,7,8-tetrachlorodibenzofuran and 1,2,4,8-tetrachlorodibenzofuran. Since no 2,3,7,8-substituted congeners were formed as dechlorination products from 1,2,3,4,7,8-HxCDF, this dechlorination represents a detoxification reaction. Tetrachloroethene (PCE) and 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) were added as additional halogenated substrates to enhance the degree of 1,2,3,4,7,8-HxCDF dechlorination. The 1,2,3,4-TeCB enhanced the extent of dechlorination of 1,2,3,4,7,8-HxCDF approximately three-fold compared to PCE or no co-substrate amendment. No dechlorination products were detected from OCDD. Bioremediation of PCDD/Fs by bacterial reductive dechlorination should address the pathway of dechlorination to ensure detoxification.

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are two groups of compounds with similar tricyclic planar structures. They are produced inadvertently and enter the environment from many sources including chemical manufacturing, pulp and paper production, and combustion processes (US EPA 2006). PCDD/Fs are hydrophobic, have low solubility and volatility, accumulate in soils, sediments and biota, and are ubiquitous in the environment (Czuczwa & Hites 1984; Brzuzy & Hites 1996; Wagrowski & Hites 2000; Mai et al. 2007, Rappolder et al. 2007). Contamination of sediment with PCDD/Fs is a serious environmental problem (Bopp et al. 1991; Kjeller & Rappe 1995; Juttner et al. 1997; Isosaari et al. 2002; Verta et al. 2007). PCDD/Fs are generally present at very low concentrations; however, they are of great concern because of their toxicity (US EPA 1989) van den Berg et al. 2006) and potential to bioaccumulate (Cai et al., 1994). Seventeen 2,3,7,8-substituted PCDD/F congeners are the focus of regulatory effort and scientific investigation owing to their high toxicity to humans and wildlife. Toxicity equivalency factors (TEFs) indicate an order of magnitude estimate of the toxicity of the 2,3,7,8-substituted PCDD/F congeners and other dioxin-like compounds including certain co-planar polychlorinated biphenyls (PCBs) (van den Berg et al. 2006). TEF values are assigned relative to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TeCDD), the PCDD/F congener considered the most toxic of these compounds (van den Berg et al. 2006). A toxic equivalent (TEQ) expressed as equivalents of 2,3,7,8-TeCDD can be computed for mixtures of 2,3,7,8-substituted PCDD/F congeners by summing the products of individual concentrations multiplied by their respective TEFs. TEFs are intended to predict the relative

toxicity of congeners in animal tissues or their diets. TEFs are also widely used to quantify TEQs for dioxin-like compounds in environmental media, e.g. sediment. Summing converted TEQs of individual congeners to a single total TEQ value is useful for comparing contaminated sediments and prioritizing remedial efforts.

Biotransformation of PCDD/Fs occurs under both aerobic and anaerobic conditions (Wittich 1998; Adriaens et al., 1995; Barkowskii & Adriaens 1996, Beurskens et al. 1995; Ballerstedt et al., 1997; Yoshida et al. 2005). Lightly chlorinated PCDD/Fs may be biotransformed or mineralized under aerobic conditions (Wittich 1998; Yoshida et al. 2005), while highly chlorinated PCDD/Fs undergo reductive dechlorination under anaerobic conditions (Adriaens et al., 1995; Barkowskii & Adriaens 1996, Beurskens et al. 1995; Ballerstedt et al., 1997; Bunge et al., 2003; Fennell et al. 2004). Recently two bacterial strains have been identified which dechlorinate PCDDs under anaerobic conditions — *Dehalococcoides* sp. strain CBDB1 (Bunge et al., 2003) and *Dehalococcoides ethenogenes* strain 195 (Fennell et al. 2004).

D. ethenogenes strain 195 dechlorinates tetrachloroethene (PCE) to vinyl chloride (VC) and ethene. It grows on PCE, trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-1,2-DCE), 1,1-DCE (Maymo-Gatell et al. 1997, 1999), selected chlorinated benzenes (Fennell et al. 2004), and chlorophenols (Adrian et al. 2007). Strain 195 debrominated commercial octa-brominated diphenyl ether (BDE) (a mixture containing hexa-BDE through nona-BDE) to a mixture of penta-, hexa-, and hepta-BDEs (He et al. 2006). In pure culture, strain 195 dechlorinated 2,3,4,5,6-PCB, 1,2,3,4-tetrachloronaphthalene, and 1,2,3,4-tetrachlorodibenzofuran (1,2,3,4-TeCDF). Specifically, it dechlorinated 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) to 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD), and subsequently to 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (Fennell et al. 2004). Notably, strain 195 did not dechlorinate 2,3,7,8-TeCDD *(23)*. It is not known if the PCBs or PCDD/Fs support its growth.

For bioremediation of contaminated sediments, not only is dechlorination desired, but more importantly, detoxification with respect to the PCDD/Fs should be achieved. We investigated dechlorination of two 2,3,7,8-substituted PCDD/F congeners, 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) and 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD) by a mixed culture containing *D. ethenogenes* strain 195. 1,2,3,4,7,8-HxCDF has a TEF of 0.1 (van den Berg et al. 2006) and is the most abundant of the hexa-CDFs from anthropogenic sources in the USA (Cleverly et al., 1997). It is a significant contributor to the total TEQ in sediments in the New York-New Jersey Harbor (Gale et al. 2000). OCDD, with a TEF of 0.0003 (van den Berg et al. 2006) has the highest mass concentration of PCDD/Fs in atmospheric deposition sources and thus generally in sediments (Bopp et al. 1991; Hites 1990). Since both compounds have fully chlorinated rings similar to the structures of 1,2,3,4-TeCDD/F, we hypothesized that strain 195 would also dechlorinate these congeners. Dechlorination of 1,2,3,4-TeCDD by *D. ethenogenes* strain 195 was observed to proceed through a lateral, followed by a peri dechlorination step. During the dechlorination of 1,2,3,4,7,8-HxCDF or OCDD, removal of chlorine from one of the 2-, 3-, 7-, or 8-positions would greatly decrease the TEQ of the total PCDD/Fs. This study examined both the pathway and extent of dechlorination of 1,2,3,4,7,8-HxCDF and OCDD by a mixed culture containing *D. ethenogenes* strain 195 under different conditions.

Materials and Methods

Chemicals. 1,2,3,4,7,8-HxCDF (98+%), 1,2,3,4-TeCDF, and 1,3,7,8-tetrachlorodibenzofuran (1,3,7,8-TeCDF) were purchased from Ultra Scientific (North Kingstown, RI, USA). The 2,2',5-trichlorobiphenyl (2,2',5-TrCB) and OCDD and standard solutions of 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF), 1,2,3,7,8-pentachlorodibenzofuran (1,2,3,7,8-PeCDF), and 2,3,7,8-tetrachlorodibenzofuran (2,3,7,8-TeCDF) were purchased from AccuStandard, Inc. (New Haven, CT, USA). A 1,2,3,4,8-pentachlorodibenzofuran (1,2,3,4,8-PeCDF) standard solution was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Standard solutions of 1,2,4,7,8-pentachlorodibenzofuran (1,2,4,7,8-PeCDF), 1,3,4,7,8-pentachlorodibenzofuran (1,3,4,7,8-PeCDF), 2,3,6,8-tetrachlorodibenzofuran (2,3,6,8-TeCDF), 1,4,7,8-tetrachlorodibenzofuran (1,4,7,8-TeCDF), 1,3,4,8-tetrachlorodibenzofuran (1,3,4,8-TeCDF), 1,2,4,7-tetrachlorodibenzofuran (1,2,4,7-TeCDF) and 1,2,4,8-tetrachlorodibenzofuran (1,2,4,8-TeCDF) were purchased from Wellington Laboratories, Inc. (Guelph, Ontario, Canada). The 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA), PCE (99.9+%), TCE (99.5+%), and butyric acid (99+%) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). The *cis*-1,2-DCE was purchased from Supelco, Inc. (Bellefonte, PA, USA). VC (99.5+%) was obtained from Fluka Chemie GmbH (Germany). Ethene (99%) was purchased from Matheson Tri-Gas, Inc. (Montgomeryville, PA, USA).

Culture Preparation. A mixed culture containing *D. ethenogenes* strain 195 was grown at 25°C on PCE and butyric acid using methods described previously (Fennell 1998; Fennell et al. 1997, 2004). Dechlorination experiments were carried out in 60-mL serum bottles. Dry sterile sediment (0.375 g) prepared as previously described (Fennell et al. 2004) was added to each bottle. The sediment was completely wetted by 0.35 mL of a 535 μM (200 mg/L) 1,2,3,4,7,8-HxCDF-toluene stock solution. The toluene was allowed to volatilize under sterile N_2, leaving behind a coating of 1,2,3,4,7,8-HxCDF on the sediment carrier. Culture (37.5 mL) was transferred to each bottle under anoxic and sterile conditions resulting in a final nominal 1,2,3,4,7,8-HxCDF concentration of 5 μM (1.87 mg/L). Each bottle also received 100 μM butyric acid as an electron donor and hydrogen source and 15 μL of a 50 g/L fermented yeast extract solution (Fennell 1998; Fennell et al. 1997) as a nutrient source on days 6, 25, 51, 74, 111, 144, and 165. A vitamin stock solution (Fennell 1998; Fennell et al. 1997) was added at set up. Four sets of triplicate treatments were established. One set of bottles received 1,2,3,4,7,8-HxCDF as the sole halogenated substrate. Because we do not know if PCDD/Fs are growth substrates for *D. ethenogenes* strain 195, in addition to the 1,2,3,4,7,8-HxCDF, one set of triplicate bottles was amended with PCE, a known growth compound, as an additional substrate on the same days when butyric acid and fermented yeast extract were amended. One set of triplicate bottles was spiked with 1,2,3,4-TeCB, which is also a growth supporting substrate for *D. ethenogenes* strain 195, but only on days 0 and 76. The nominal concentrations of PCE and 1,2,3,4-TeCB added to the culture bottles were 25 μM. The fourth set of triplicate bottles was autoclaved for one hour on each of three consecutive days to serve as killed controls. Parallel treatments were prepared with OCDD as the PCDD/F substrate at 5 μM, using the same experimental protocol as described for 1,2,3,4,7,8-HxCDF. The bottles were shaken in the dark at 120 rpm at 28°C and sampled periodically over 195 days.

To ascertain the dechlorination intermediates of 1,2,3,4,7,8-HxCDF, two separate experiments were performed using 1,2,4,7,8-PeCDF or 1,3,4,7,8-PeCDF as the halogenated substrate. In the first experiment, 28 mL tubes were spiked with toluene stock solutions of either 1,2,4,7,8-PeCDF or 1,3,4,7,8-PeCDF, and toluene was allowed to volatilize. Three mL of culture was added and the final nominal concentration of PeCDF was 2 μM. Tubes were sacrificed at set up and after 1 and 2 months. Two active tubes and one autoclaved control tube were prepared for each time point for both of the PeCDF congeners. In the second experiment, dry sediment (0.15 g) was added to 60 mL serum bottles followed by spiking 0.25 mL of a 147 μM (50 mg/L) stock solution of 1,2,4,7,8-PeCDF or 1,3,4,7,8-PeCDF (in toluene), respectively. After volatilization of toluene, 15 mL culture was transferred to each bottle under sterile and anaerobic conditions to achieve a final nominal concentration of 1,2,4,7,8-PeCDF or 1,3,4,7,8-PeCDF of 2.45 μM. At set up, cultures in both experiments received 12 μM 1,2,3,4-TeCB and butyric acid, fermented yeast extract and vitamin stock solution at the same concentrations as the experiment with 1,2,3,4,7,8-HxCDF. The cultures were shaken in the dark at 120 rpm at 28°C.

Analytical Methods. Headspace samples (0.1 mL) were analyzed for chloroethenes and ethene using an Agilent 6890 gas chromatograph equipped with a GS-GasPro (Agilent Technologies, Inc. Santa Clara, CA) column (30 m × 0.32 mm I.D.) and a flame ionization detector. The oven temperature program was: 50°C for 2 min; increased at 15°C/min to 180°C; and then held at 180°C for 4 min.

Samples of 1 or 2 mL of culture/sediment mixture were removed for PCDD/F extraction using a sterile anoxic syringe with an 18 gauge needle. [The entire 3 mL culture in the tube experiment examining 1,2,4,7,8 and 1,3,4,7,8-PeCDF dechlorination was extracted.] Samples were extracted and prepared for analysis as described previously (Vargas et al. 2001). Briefly, after sample centrifugation, the aqueous phase was removed to a separate vial, 2,2',5-TrCB was added as a surrogate standard to the sediment residue, and then the sediment phase was rinsed with 1 mL of acetone to remove water. The solid phase was extracted overnight with 3 mL of 2:1 volume:volume (vol:vol) toluene:acetone solution, then for 4 h with 1 mL of 2:1 vol:vol toluene:acetone solution, and was then rinsed with 1 mL toluene. After each step, the solvent phase was combined with the aqueous phase. Finally, the pooled solvent was back extracted by adding NaCl. Interfering organic compounds were removed by passing the solvent phase through a 2 mL glass pipette filled with Florisil (Sigma-Aldrich, St. Louis, MO) and eluting with three volumes of toluene. The extract was then concentrated to about 2 mL. PCDD/Fs and chlorobenzenes were analyzed using an Agilent 6890 gas chromatograph equipped with a 5973N mass selective detector (GC-MS) (Agilent Technologies, Inc. Santa Clara, CA) and a HP-5MS (Agilent Technologies, Inc. Santa Clara, CA, USA) column (60 m × 0.25 mm I.D.). The temperature program was: initial temperature 70°C; increased by 10°C/min to 170°C; increased by 2°C/min to 200°C; increased by 5°C/min to 220°C and held for 16 min; increased by 5°C/min to 235°C and held for 7 min; and finally increased by 5°C/min to 280°C. The post run temperature was 300°C, for 5 min. PCDD/Fs were detected and identified based on the retention times of standards and their molecular ions (*m/z*: 460, OCDD; 374, 1,2,3,4,7,8-HxCDF; 340, PeCDFs; 306, TeCDFs; and 186, 2,2',5-TrCB). A qualifying ion was monitored to assure the correct identification (458, OCDD; 376, 1,2,3,4,7,8-HxCDF; 342, PeCDFs; 304, TeCDFs; and 256, 2,2',5-TrCB). The response factors of each PCDD/F congener compared to the surrogate,

2,2',5-TrCB, were calculated over five concentration levels as a linear calibration curve. The approximate detection limit for the PCDD/Fs was 1 ppb.

The PCDD/Fs were quantified based on the calibration curve and presented as averages of duplicate or triplicate data points plus or minus one standard deviation. The 1,3,4,7,8-PeCDF and 1,2,4,7,8-PeCDF were co-eluted and it was not possible to quantify them separately. We therefore used the calibration curve of 1,3,4,7,8-PeCDF to quantify the PeCDF peak detected in samples. The 1,2,4,7,8-PeCDF and 1,3,4,7,8-PeCDF had similar response factors. Results are presented as a molar fraction of an individual congener of the total moles of all PCDFs present. Presentation of PCDFs as mole percent assumed no anaerobic degradation of the dibenzofuran structure and that the PCDFs underwent no significant reactions other than dechlorination.

Table 7-1. Retention times of PCDF standards and metabolites on an Agilent 6890 gas chromatograph equipped with a 5973N mass selective detector (GC-MS) (Agilent Technologies, Inc. Santa Clara, CA) and a HP-5MS (Agilent Technologies, Inc. Santa Clara, CA, USA) column (60 m x 0.25 mm I.D.). [The temperature program was: initial temperature 70°C; increased by 10°C/min to 170°C; increased by 2°C/min to 200°C; increased by 5°C/min to 220°C and held for 16 min; increased by 5°C/min to 235°C and held for 7 min; and finally increased by 5°C/min to 280°C.]

Congener	Retention Time (min)
1,3,7,8-TeCDF	39.69
1,2,4,7-TeCDF	39.69
First TeCDF metabolite	39.69
1,3,4,8-TeCDF	40.29
1,2,4,8-TeCDF	40.34
Second TeCDF metabolite	40.34
1,4,7,8-TeCDF	41.06
2,3,6,8-TeCDF	41.71
1,2,3,4-TeCDF	42.38
2,3,7,8-TeCDF	43.90
1,3,4,7,8-PeCDF	51.05
1,2,4,7,8-PeCDF	51.06
PeCDF metabolite	51.05
1,2,3,4,8-PeCDF	53.06
1,2,3,7,8-PeCDF	53.26
2,3,4,7,8-PeCDF	55.59
1,2,3,4,7,8-HxCDF	62.18

Results

Dechlorination Pathway. Dechlorination of 1,2,3,4,7,8-HxCDF by the mixed culture produced a PeCDF metabolite peak with a retention time of 51.05 min and two TeCDF metabolite peaks at 39.69 min and 40.34 min, based on their mass spectra. No lesser chlorinated products were detected. The PeCDF peak was identified as 1,3,4,7,8-PeCDF and/or 1,2,4,7,8-PeCDF and the TeCDFs were identified as 1,3,7,8-TeCDF and 1,2,4,8-TeCDF based on retention time and co-injection comparison with standards (**Table 7-1**). The dechlorination pathway of 1,2,3,4,7,8-HxCDF is shown in **Figure 7-1**. One route was 1,2,3,4,7,8-HxCDF to 1,3,4,7,8-PeCDF and further to 1,3,7,8-TeCDF. The other route was from 1,2,3,4,7,8-HxCDF to 1,2,4,8-TeCDF via 1,2,4,7,8-PeCDF. No potential 2,3,7,8-substituted daughter products—i.e., 2,3,4,7,8-PeCDF, 1,2,3,7,8-PeCDF or 2,3,7,8-TeCDF—were formed based on comparisons between the retention times of the metabolites and the standards (**Table 7-1**). The 2,3,7,8-substituted parent compound was dechlorinated to non 2,3,7,8-substituted PCDFs, thus, dechlorination of 1,2,3,4,7,8-HxCDF by the mixed culture containing *D. ethenogenes* strain 195 was a detoxification process. The 1,2,3,4,8-PeCDF was also excluded as a metabolite based on the retention time in comparison to the standard.

Because standards of 1,2,4,7,8-PeCDF and 1,3,4,7,8-PeCDF had the same retention time as the PeCDF metabolite in the samples (**Table 7-1**), we identified the PeCDF metabolite(s) as 1,2,4,7,8- and/or 1,3,4,7,8-PeCDF. The 1,2,4,7,8- and 1,3,4,7,8-PeCDF could not be resolved using our GC method. The formation of 1,2,4,7,8-PeCDF and/or 1,3,4,7,8-PeCDF resulted from a lateral chlorine removal from the fully chlorinated ring of 1,2,3,4,7,8-HxCDF. We determined

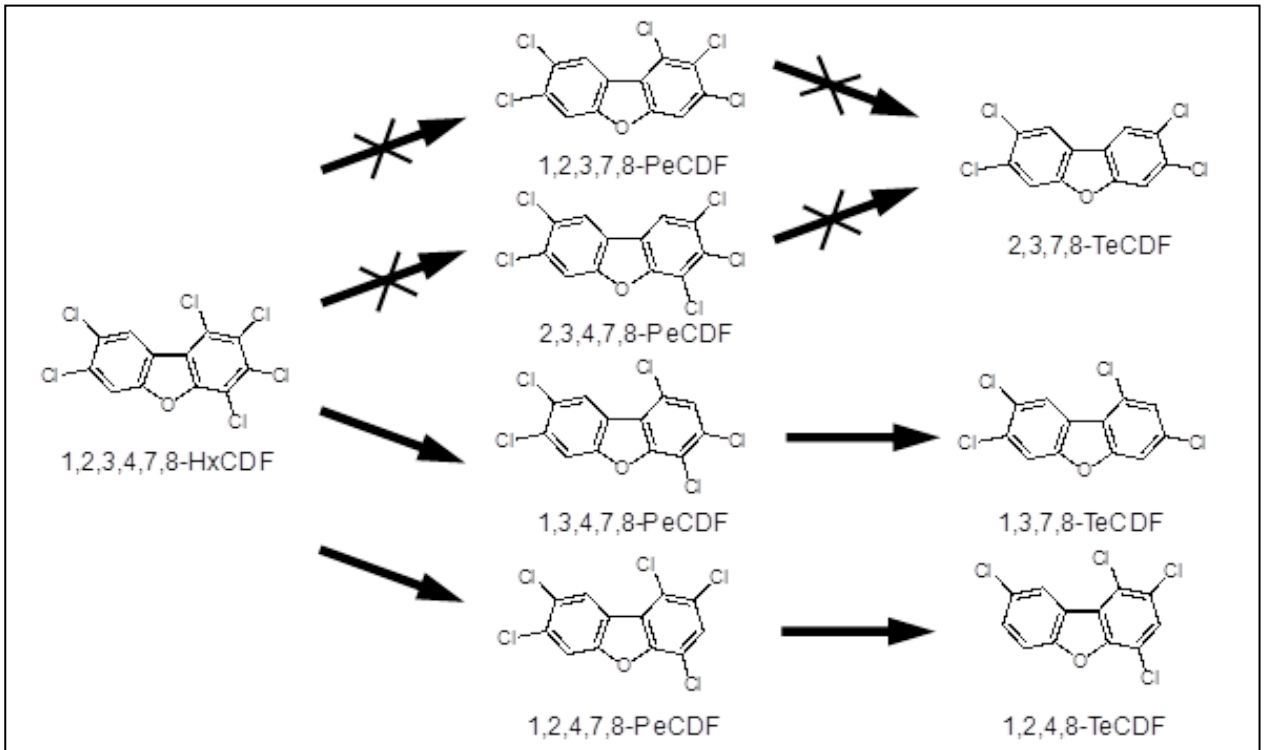


Figure 7-1. Pathways of the dechlorination of 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) by a mixed culture containing *Dehalococcoides ethenogenes* strain 195.

that 1,3,7,8-TeCDF was the first eluted TeCDF metabolite, which also indirectly confirmed the formation of 1,3,4,7,8-PeCDF from the dechlorination of 1,2,3,4,7,8-HxCDF. The 1,3,7,8-TeCDF was produced through removal of a chlorine from a flanked peri position of 1,3,4,7,8-PeCDF. This dechlorination pattern, first removal from a lateral position, followed by a removal from a peri position, was consistent with the dechlorination pattern of 1,2,3,4-TeCDD reported previously for *D. ethenogenes* strain 195 (Fennell et al., 2004).

We further narrowed the number of TeCDFs to be examined using the retention indices of PCDFs developed by Hale et al. (1985). The order of elution of the TeCDF congeners tested in our study and that of Hale et al. (1985) were almost identical. Through a process of elimination using results from the PeCDF-spiked cultures, expected elution order, theoretically possible dechlorination products, and finally, comparison of the metabolite retention time to that of known standards, the second eluted TeCDF metabolite was identified as 1,2,4,8-TeCDF (**Table 7-1**), a metabolite from the dechlorination of 1,2,4,7,8-PeCDF. The formation of 1,2,4,7,8-PeCDF was a result of a chlorine removal from a lateral position of 1,2,3,4,7,8-HxCDF, which followed the expected pattern. However, the second dechlorination, 1,2,4,8-TeCDF formation by removal of a chlorine from a lateral position of 1,2,4,7,8-PeCDF, instead of a peri position, was not expected.

In cultures spiked with 1,3,4,7,8-PeCDF, 1,3,7,8-TeCDF was formed as a dechlorination product in both sets of experiments. The cultures spiked with 1,2,4,7,8-PeCDF formed a trace of 1,2,4,8-TeCDF in the second experiment only, also confirming the second dechlorination route of 1,2,3,4,7,8-HxCDF to 1,2,4,7,8-PeCDF to 1,2,4,8-TeCF. The 1,2,4,7-TeCDF, which had a retention time identical to 1,3,7,8-TeCDF was not detected as a metabolite from 1,2,4,7,8-PeCDF, excluding it as a TeCDF daughter product in the 1,2,3,4,7,8-HxCDF spiked cultures. We detected no dechlorination products from OCDD in any active treatments or in the killed controls over 195 days. In all experiments where they were added, PCE was dechlorinated to primarily ethene and 1,2,3,4-TeCB was dechlorinated to a mixture of tri- and dichlorobenzene (data not shown).

Dechlorination and Effects of Additional Substrates. Dechlorination of 1,2,3,4,7,8-HxCDF occurred in all live treatments, regardless of amendments of additional substrates (**Fig. 7-2**). No dechlorination daughter products were detected in killed controls (data not shown). However, the extent of dechlorination varied in different treatments. At day 38, a PeCDF peak was observed in all live treatments spiked with 1,2,3,4,7,8-HxCDF. At day 70, in 1,2,3,4-TeCB-amended cultures, the two tetrachlorodibenzofuran (TeCDF) peaks were observed. At the end of incubation, the treatment amended with 1,2,3,4-TeCB contained (**Fig. 7-2C**): 57.6 ± 1.5 mol % parent compound 1,2,3,4,7,8-HxCDF; 32.2 ± 0.7 mol % PeCDF intermediates; and 5.8 ± 0.4 mol % 1,3,7,8-TeCDF and 4.4 ± 0.4 mol % 1,2,4,8-TeCDF, the final products. While dechlorination occurred in the other two active treatments, their dechlorination proceeded less extensively than the treatment with 1,2,3,4-TeCB addition. In the bottles spiked only with 1,2,3,4,7,8-HxCDF (**Fig. 7-2A**), the mole % of PCDF congeners at day 195 were 87.2 ± 0.7%, 12.1 ± 0.4%, and 0.3 ± 0.2% and 0.3 ± 0.1%, for 1,2,3,4,7,8-HxCDF, the PeCDF congeners and the two TeCDF congeners, respectively. Similarly, the final mole % of PCDFs in the treatment amended with PCE (**Fig. 7-2B**) were 84.1 ± 4.4%, 15.5 ± 4.2%, 0.3 ± 0.1%, and 0.2 ± 0.0% for the parent compound, intermediates and final products.

In cultures spiked with 1,3,4,7,8-PeCDF and 1,2,3,4-TeCB, 2.9 ± 2.8 mol % 1,3,7,8-TeCDF was formed after 2 months, and in the second experiment, 8.3 mol % 1,3,7,8-TeCDF was formed, in one replicate only, after 3 months. The cultures spiked with 1,2,4,7,8-PeCDF and 1,2,3,4-TeCB formed approximately 1 mol % 1,2,4,8-TeCDF in the second experiment only after 3 months.

Discussion

The 1,2,3,4-TeCB stimulated the most extensive dechlorination of 1,2,3,4,7,8-HxCDF to 1,2,4,7,8-/1,3,4,7,8-PeCDF with formation of TeCDF products. In the other two treatments, dechlorination of 1,2,3,4,7,8-HxCDF to PeCDF(s) was less extensive and the formation of TeCDFs was negligible. Although both PCE and 1,2,3,4-TeCB are growth substrates for *D. ethenogenes* strain 195 (Fennell et al. 2004; Maymo-Gatell et al. 1997), 1,2,3,4-TeCB stimulated more dechlorination of 1,2,3,4,7,8-HxCDF than PCE, even though it was amended less often than PCE and presumably supported less overall growth. This could mean that the 1,2,3,4-TeCB may have a greater role than just as a growth substrate. For example, it may induce expression of a dehalogenase with a higher affinity for PCDFs. The complete genome sequence of *D. ethenogenes* strain 195 has 17 coding sequences that encode putative reductive dehalogenases (Seshadri et al.

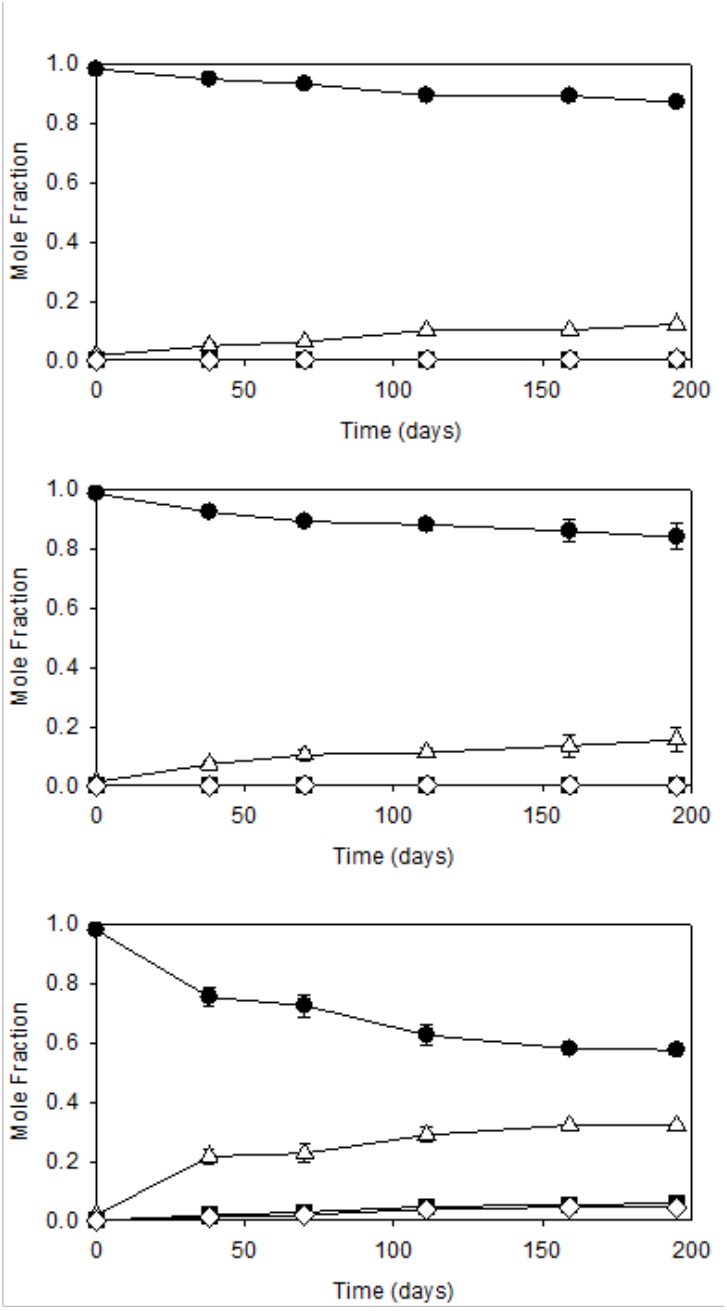


Figure 7-2. Dechlorination of 1,2,3,4,7,8-HxCDF in a mixed culture containing *Dehalococcoides ethenogenes* strain 195 (A) when added as sole halogenated substrate; (B) when added with PCE as an additional substrate; and (C) when added with 1,2,3,4-TeCB as an additional substrate. (●) 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF); (△) 1,3,4,7,8-pentachlorodibenzofuran (1,3,4,7,8-PeCDF)/ 1,2,4,7,8-pentachlorodibenzofuran (1,2,4,7,8-PeCDF); (■) 1,3,7,8-tetrachlorodibenzofuran (1,3,7,8-TeCDF); and (◇) 1,2,4,8-tetrachlorodibenzofuran. Symbols are averages of triplicates and error bars are one standard deviation.

2005), implying a diverse dehalogenation ability. A broad spectrum of dechlorination processes for strain 195 has been confirmed experimentally (Fennell; et al. 2004, Maymo-Gatell et al., 1997, 1999; Adrian et al., 2007, he et al., 2006).

The 1,2,3,4-TeCB has been shown to enhance dechlorination of PCDD/Fs in sediments (Ahn et al., 2005) and could perhaps induce a reductive dehalogenase which is able to dechlorinate both 1,2,3,4-TeCB and 1,2,3,4,7,8-HxCDF. In contrast, while the dechlorination of PCE supplies energy for the growth of strain 195, it may not induce the enzyme instrumental in the dechlorination of 1,2,3,4,7,8-HxCDF. It is also possible that PCE suppressed the enzyme needed for dechlorination of 1,2,3,4,7,8-HxCDF, or that PCE competed as a substrate for enzyme or reducing equivalents since it was added more frequently than 1,2,3,4-TeCB, albeit with an electron donor. Two membrane-bound reductive dehalogenases (RDs) were responsible for the dechlorination of PCE to ethene (Magnuson et al. 1998), PCE–RD (PceA) and TCE–RD (TceA). Rahm et al. (2006) found that genes encoding four reductive dehalogenases, *tceA*, DET0162, DET0318 (*pceA*), and DET1559, in strain 195 had high expression in the mixed culture containing strain 195 (the original source of the culture we used) fed with PCE and butyrate. Fung et al. (2007) observed that when strain 195 was grown with 2,3-dichlorophenol, the genes *pceA* and DET0162 had high transcript levels and proteomic analysis detected PceA with high peptide coverage. Since we do not know which enzyme(s) mediates PCDD/F dechlorination it is not possible to understand the importance of additional halogenated substrates on regulation of 1,2,3,4,7,8-HxCDF dechlorination. However, the amendment with 1,2,3,4-TeCB enhanced the extent of dechlorination of 1,2,3,4,7,8-HxCDF approximately three-fold, compared to when 1,2,3,4,7,8-HxCDF was added alone, which may further support the importance of alternate halogenated electron acceptors in the biotransformation of PCDD/Fs (Ahn et al., 2005).

OCDD was not dechlorinated after 195 days. The extremely low aqueous solubility and high hydrophobicity, or the planar structure with eight bulky chlorine substituents might hinder OCDD dechlorination. Similarly, strain 195 did not debrominate deca-BDE (He et al., 2006).

Environmental Relevance. The dechlorination of 1,2,3,4,7,8-HxCDF, an environmentally relevant PCDF congener was demonstrated by a mixed culture containing *D. ethenogenes* strain 195. We confirmed that one route of dechlorination of 1,2,3,4,7,8-HxCDF was through 1,3,4,7,8-PeCDF to 1,3,7,8-TeCDF and the other route was through 1,2,4,7,8-PeCDF to 1,2,4,8-TeCDF. Production of 2,3,7,8-substituted PCDF congeners was excluded, thus this reaction resulted in detoxification. *D. ethenogenes* strain 195 was previously shown to dechlorinate 1,2,3,4-TeCDF to a trichlorodibenzofuran which was not identified because of lack of standards for trichlorinated congeners (Fennell et al. 2004). The lack of dechlorination of OCDD is also significant. OCDD has a low TEF (0.0003) but it is often the dominant congener of the seventeen 2,3,7,8-substituted PCDD/Fs (Czuczwa & Hites 1984; Brzuzy & Hites 1996; Wagrowski & Hites 2000; Bopp et al. 1991; Hites 1990). OCDD dechlorination to multiple less chlorinated congeners was reported for microcosms of Passaic River, NJ sediments (Barkovskii & Adriaens 1996).

Dechlorination does not always achieve the goal of detoxification. In early studies on microbial dechlorination of PCE and TCE, the production of VC, a potent human carcinogen was

recognized as a potential limitation for remediation. In a critical study, Freedman and Gossett demonstrated dechlorination completely to ethene, a benign product (Freedman & Gossett 1989). Dehalogenation resulting in increased system toxicity was also observed for PBDEs when an octa-BDE mixture was dechlorinated to more toxic hexa-, penta- and tetra-BDEs by *Dehalococcoides*-containing cultures (He et al., 2006). Similarly, Adriaens & Grbic-Galic (1994) found that 1,2,3,4,6,7,8-HeptaCDF was dechlorinated to 1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF by a peri chlorine removal in aquifer microcosms. This resulted in increased toxicity because the produced HxCDFs both have TEFs of 0.1, ten-fold higher than that of the parent compound. *Dehalococcoides* sp. strain CBDB1 dechlorinated 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD) with a TEF of 1, slowly (2.8 mol % within 104 days) to mainly 2,3,7,8-TeCDD, also with a TEF of 1, and small amounts of 1,3,7,8-TeCDD (Bunge et al., 2003). Strain CBDB1 further detoxified the 2,3,7,8-TeCDD intermediate, forming 2,3,7-TrCDD, 2,7-DiCDD and/or 2,8-DiCDD (Bunge et al., 2003). This pattern of dechlorination was similar to what had been demonstrated by strain CBDB1 with 1,2,3,4-TeCDD. A pure culture of *D. ethenogenes* strain 195 did not dechlorinate 2,3,7,8-TeCDD after 249 days (Fennell et al. 2004).

Considering the generally slow rates of dechlorination of PCDD/Fs by dechlorinating bacteria (Adriaens et al. 1995; Adriaens & Grbic-Galic 1994; Barkovskii & Adriaens 1996; Beurskens et al., 1995; Ballerstedt et al., 1997; Bunge et al., 2003; Fennell et al. 2004; this study*),* transient intermediates might exist in the environment for years and have great environmental impact. The formation of equally or more toxic intermediates will not reduce the threat to the environment and biota. The TEF values of the seventeen 2,3,7,8-substituted PCDD/Fs normally increase with decreasing number of chlorines on the carbon backbone. Dechlorination from a peri position mostly increases the toxicity in the system. On the contrary, dechlorination from a lateral position greatly reduces the toxicity of the 2,3,7,8-substituted congeners. The TEF of 1,2,3,4,7,8-HxCDF is 0.1, while the TEFs of potential 2,3,7,8-substituted products 2,3,4,7,8-PeCDF, 1,2,3,7,8-PeCDF and 2,3,7,8-TeCDF are 0.3, 0.03 and 0.1, respectively. Dechlorination of 1,2,3,4,7,8-HxCDF to 2,3,4,7,8-PeCDF would be a detrimental rather than favorable process and the system TEQ would increase. If 1,2,3,7,8-PeCDF or 2,3,7,8-TeCDF, were formed from dechlorination, the TEQ would decrease or remain the same. We have demonstrated detoxification during the dechlorination of an environmentally relevant 2,3,7,8-substituted PCDF congener by a mixed culture containing *D. ethenogenes* strain 195, and an additional halogenated substrate, 1,2,3,4-TeCB, enhanced this process. Here, TEQ in 1,2,3,4,7,8-HxCDF spiked systems decreased from 187 µg/L at time 0 to 108 µg/L, 157 µg/L, and 163 µg/L in the treatments amended with 1,2,3,4-TeCB, PCE, and no additional halogenated substrate, respectively. *Dehalococcoides* spp. and closely related *Chloroflexi* have been implicated in PCDD/F dechlorination in environmental samples (Yoshida et al., 2005; Ahn et al., 2007). Obtaining more information about the bacteria and dehalogenases involved in PCDD/F dechlorination could enable advances in using this process for bioremediation.

8. Carbon Isotope Fractionation during Dechlorination of 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin by a *Dehalococcoides* - Containing Culture

(Liu F, Cichocka D, Nijenhuis I, Richnow H.H, Fennell DE. 2010. Carbon isotope fractionation during dechlorination of 1,2,3,4-tetrachlorodibenzo-p-dioxin by a Dehalococcoides-containing culture. Chemosphere 80, 1113–1119)

Abstract

Carbon isotope fractionation was observed during dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) by a mixed culture containing *Dehalococcoides ethenogenes* strain 195. Fractionation was examined when 1,2,3,4-TeCDD was added as the only chlorinated compound and when 1,2,3,4-TeCDD was added with a known growth substrate, tetrachloroethene (PCE). The 1,2,3,4-TeCDD was dechlorinated to 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) which was enriched in ^{13}C relative to 1,2,3,4-TeCDD with isotope separation factors, ε_C, of 1.3 ± 0.2‰ and 1.7 ± 0.4‰ (average ± 95% confidence interval (CI)) in cultures with and without PCE, respectively. The 1,2,4-TrCDD was further dechlorinated to 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) which was depleted in ^{13}C relative to 1,2,4-TrCDD with ε_C of -2.4 ± 0.4‰ and -2.9 ± 0.8‰ (average ± 95% CI) in cultures with and without PCE, respectively. This demonstrates carbon isotope fractionation during sequential reductive dechlorination of PCDDs, where isotope fractionation during dechlorination of the intermediate was substantial and a ^{13}C depleted lightly chlorinated PCDD congener was ultimately formed during dechlorination of more highly chlorinated PCDD congeners. Despite reproducible, statistically significant differences between isotope compositions of the parent, 1,2,3,4-TeCDD and daughter, 1,2,4-TrCDD and 1,3-DCDD congeners in triplicate bottles of both treatments, fractionation factors for 1,2,3,4-TeCDD could not be determined for all replicates by regression analysis of the plot of the Rayleigh equation. It is possible that dissolution of 1,2,3,4-TeCDD imposed a kinetic limitation on dechlorination, thus masking isotope fractionation during its dechlorination.

Introduction

A promising tool for monitoring biotransformation of pollutants is compound specific isotope analysis (CSIA) (Schmidt et al., 2004, Meckenstock et al., 2004). CSIA detects fractionation resulting from the kinetic isotope effect (KIE) caused by different reaction rates for a compound containing the heavy isotope versus the light isotope at the location of the reaction, and from other environmental processes (Schmidt et al., 2004). CSIA was examined for fuel components (Meckenstock, 1999; Hunkeler et al., 2001; Gray et al., 2002; Somsamak et al., 2005); aromatic hydrocarbons (Richnow et al., 2003; Yanik et al., 2003; Hall et al., 1999); and halogenated compounds (Hunkeler et al., 1999; Bloom et al., 2000; Sherwood Lollar et al., 2001; Drenzek et al., 2001; Nijenhuis et al., 2005; Nijenhuis, et al., 2007, Cichocka et al., 2008). CSIA could be useful for monitoring fate of the polychlorinated dibenzo-*p*-dioxins (PCDDs). PCDDs occur as mixtures of up to 75 congeners, are hydrophobic and accumulate in sediments and biota (Czuczwa et al., 1984; Hites 1990; Bopp et al., 1991; Cai et al., 1994; Koistinen et al., 1995; Wagrowski et al, 2000). The 2,3,7,8-substituted PCDDs are highly toxic (Van den Berg et al., 2006) and environmental problems are global. For example, 500 ppb PCDDs were detected in the River Elbe, Germany (Götz et al., 2007); 50 ppb in the Gulf of Finland (Verta et al., 2007);

20 to 40 ppb in the Passaic River, NJ, USA (Bopp et al., 1991); and up to 90 ppb in Newark Bay, NJ, USA (Chaky, 2003). Freshly added (Adriaens et al., 1995; Beurskens et al., 1995; Ballerstedt et al., 1997; Vargas et al., 2001; Ahn et al., 2005) and existing PCDDs (Albrecht et al., 1999; Yoshida et al., 2005) were dechlorinated under anaerobic conditions. However, with few exceptions (Lohmann et al., 2000; Bunge et al., 2007), lightly chlorinated PCDDs are not routinely measured in environmental samples, thus there is little historical data documenting environmental PCDD dechlorination. CSIA could be used to compare isotopic signatures between PCDD parent and daughter congeners or between homolog groups as a marker for biodechlorination. Ewald et al. (2007) reported carbon stable isotope fractionation during dechlorination of trichlorodibenzo-*p*-dioxins (TrCDD) by a culture containing bacteria with high similarity to *Dehalococcoides* sp. strain CBDB1 (Bunge et al, 2003). It is important to document CSIA with different PCDD congeners and environmental conditions. Here, we examined carbon stable isotope fractionation of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) by a PCE-grown mixed culture containing *Dehalococcoides ethenogenes* strain 195 (Maymó-Gatell et al., 1997). *S*train 195 grows on PCE (Maymó-Gatell et al., 1997) and the mixed culture containing strain 195 dechlorinates selected PCDD/Fs — 1,2,3,4-TeCDD, 1,2,3,4-tetrachlorodibenzofuran and 1,2,3,4,7,8-hexachlorodibenzofuran (Fennell et al., 2004; Liu and Fennell, 2008). It is not known if strain 195 obtains energy for growth on PCDD/Fs or which of its reductive dehalogenase(s) (Seshadri et al., 2005) mediates their dechlorination.

We used 1,2,3,4-TeCDD as a model PCDD, because it is dechlorinated at relatively high rates (Fennell et al., 2004), has low toxicity (Van den Berg et al., 2006) and could be added at high concentrations to enhance detection of fractionation. This congener also has similar physical-chemical properties to 2,3,7,8-TeCDD, one of the most toxic PCDD congeners. The 1,2,3,4-TeCDD is dechlorinated to 1,2,4-TrCDD and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) by strain 195 (Fennell et al., 2004). We found that PCDD dechlorination was most rapid when PCDD was loaded on sediment (Fennell et al., 2004), thus 1,2,3,4-TeCDD was loaded on sediment carrier for all experiments. This approach mimicked realistic conditions since PCDDs are likely sorbed to sediment particles in the environment (Shiu et al., 1988; Hites, 1990).

Materials and Methods

Chemicals. 1,2,3,4-TeCDD, 1,2,4-TrCDD, 1,3-DCDD, and 2,2',5-trichlorobiphenyl (2,2',5-TrCB) were from AccuStandard (New Haven, CT). PCE (99.9+%), trichloroethene (TCE) (99.5+%), *cis*-1,2-dichloroethene (*cis*-1,2-DCE) and butyric acid (99+%) were from Aldrich Chemical Company (Milwaukee, WI, USA). Vinyl chloride (VC) (≥99.97%) was from Linde AG (Leuna, Germany). Ethene (99%) was from Matheson Tri-Gas, Inc. (Montgomeryville, PA, USA).

Experimental Setup. Carbon stable isotope fractionation of 1,2,3,4-TeCDD and PCE was investigated in a mixed culture containing *D. ethenogenes* strain 195. Culture was pregrown on PCE and butyric acid at 34°C for PCE studies or at 25°C for PCDD studies (Fennell et al., 1997). In the 1,2,3,4-TeCDD study, 1,2,3,4-TeCDD was present both with and without the addition of PCE as additional halogenated compound, because we do not know if 1,2,3,4-TeCDD is a growth substrate for strain 195. In the PCE study, PCE was added as the only halogenated

substrate. Isotope fractionation of PCE was analyzed only in cultures where PCE was added as the only halogenated substrate.

In 1,2,3,4-TeCDD experiments one g sterile sediment (Arthur Kill, NJ, USA; 5.4% total carbon) was added to 160-mL serum bottles (Vargas et al., 2001; Fennell et al., 2004); was wetted by addition of 0.5 mL of 6200 μM 1,2,3,4-TeCDD-toluene solution; and toluene was volatilized under sterile N_2, leaving a coating of 1,2,3,4-TeCDD on the sediment. Next, 100 mL culture was added under anoxic, sterile conditions to achieve a final concentration of 31 μM 1,2,3,4-TeCDD on a bulk volume basis. We routinely recovered only about 6 μM 1,2,3,4-TeCDD, because two mL of the mixed slurry was removed at each sampling from a total 100 mL slurry, resulting in incomplete sampling of the PCDDs sorbed to the bottle surfaces. [Note that complete extraction of 10 mL sediment-culture slurries from 28 mL glass tubes recovered 70 % of PCDDs using the extraction method described here (Liu, 2007).] The sediment to culture ratio was 10 g/L. Assuming a maximum aqueous-phase solubility of 1.95 nM 1,2,3,4 TeCDD (Govers and Krop, 1998), the aqueous-phase 1,2,3,4-TeCDD would account for 0.0063% of added 1,2,3,4-TeCDD, with greater than 99.99% sorbed to sediment, colloid matter, and the vessel.

One set of triplicate cultures received 1,2,3,4-TeCDD as the only chlorinated substrate. A second set of triplicate cultures was additionally amended with 110 μM PCE on days 0, 32, 51, 81, 111, and 132, because we do not know if 1,2,3,4-TeCDD supports growth of strain 195 and we were unsure of the ability of the culture to dechlorinate 1,2,3,4-TeCDD in the absence of a known growth substrate. Cultures received 440 μM butyrate as a hydrogen source and 40 μL of 50 g/L yeast extract, at the same time PCE was added. A third set of cultures with 1,2,3,4-TeCDD only was autoclaved at 121°C as killed controls. Incubation was in the dark at 28°C at 120 rpm. PCDDs were analyzed on days 0, 7, 27, 39, 55, 77, 98, 120 and 153.

The PCE study was performed in 250 mL serum bottles, containing 100 mL medium inoculated with 10% volume:volume (vol:vol) culture after growth on 500 μM PCE. Cultures were amended with 500 μM PCE and 2.75 mM butyrate and incubated at 34°C. On days 0, 1, 4, 8, 11, 14 and 17, 0.5 mL headspace samples were removed from the bottles and added to helium-flushed autosampler vials sealed with Teflon® coated butyl rubber septa for determination of chloroethenes and ethene concentrations. Simultaneously, three 0.5 mL headspace samples were removed sequentially from each bottle for CSIA.

Analytical Methods. Two mL of culture-sediment mixture was removed from each bottle for PCDD analysis and placed into 7 mL glass vials. Samples were centrifuged, the aqueous portion was removed to a separate vial, 2,2',5-TrCB was added as a surrogate standard to the sediment residue, and the sediment phase was rinsed with 1 mL of acetone to remove water. The sediment phase was extracted overnight with 3 mL of 2:1 vol:vol toluene:acetone, then for 4 h with 1 mL of 2:1 vol:vol toluene:acetone, and then rinsed with 1 mL toluene. The solvent phases were combined with the aqueous phase and back extracted by NaCl. The solvent phase was passed through a 2 mL pipette filled with Florisil (Sigma-Aldrich, St. Louis, MO, USA) and eluted with three volumes toluene per volume extract. The extract was concentrated to 2 mL. PCDDs were analyzed using an Agilent 6890 GC equipped with a 5973N mass selective detector (GC-MS) (Agilent Technologies, Inc. Santa Clara, CA) and a HP-5MS (Agilent Technologies, Inc. Santa Clara, CA, USA) column (30 m × 0.25 mm I.D.). PCDDs were detected based on retention times

of standards and identification of molecular ions (*m/z*: 1,2,3,4-TeCDD, 322; 1,2,4,-TrCDD, 286; 1,3-DCDD, 252; and 2,2',5-TrCB, 256). A qualifying ion was monitored to assure correct identification of congeners (1,2,3,4-TeCDD, 320; 1,2,4-TrCDD, 288; 1,3-DCDD, 254; and 2,2',5-TrCB, 186). The response factors for PCDD congeners compared to the surrogate, 2,2',5-TrCB, were calculated over four concentrations from 0.5 to 10 µM as a linear calibration curve. Ethene and chloroethenes were quantified using a Varian Chrompack CP-3800 gas chromatograph (GC) (Middelburg, the Netherlands) with flame ionization detection (FID) using a 30 m x 0.53 mm GS-Q column (J&W Scientific, Waldbronn, Germany) (Cichocka et al., 2008).

Isotope compositions of PCDDs and chloroethenes was by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) using an Agilent 6890 GC (Agilent Technologies, Inc. Santa Clara, CA) connected to a Finnigan MAT 252 mass spectrometer via a Finnigan GC-C/TC III Interface (Richnow et al., 2003; Nijenhuis et al., 2007; Cichocka et al., 2008). PCDDs were separated on a ZB-5 column (60 m × 0.32 mm I.D. with 0.25 µm thickness) (Phenomenex, Torrance, CA, USA). The temperature program was: 100°C for 1 min; increased at 15°C/min to 220°C; held for 5 min; increased at 6°C/min to 320°C; and held for 10 min. The injector was 280°C, operated in a pulsed splitless mode with a 0.8 min splitless time, and a 10 mL/min purge flow. The injection pulse pressure was 3.10 x10^5 Pa and the pulse time was 1 min. Helium was the carrier gas at 1.5 mL/min. Each sample extract was analyzed at least three times and isotope compositions obtained were averaged. Each CSIA data point for individual replicates represents an average of three injections plus or minus one standard deviation (1σ). For two replicates of the bottle set amended with 1,2,3,4-TeCDD plus PCE, we added an internal standard, 1,2,4,7,8-PeCDD at 10 to 70 µM to extracts prior to CSIA. Each replicate bottle was sampled at nine time points. Samples obtained at each time point were measured three times and the average carbon isotope ratio was reported.

Chlorinated ethenes and ethene were analyzed as described previously (Cichocka et al., 2008). Each headspace was sampled and analyzed at least three times and the average and one standard deviation (1σ) were reported.

Carbon Stable Isotope Calculations. Isotope compositions are reported in standard δ notation in parts per thousand (‰):

$$\delta^{13}C(‰) = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000 \qquad \text{(Equation 1)}$$

where R_{sample} is the ratio of $^{13}C/^{12}C$ in the sample and $R_{standard}$ is the ratio of $^{13}C/^{12}C$ in the standard, Vienna Peedee belemnite (Coplen et al., 2006).

A $\delta^{13}C$ value for each compound detected in each sample was obtained by averaging the results of triplicate GC-C-IRMS injections. The $\delta^{13}C$ average ± 1σ thus obtained for each time point (PCDD study: days 0, 7, 27, 39, 55, 77, 98, 120 and 153; PCE study: days 0, 1, 4, 8, 11, 14 and 17) were presented individually for each replicate culture of the PCDD study but were averaged to obtain one data set for the PCE study. Analysis of variance (ANOVA) was performed in Microsoft Excel (Microsoft Corp., Redmond, WA, USA) to compare the average $\delta^{13}C$ values of

1,2,3,4-TeCDD and its dechlorination products. The number of data points for the 1,2,3,4-TeCDD treatment without PCE were n=26 for 1,2,3,4-TeCDD, n=20 for 1,2,4-TrCDD and n=13 for 1,3-DCDD and for the 1,2,3,4-TeCDD treatment with PCE were n=27 for 1,2,3,4-TeCDD, n=16 for 1,2,4-TrCDD and n=13 for 1,3-DCDD.

The Rayleigh model was used to correlate changes in concentration to changes in isotope composition (Rayleigh 1896; Mariotti et al., 1981)

$$\ln(\frac{R_t}{R_0}) = (\alpha C - 1)\ln(\frac{C_t}{C_0}) \qquad \text{(Equation 2)}$$

where R_t and R_0 are the isotopic compositions (ratio of $^{13}C/^{12}C$) of the substrate at time t and time 0; C_t and C_0 are the concentrations of ^{12}C in the substrate at time t and time 0; and αC is the carbon isotope fractionation factor. C_t and C_0 were approximated as the total concentration of ^{13}C plus ^{12}C in the substrate at time t and time 0. $\text{Ln}(R_t/R_0)$ was plotted against $\ln(C_t/C_0)$ and the slope (αC-1) was determined by least-squares regression.

The isotope fractionation was reported as an isotope enrichment factor, εC,

$$\varepsilon C = (\alpha C - 1) \times 1000 \qquad \text{(Equation 3)}$$

Regression of the plot of the Rayleigh equation was performed in SigmaPlot (Systat Software, Inc., San Jose, CA, USA). A 95% confidence interval (95% CI; 1.96∗standard error) was reported for εC. The significance of the linear relationship was evaluated by ANOVA.

An intrinsic isotope enrichment factor $\varepsilon C_{intrinsic}$ normalizes the observed isotope fractionation of a molecule to the kinetic isotope effect of bond cleavage by subtracting dilution of non-reactive positions *[Supporting Information]* and was calculated as (Elsner et al., 2005; Morasch et al., 2004)

$$\varepsilon C_{intrinsic} = \varepsilon C \times n \qquad \text{(Equation 4)}$$

where n is the number of the atoms of interest, i.e., n=12 for PCDDs and 2 for chloroethenes (Elsner et al., 2005).

An estimate of the isotope separation factor (Whiticar, 1999) was approximated on a point by point basis from the time progression data as:

$$\varepsilon_C \approx \delta_p - \delta_s \qquad \text{(Equation 5)}$$

where the subscripts p and s denote product and substrate with respect to a specific reaction and applies for low fractionation and low transformation rate (Hunkler et al., 1999; Ewald et al., 2007; Elsner et al., 2005; Whiticar, 1999) [*Supporting Information*]. The ε_C was calculated for dechlorination of 1,2,3,4-TeCDD to 1,2,4-TrCDD and 1,2,4-TrCDD to 1,3-DCDD for each time point where paired data were available and an average ± 95% CI is reported.

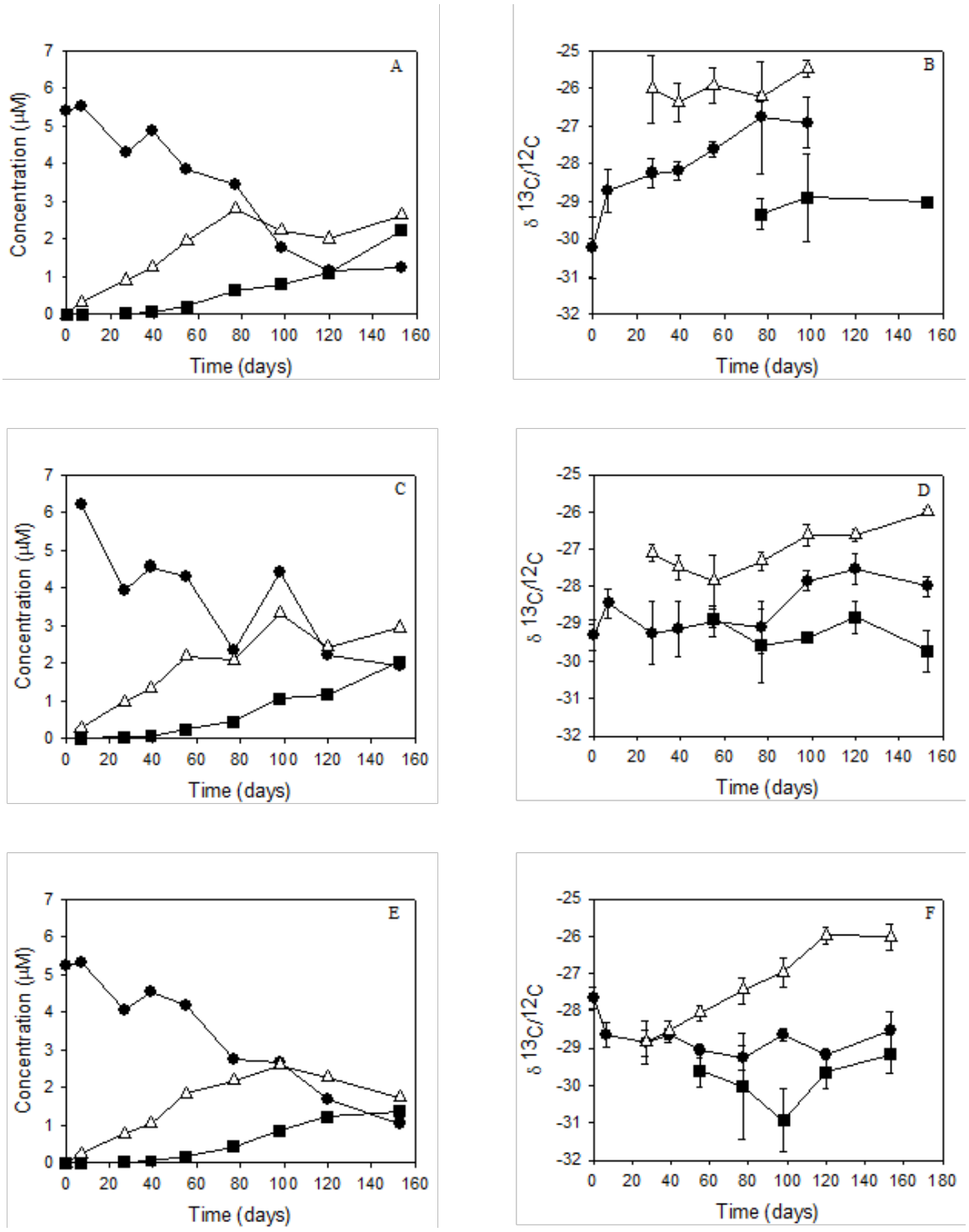


Figure 8-1. Concentrations (A, C, E) and carbon isotope compositions (B, D, F) of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (•)1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) (Δ) and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (■) during reductive dechlorination by a mixed culture containing *Dehalococcoides ethenogenes* strain 195 amended with 1,2,3,4-TeCDD as the sole chlorinated substrate in replicate 1 (A, B), replicate 2 (C, D) and replicate 3 (E, F).

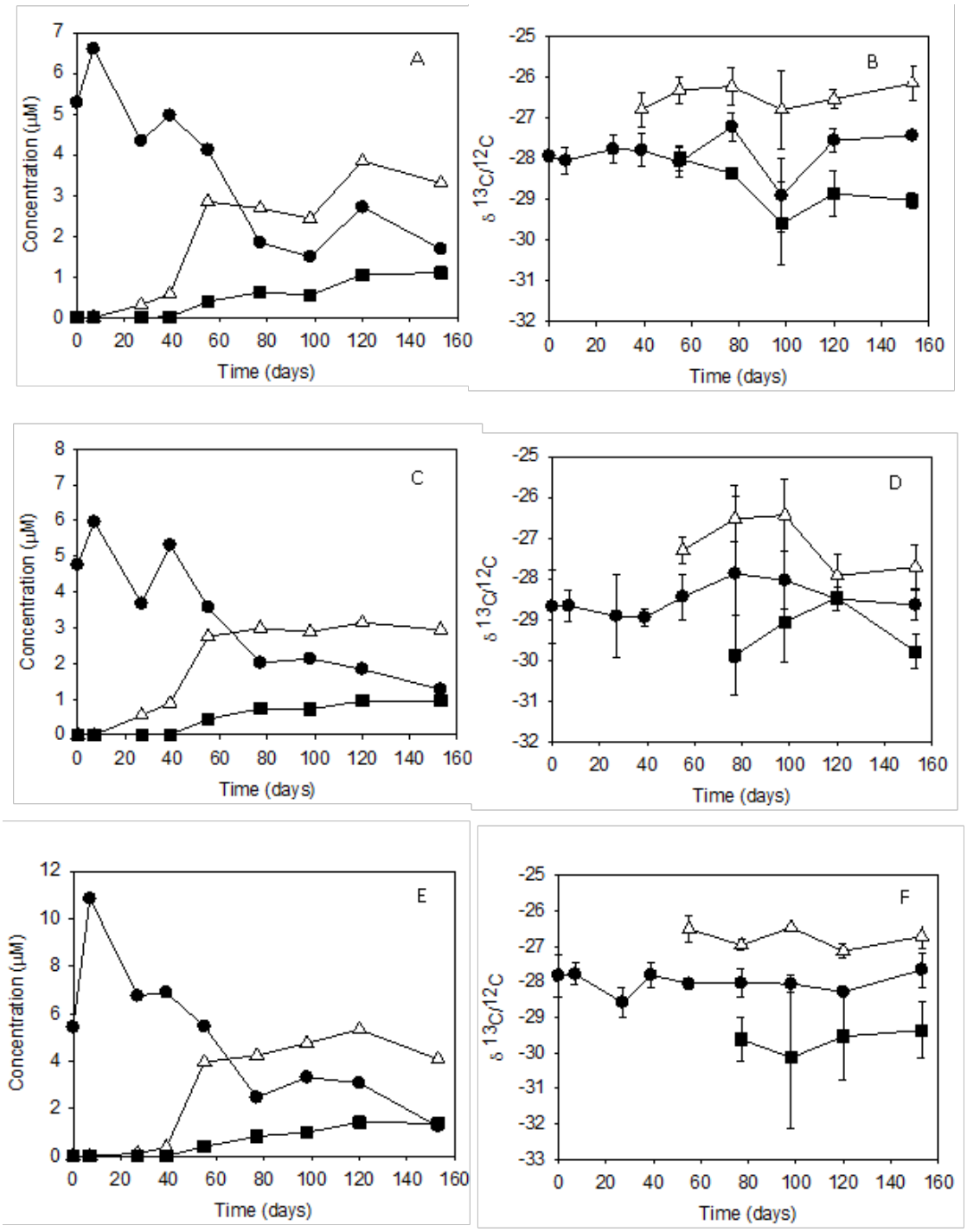


Figure 8-2. Concentrations (A, C, E) and carbon isotope compositions (B, D, F) of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (•) and its products 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) (Δ) and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (■) during reductive dechlorination by a mixed culture containing *Dehalococcoides ethenogenes* strain 195. Shown are replicates 1 (A, B), 2 (C, D) and 3 (E, F) from the treatment with 1,2,3,4-TeCDD plus PCE as an additional chlorinated substrate.

Results and Discussion

Dechlorination and Fractionation. The 1,2,3,4-TeCDD was dechlorinated in treatments with and without added PCE. Dechlorination of 1,2,3,4-TeCDD in triplicates without PCE is shown in **Fig 8-1A, C and D**. Dechlorination of 1,2,3,4-TeCDD in triplicates with PCE is shown in **Figure 8-2**. By day 153 in bottles without PCE, total PCDDs consisted of 1,2,3,4-TeCDD, 1,2,4-TrCDD and 1,3-DCDD at 24 ± 3.8, 42 ± 0.6 and 33 ± 3.4 mol% (average ± 1σ), respectively. In the bottles with PCE, total PCDDs consisted of 1,2,3,4-TeCDD, 1,2,4-TrCDD and 1,3-DCDD at 23 ± 4.5, 57 ± 3.2 and 19 ± 1.3 mol% (average ± 1σ), respectively. Thus cultures with and without PCE exhibited different rates and extents of product formation with greater 1,3-diCDD formation in the cultures amended with 1,2,3,4-TeCDD alone. A lag in 1,2,4-TrCDD production was observed when PCE was added as an additional chlorinated substrate (**Figure 8-2A, C, and E**). These differences may have been caused by the periodic addition of the competing electron acceptor, PCE. In cultures amended with 1,2,3,4-TeCDD and PCE, PCE was dechlorinated to VC and ethene within a few days (data not shown). In killed controls 1,2,4-TrCDD and 1,3-DCDD were detected in trace amounts only at the final time point (**Figure 8-2**).

Figures 8-1B, D and F show isotope compositions of PCDDs for triplicate culture bottles without PCE amendment. Data for triplicates with PCE are shown in **Figure 8-2**. **Table 8-1** shows averages ± 1σ of $\delta^{13}C$ values of PCDDs. The 1,2,4-TrCDD was enriched in ^{13}C compared to 1,2,3,4-TeCDD. The 1,3-DCDD was depleted in ^{13}C relative to both 1,2,3,4-TeCDD and the intermediate, 1,2,4-TrCDD. This pattern was observed in all replicates of both treatments (**Fig. 8-1 and 8-2**). The carbon isotopic compositions (averages ± 1σ) of total PCDDs in the system varied from -28.6 ± 0‰ to -27.5 ± 0.8‰

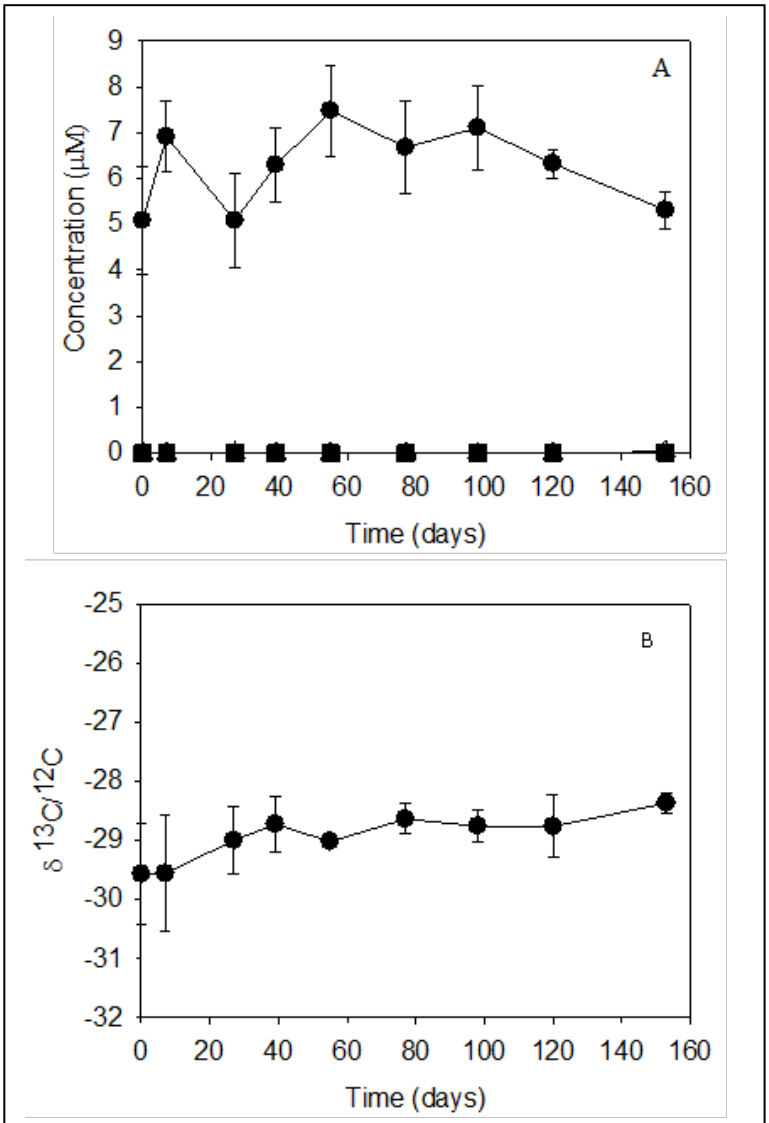


Figure 8-3. Average concentrations (A) of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (•) and its products 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) (Δ) and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (■) and carbon isotope composition (B) of 1,2,3,4-TeCDD in a killed (autoclaved) control.

Table 8-1. Carbon stable isotope compositions (average ± 1 standard deviation) of polychlorinated dibenzo-*p*-dioxins (PCDDs), isotope balance, and p value from analysis of variance (ANOVA) of the isotope compositions of the individual PCDD congeners.

Experimental Condition →		**1,2,3,4-TeCDD**	**1,2,3,4-TeCD plus PCE**
1,2,3,4-TeCDD	Average	-28.5 ± 0.8	-28.1 ± 0.5
1,2,4-TrCDD	Average	-26.8 ± 1.0	-26.8 ± 0.5
1,3-DCDD	Average	-29.5 ± 0.6	-29.2 ± 0.6
Isotope balance		-27.7 ± 0.7	-27.5 ± 0.4
p value		2.57E-12	1.68E-16

for the treatment amended only with 1,2,3,4-TeCDD and -28.2 ± 0.5‰ to -27.3 ± 0.4‰ for the treatment amended with 1,2,3,4-TeCDD plus PCE (**Fig. 8-4**). The *p* values from ANOVA of $\delta^{13}C$ values observed for each congener were < 0.01 indicating significant differences between isotope compositions of the parent, 1,2,3,4-TeCDD and daughter, 1,2,4-TrCDD and 1,3-DCDD congeners. The isotope ratio of 1,2,3,4-TeCDD in killed controls was -28.9 ± 0.4‰ (average ± 1σ) (**Fig. 8-3**).

The isotope separation factors, ε_C, for PCDDs was determined (equation 5). For dechlorination of 1,2,3,4-TeCDD to 1,2,4-TrCDD, the ε_C (average ± 95% CI) (equation 5) were 1.3 ± 0.2‰ and 1.7 ± 0.4‰ for treatments with and without PCE, respectively. In the second dechlorination step of 1,2,4-TrCDD to 1,3-DCDD the ε_C (average ± 95% CI) were -2.4 ± 0.4‰ and -2.8 ± 0.5‰ for treatments with and without PCE, respectively. Ewald et al. (2007) reported an isotope discrimination (depletion) between the intermediate 1,3-DCDD and the final product 2-monochlorodibenzo-*p*-dioxin (2-MCDD), expressed using the convention of (equation 5) (Whiticar, 1999), of -2.5 to -3.6‰ in cultures dechlorinating 1,2,4-TrCDD.

The isotope composition of the internal standard, 1,2,4,7,8-PeCDD, analyzed with samples was –28.8 ± 0.4‰ (average ± 1σ, n=18) and the isotope ratio of 1,2,3,4-TeCDD in killed controls was -28.9 ± 0.4‰ (average ± 1σ, n=9) (**Fig. 8-3**). These findings indicate low variability in analytical measurement and support the proposition that the observed isotope discrimination between

1,2,3,4-TeCDD and its daughter products during dechlorination were reflective of isotope fractionation by the biological mechanism.

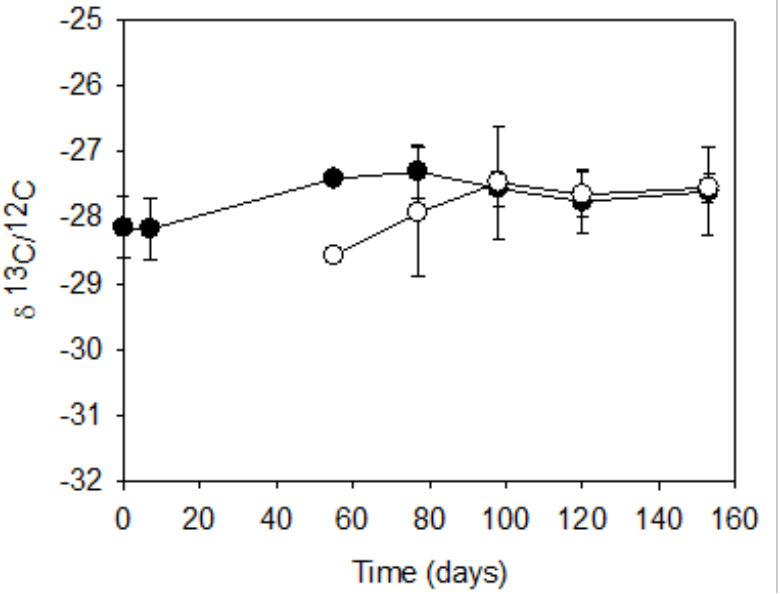


Figure 8-4. Average isotope mass balance of polychlorinated dibenzo-*p*-dioxins in treatments amended with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) alone (○) and 1,2,3,4-TeCDD plus tetrachloroethene (PCE) (●).

Although, there were statistically significant and reproducible differences between isotope compositions of the parent, 1,2,3,4-TeCDD and daughter, 1,2,4-TrCDD and 1,3-DCDD congeners in all replicates of both treatments, regression analysis of the plot of the Rayleigh equation (equation 2) could be used successfully ($R^2 \geq 0.5$ and $p < 0.05$) for only two replicates (**Fig. 8-1B and 8-1D**) amended with 1,2,3,4-TeCDD alone. The εC were -2.9 ± 1.4‰ and -1.1 ± 0.78‰ (average ± 95% CI), respectively. The regression of the plot of the Rayleigh equation for the third replicate (**Fig. 8-1F**) yielded a positive value for εC (data not shown). The isotope regression of the plot of the Rayleigh equation could not be used for cultures amended with 1,2,3,4-TeCDD plus PCE because there was little change in the isotope composition over time (**Fig. 8-2**). This could be related to a lag in 1,2,3,4-TeCDD dechlorination in PCE-amended cultures (**Fig. 8-2A, C and E**) in contrast to cultures that received no PCE (**Fig. 8-1A, C and E**). Liu (2007) observed two-fold lower rates of 1,2,3,4-TeCDD dechlorination when PCE was present as an additional substrate. It is also possible that a different dehalogenase dechlorinates 1,2,3,4-TeCDD when PCE is present, or that competition between PCE and 1,2,3,4-TeCDD for dehalogenase(s) occurs, and these factors could affect the fractionation. Ewald et al. (2007) reported an εC of -0.92 ± 0.18‰ for dechlorination of 1,2,3-TrCDD by a *Dehalococcoides* mixed culture, however, in their study, regression of the plot of the Rayleigh equation could not be used to determine the εC for dechlorination of 1,2,4-TrCDD.

The $\varepsilon C_{intrinsic}$ of 1,2,3-TrCDD dechlorination by a *Dehalococcoides* containing culture (Ewald et al., 2007) was -11.0 ± 2.2‰ whereas $\varepsilon C_{intrinsic}$ for 1,2,3,4-TeCDD dechlorination determined here were -34.8 ± 16.8‰ and -13.3 ± 9.4‰, for replicates. In comparison, the $\varepsilon C_{intrinsic}$ reported for dechlorination of trichlorobenzenes by *Dehalococcoides* sp. strain CBDB1 was approximately -20‰ (Griebler et al., 2004).

The mixed culture containing *D. ethenogenes* strain 195 dechlorinated PCE to VC and ethene via TCE and *cis*-DCE when PCE was added as the only halogenated substrate (data not shown). Carbon isotope fractionation during dechlorination of PCE yielded a εC of -7.1 ± 0.98‰

(average ± 95% CI) for PCE. The $\varepsilon C_{intrinsic}$ of PCE, -14.2 ± 2‰, was similar to that measured for a pure culture of *D. ethenogene*s strain 195, -12 ± 1.4‰ (Cichocka et al., 2008).

Limitations on Quantification of 1,2,3,4-TeCDD Fractionation. The isotope composition data show repeatable, statistically significant isotope discrimination between the substrate and the products of dechlorination and support the occurrence of carbon isotope fractionation during dechlorination of PCDDs. The enrichment of ^{13}C in 1,2,4-TrCDD is unlikely to occur as a result of carbon-chlorine bond cleavage in the step from 1,2,3,4-TeCDD to 1,2,4-TrCDD. Rather a greater isotope fractionation for dechlorination of 1,2,4-TrCDD to 1,3-DCDD than for 1,2,3,4-TeCDD to 1,2,4-TrCDD may explain the enrichment of ^{13}C in 1,2,4-TrCDD. Ewald et al. (2007) reported a similar pattern for 1,2,4- and 1,2,3-TrCDD dechlorination where the first transformation step to DCDDs was thought to be associated with a lower isotope fractionation than the subsequent step to 2-MCDD. Sorption-desorption of PCDDs may be a rate-limiting step during dechlorination of PCDDs (Adriaens et al., 1995) and mask the magnitude of isotope fractionation (Elsner et al., 2005). Kampara et al. (2008) showed that low bioavailability of contaminants can impose kinetic limitations on the biodegradation and could significantly reduce observed kinetic isotope fractionation. Since the aqueous solubility of 1,2,4-TrCDD is ten-fold higher than that of 1,2,3,4-TeCDD (Shiu et al., 1988), and because it was likely produced at the cell (Seshadri et al., 2005), it likely had greater bioavailability than 1,2,3,4-TeCDD. This may have contributed to greater observed fractionation during dechlorination of 1,2,4-TrCDD to 1,3 DCDD and the enrichment of ^{13}C in 1,2,4-TrCDD. Isotope fractionation could also occur during sorption-desorption (Slater et al., 2000; Kopinke et al., 2005), however, we expect that sorption-desorption itself was less important as a signal of fractionation than the effect from kinetic limitation caused by sorption-desorption. Future experiments must address and solve these issues to obtain quantitatively useful isotope fractionation factors for PCDDs.

Environmental Implications. Drenzek et al. (2001) investigated isotope fractionation during dechlorination of 2,3,4,5-tetrachlorobiphenyl (2,3,4,5-TeCB) to 2,3,5-trichlorobiphenyl (2,3,5-TrCB) and observed that isotope compositions of 2,3,4,5-TeCB and 2,3,5-TrCB remained nearly constant over 90 days, with 2,3,5-TrCB 0.3‰ heavier than 2,3,4,5-TeCB. We observed isotope discrimination during the first dechlorination step, with a greater discrimination observed during a second dechlorination step perhaps because of greater bioavailability of the metabolite. In Drenzek et al.'s (2001) study only one dechlorination step occurred, thus 2,3,5-TrCB was not subject to additional fractionation as our first metabolite, 1,2,4-TrCDD, was. It would be interesting to determine whether fractionation of PCBs could be observed if the first dechlorination product were subsequently dechlorinated.

PCDD fate in the environment is influenced by multiple physical, chemical and biological processes that could affect isotope composition. Further, originally released PCDDs were formed by different processes and may have different initial isotope contents. For CSIA to be useful for PCDDs, the effect of physical-chemical processes on isotope fractionation should be examined and more sensitive detection of fractionation must be achieved.

Conclusions. Carbon isotope discrimination was observed between the parent and daughter products during dechlorination of 1,2,3,4-TeCDD to 1,2,4-TrCDD and then to 1,3-DCDD by a mixed culture containing *Dehalococcoides ethenogenes* strain 195. Because 1,2,3,4-TeCDD is

hydrophobic and sparingly soluble, it is possible that dissolution of 1,2,3,4-TeCDD may act as a kinetic limitation on dechlorination and thus lower kinetic isotope fractionation of 1,2,3,4-TeCDD to some extent. This may have resulted in inability to reproducibly quantify the isotopic fractionation by regression analysis of the plot of the Rayleigh equation. A greater isotope fractionation for dechlorination of 1,2,4-TrCDD to 1,3-DCDD than for 1,2,3,4-TeCDD to 1,2,4-TrCDD may be explained by the formation of more soluble, bioavailable 1,2,4-TrCDD at the cell resulting in less kinetic limitation and greater apparent enrichment of ^{13}C in the final dechlorination product 1,3-DiCDD. To use CSIA to document on-going *in situ* biological dechlorination of PCDDs at contaminated sites, it is necessary to follow on-going processes in the environment. To do this, sensitive detection limits on the stable isotope content of individual, environmental PCDD congeners or homolog groups are needed. Documentation of lightly chlorinated products that are depleted in ^{13}C when compared to potential precursors that are more heavily chlorinated could be a signal for biological dechlorination. Further, a better understanding of all biotic and abiotic factors controlling isotope fractionation of PCDDs in the environment is required.

Supporting Information

Carbon stable isotope calculations. The Rayleigh model (Rayleigh, 1896) (equation 2) is applicable for characterizing the isotope fractionation of an initial dehalogenation step. For sequential dehalogenation reactions where a compound is simultaneously formed and further dehalogenated — e.g. for dehalogenation of 1,2,3,4-TeCDD to form 1,2,4-TrCDD and subsequently 1,3-DCDD — a point by point calculation may be used to estimate the isotope fractionation related to the individual dehalogenation steps (Hunkler et al., 1999). In this case, isotope fractionation can be calculated as an isotope equilibrium ratio between substrate and product which is acceptable for relatively small isotope fractionation and low transformation (Clark and Fritz, 1997; Hoefs, 1997; Hunkler et al., 1999). The isotope fractionation can be described with the assumption that the isotope composition of substrate and the accumulating product is not strongly changing during the reaction. Isotope fractionation factors may be calculated as:

$$\alpha C = \frac{R_p}{R_s} \qquad \text{(Equation S1)}$$

where R_p and R_s are the ratios of $^{13}C/^{12}C$ in the product and substrate, respectively. This may also be expressed as:

$$\alpha C = \frac{\delta_p + 1000}{\delta_s + 1000} \qquad \text{(Equation S2)}$$

Equation S2 may then be substituted into equation 3:

$$\varepsilon_{p-s} = \frac{\delta_p - \delta_s}{\delta_s + 1000} \times 1000 \qquad \text{(Equation S3)}$$

An approximation for ε_C, also referred to as an isotope separation factor, applied to systems exhibiting low fractionation factors and low transformation rates (Hunkler et al., 1999; Whiticar, 1999) is:

$$\varepsilon_C = \varepsilon_{p-s} \approx \delta_p - \delta_s \qquad \text{(Equation S4)}$$

Although equation S4 is used primarily to calculate the isotope fractionation of one element at equilibrium among different phases or substances, it has been applied for kinetic isotope fractionation (Hunkler et al., 1999; Whiticar, 1999; Elsner et al., 2005) to give an estimation of the magnitude of the fractionation when the Rayleigh equation was not applicable. Note that αC may be defined as the isotope ratio of product to substrate or the reverse, where the resulting equation S4 would differ by sign. Mariotti et al. (1981) reasoned that equation S4 was applicable for quantifying kinetic isotope fractionation in the initial phase of a reaction where concentrations have not changed substantially due to substrate depletion and accumulation of the product.

Calculation of the Intrinsic Kinetic Isotope Effect. The intrinsic kinetic isotope effect (KIE) is defined as the ratio of the first order rate constants of the lighter to the heavier isotope species (Elsner et al., 2005)

$$KIE = \frac{{}^{l}K}{{}^{h}K} \qquad \text{(Equation S5)}$$

where ${}^{l}K$ is the first order rate constant of the lighter isotope species and ${}^{h}K$ is that of the heavier isotope species.

The isotope fractionation factor α can be defined as

$$\alpha = \frac{{}^{h}K}{{}^{l}K} \qquad \text{(Equation S6)}$$

Thus

$$\alpha = \frac{1}{KIE} \qquad \text{(Equation S7)}$$

Further,

$$\varepsilon = (\frac{1}{KIE} - 1) \times 1000 \qquad \text{(Equation S8)}$$

The ε calculated from equation S8 is the intrinsic enrichment factor which reveals the real difference between the reaction rates of molecules containing different isotope species at the reactive site. However, the enrichment factor ε calculated based on the Rayleigh equation from experimental data is the average isotope effect observed for the total molecule. Elsner et al. (2005) discussed factors that mask the intrinsic kinetic isotope effect—i.e., the dilution effect from nonreactive position where the isotope presents in the molecule other than the reactive site, the intramolecular competition of isotopes in indistinguishable reactive positions, and the influence of rate limiting steps prior to bond breakage/commitment to catalysis.

The dilution effect of nonreactive positions is eliminated by converting the bulk enrichment factor to a position-specific enrichment factor

$$\varepsilon_{reactive\ \ position} = {}^{n}\!/_{x} \bullet \varepsilon_{bulk} \qquad \text{(Equation S9)}$$

where $\varepsilon_{reactive\ position}$ is the position-specific enrichment factor, ε_{bulk} is the bulk enrichment factor derived from experimental data, and n is the number of atoms of the element of interest where x is the number of atoms located at the reactive site.

Further, intramolecular competition could be eliminated by calculation of $\varepsilon_{intrinsic}$

$$\varepsilon_{intrinsic} = z \bullet \varepsilon_{reactive\ \ position} \qquad \text{(Equation S10)}$$

where z is the number of indistinguishable reactive positions in one molecule that the isotope of interest might occupy. For the primary isotope effect in non-concerted reactions, $z = x$. Therefore, equation S10 may also be expressed as

$$\varepsilon_{intrinsic} = n \bullet \varepsilon_{reactive\ \ position} \qquad \text{(Equation S11)}$$

The concept of $\varepsilon_{intrinsic}$ was defined by Morasch (2004) as the bulk enrichment factor times the number of atoms of element of interest in a molecule. Although the reasoning was done with a different approach, the final equation for calculation of the intrinsic enrichment factor is the same.

For a symmetrical molecule like tetrachloroethene (PCE), $\varepsilon_{reactive\ position} = \varepsilon_{bulk}$. Only intramolecular competition needs to be considered. So the intrinsic enrichment factor of PCE is 2 × ε_{bulk}, since the two carbon atoms are indistinguishable reactive sites for the reductive dechlorination of PCE.

For 1,2,3,4-tetrachlorodibenzo-p-dioxin (1,2,3,4-TeCDD), n = 12 and x = 1. There is no intramolecular competition in the molecule of 1,2,3,4-TeCDD, thus the intrinsic enrichment factor of 1,2,3,4-TeCDD is 12× ε_{bulk}.

Linearity of GC-C-IRMS Response for PCDDs. The linearity of the detector response of the gas chromatography-combustion-isotope ratio mass spectrometer (GC-C-IRMS) was assessed for 1,2,3,4-TeCDD over a detector output range of 80 to 1400 mV (**Fig. 8-5**). A linear regression of the detector response had a slope of 0.82, indicating that the nonlinearity of

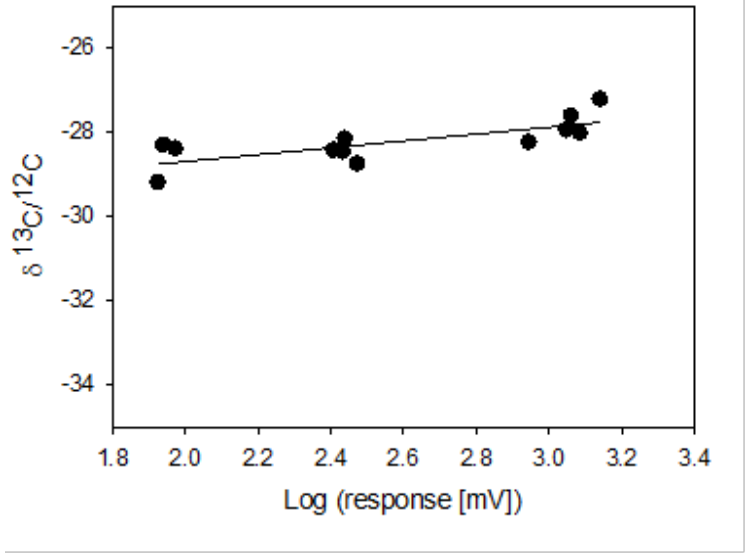


Figure 8-5. Linearity of the GC-C-IRMS detector response for the 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) standard over a detector output range of 80 to 1400 mV.

the instrument during the analysis of 1,2,3,4-TeCDD was about 0.8 ‰ per 10-fold increase in the detector response between 80 and 1400 mV. This systematic error is higher than that reported for other compounds. For example, the detector response for toluene exhibited a nonlinearity of 0.1‰ per 10-fold increase in the detector response between 45 and 3500 mV (Richnow et al., 2003). This could be a result of the poor combustibility of the polychlorinated dibenzo-*p*-dioxins (PCDDs) related to their high molecular weight and stable molecular structure. These characteristics may result in higher error during GC-C-IRMS measurement of the stable carbon isotope ratios of the PCDDs. The values were not corrected for linearity, however isotope values were reported for measurements where concentrations were of the same order of magnitude and thus comparable wherever possible.

Carbon Stable Isotope Fractionation of PCDDs. Dechlorination and isotope composition of PCDDs in three replicates from the treatment with 1,2,3,4-TeCDD plus PCE as an additional substrate are shown in **Figure 8-3**. The concentration and isotope data for 1,2,3,4-TeCDD in the killed controls is shown in **Figure 8-4**.

Carbon Stable Isotope Mass Balance. In a closed system, the mass balance on the isotopes of carbon in different compounds in the system is expressed as

$$R_{Total,t} = \sum R_{i,t} \times f_{i,t} = \sum R_{i,0} \times f_{i,0} \qquad \text{(Equation S13)}$$

where $R_{Total,t}$ is the carbon isotope ratio of the total pool of parent compound and its metabolites in the system at time t. Theoretically, $R_{Total,t}$ should be constant over the course of the reaction. $R_{i,0}$ is the carbon isotopic composition of a chemical *i* at time 0 and $f_{i,0}$ is the fraction of chemical *i* at time 0. Similarly, $R_{i,t}$ and $f_{i,t}$ are its carbon isotopic composition and fraction at time *t*. The average isotope mass balance was calculated from triplicate data for each treatment (with and without PCE addition) for time points where detection and quantification of all PCDDs were possible (**Fig. 8-5**).

9. Reductive Dechlorination of Dioxins and Aroclors by a Mixed Culture Containing *Dehalococcoides ethenogenes* strain 195

Abstract

A mixed culture containing *Dehalococcoides ethenogenes* strain 195 was tested for its ability to dechlorinate 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD),the dioxin-like polychlorinated biphenyl, PCB 114 (2345-4 pentachlorobiphenyl); and Aroclors 1242, 1254, and 1260. The culture was grown on tetrachloroethene and butyric acid as an electron donor/hydrogen source at 25ºC. To assess dechlorination, aliquots of the culture were amended with one of the PCBs or the PCDD which had been coated on sediment. Dechlorination was examined at 25ºC and 35ºC and in the presence or absence of co-amended 1,2,3,4-trichlorobenzene (1,2,3,4-TeCB), a known growth substrate of strain 195, which has been shown to enhance its ability to dechlorinate polychlorinated dibenzofurans in a previous study. Additionally, 3 % and 20 % (volume:volume) dilutions of the culture were established and amended with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) or PCB 114 in an attempt to establish whether the culture could sustain growth on these compounds as sole electron acceptors. The dilutions were amended with the PCB 114 or 1,2,3,4-TeCDD coated either on a reference sediment or on silica powder. PCDD and PCB congener specific analyses were accomplished by gas chromatography mass spectrometry and tandem quadrupole gas chromatography mass spectrometry, respectively.

The mixed culture with strain 195 dechlorinated 1,2,3,7,8-PeCDD, PCB114 and selected highly chlorinated PCB congeners in Aroclors 1254 and 1260. The 1,2,3,7,8-PeCDD was dechlorinated to 1,3,7,8-TeCDD and further to 1,3,7-TrCDD — a detoxification pathway since no 2,3,7,8-TeCDD was formed. The rate of dechlorination of 1,2,3,7,8-PeCDD at 35ºC (the temperature at which strain 195 was originally enriched) was greater than that observed at 25ºC. The mixed culture with strain 195 dechlorinated PCB 114 and Aroclors 1254 and 1260 mainly by removing doubly flanked chlorines at the *meta* and *para* positions. Minimal dechlorination of Aroclor 1242 was observed. The rates of dechlorination of PCDDs and PCBs were stimulated by the addition of 1,2,3,4-TeCB. Dilutions established to determine the growth of the mixed culture with strain 195 on PCBs and PCDDs as sole electron acceptors exhibited slow rates of dechlorination (< 0.1% transformation over 120 days) indicating no or very slow growth with this PCB or PCDD as sole substrates. Strain 195 can dechlorinate many chlorinated aromatic organic compounds of high environmental relevance, often to less toxic daughter products. However, its use as a bioaugmentation agent may be limited to cases where multiple amendments of the organism or amendment of the organism with an alternate halogenated electron acceptor such as 1,2,3,4-tetrachlorobenzene could be performed, since it does not appear to grow with selected PCDDs or PCBs.

Introduction

The chlororespiring bacteria *Dehalococcoides* spp. are known to be widely involved in the dehalogenation of anthropogenic halogenated compounds and have been utilized successfully for bioaugmentation of chloroethene contaminated aquifers (see for example Lendvay et al., 2003*). Dehalococcoides*-containing cultures are sold commercially for this purpose [e.g., KB-1®

http://www.siremlab.com/kb1bioaugmentation.html and Bio-Dechlor INOCULUM® Plus http://www.regenesis.com/contaminated-site-remediation-products/bioaugmentation/bio-dechlor/default.aspx, accessed online August 2011].

In light of their ability to utilize diverse aromatic chlorinated organic substrates, cultures containing *Dehalococcoides* spp. could be used to treat sediments contaminated with pollutants such as polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) and polychlorinated biphenyls (PCBs). One specific advantage of using *Dehalococcoides* spp. with diverse dehalogenating capabilities for bioaugmentation for PCB- and PCDD/F-contaminated environments is that these organisms can be grown in mixed culture on relatively soluble substrates such as chloroethenes or chlorobenzenes which allows production of larger quantities of cells for bioaugmentation. Further, the volatile daughter products of dechlorination of these alternate halogenated compounds could be removed from the cultures via volatilization prior to amendment to the environment.

In this study, we continued to investigate *D. ethenogenes* strain 195as grown in a mixed culture (Fennell et al., 2004). Strain 195 dechlorinates tetrachloroethene (PCE) to vinyl chloride (VC) and ethene. It grows on PCE, trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-1,2-DCE), 1,1-DCE (Maymó-Gatell et al. 1997, 1999), selected chlorinated benzenes (Fennell et al. 2004), and chlorophenols (Adrian et al. 2007). Strain 195 has been documented to exhibit diverse dechlorination capability. In pure culture, strain 195 dechlorinated 2,3,4,5,6-PCB, 1,2,3,4-tetrachloronaphthalene, and 1,2,3,4-tetrachlorodibenzofuran (1,2,3,4-TeCDF). Specifically, it dechlorinated 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) to 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD), and subsequently to 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (Fennell et al. 2004). Notably, strain 195 did not dechlorinate 2,3,7,8-TeCDD. The strain also debrominates commercial octa-brominated diphenyl ether (BDE) (a mixture containing hexa-BDE through nona-BDE) to a mixture of penta-, hexa-, and hepta-BDEs (He et al. 2006). The mixed culture containing strain 195 also dechlorinated the environmentally relevant PCDF, 1,2,3,4,7,8-hexachlorodibenzofuran, to non-2,3,7,8-substituted daughter products (Liu and Fennell, 2008). We previously reported that rates of dechlorination of weathered PCBs over 415 days of incubation in Anacostia River, Washington, DC microcosms were greater in those microcosms amended with pentachloronitrobenzene (PCNB), bioaugmentation with the mixed culture containing *D. ethenogenes* strain 195, or combined PCNB plus bioaugmentation, relative to other treatments (Krumins et al., 2009). It is not known if strain 195 obtains energy for growth on PCDD/Fs or which of its reductive dehalogenase(s) (Seshadri et al., 2005) mediates their dechlorination. Krumins et al. (2009) observed slow disappearance of strain 195 from Anacostia sediments as determined by PCR-DGGE over 281 days after bioaugmentation. Park et al. (2011) quantified and sequenced dehalogenase genes from samples recovered on day 135 of that study. Dehalogenase genes in the bioaugmented microcosms had sequence similarity to those from strain 195. Two dehalogenase genes were detected in higher abundance in active PCB dechlorinating microcosms, designated rdh 04 and rdh 05. The rdh 04 gene fragments (193 bp) recovered from bioaugmented or bioaugmented plus PCNB-treated microcosms were 100% identical to DET0306 in strain 195, while the 105 bp rdh 05 gene fragments from bioaugmented or bioaugmented plus PCNB-treated microcosms were 100% identical to DET0311 in strain 195. A better understanding of the ability of *Dehalococcoides* spp. for beneficial biotransformation of halogenated pollutants will allow their further exploitation in environmental restoration

applications. Further, documenting the ability of these organisms to grow on PCBs or PCDD/Fs would justify their use as bioaugmentation agents which could theoretically be amended at a single time point to accomplish remedial goals. Bunge et al. (2008) have recently shown that *Dehalococcoides* sp. strain CBDB1, which was originally cultivated on trichlorobenzene, dehalogenates and can be transferred on selected chlorinated dibenzo-*p*-dioxins and *Dehalococcoides* sp. in culture JN1 also grew on Aroclor 1260 (Bedard et al., 2008) .

The goal of this study was to further delineate the substrate range of a mixed culture containing *D. ethenogenes* strain 195 and to assess growth of the strain on a selected PCB and TCDD congener. We amended mixed cultures containing *D. ethenogenes* strain 195 with 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD), the dioxin-like polychlorinated biphenyl (PCB) 114 (2345-4 pentachlorobiphenyl) and Aroclors 1242, 1254, and 1260 to determine the capability for dehalogenation. Dechlorination was examined at 25ºC and 35ºC and in the presence or absence of co-amended 1,2,3,4-trichlorobenzene (1,2,3,4-TeCB), a known growth substrate of strain 195 (Fennell et al., 2004) which has been shown to enhance its ability to dechlorinate PCDFs previously (Liu and Fennell, 2008). Additionally, dilutions of the culture were established on 1,2,3,4-TeCDD and PCB 114 in an attempt to establish whether the culture could sustain growth on these compounds.

Materials and Methods

Chemicals. 1,2,3,7,8-PeCDD, 1,2,7,8-tetrachlorodibenzo-*p*-dioxin (1,2,7,8-TeCDD), 1,3,7,8-tetrachlorodibenzo-*p*-dioxin (1,3,7,8-TeCDD), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TeCDD), 1,7,8-trichlorodibenzo-*p*-dioxin (1,7,8-TrCDD), 2,3,7-trichlorodibenzo-*p*-dioxin (2,3,7-TrCDD), PCB 114 (2345-4 pentachlorobiphenyl), and Aroclors 1260, 1254 and 1242 were purchased from AccuStandard, Inc. (New Haven, CT). 1,3,7-trichlorodibenzo-*p*-dioxin (1,3,7-TrCDD) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). For growing and monitoring the mixed culture, PCE (99.9+%), TCE (99.5+%), and butyric acid (99+%) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). The *cis*-1,2-DCE was purchased from Supelco, Inc. (Bellefonte, PA, USA). VC (99.5+%) was obtained from Fluka Chemie GmbH (Germany). Ethene (99%) was purchased from Matheson Tri-Gas, Inc. (Montgomeryville, PA, USA).

Culture preparation - Dechlorination assessment. A mixed culture containing *Dehalococcoides ethenogenes* strain 195 was grown at 25 °C on PCE and butyric acid, and was monitored for chloroethene dechlorination as described previously (Fennell et al., 2004; Liu and Fennell, 2008). 1 g dry sterile sediment (Fennell et al., 2004) was added to a 160 mL serum bottle and wetted by adding 0.5 mL of 200 mg/L 1,2,3,7,8-PeCDD-toluene stock solution, 1 mL of 400 mg/L PCB114 stock solution or 0.1 mL of 1000 mg/L Aroclor-acetone stock solution. The organic solvents were evaporated overnight and the bottles were sealed with a Teflon®-coated gray butyl rubber septum and crimped with an aluminum crimp cap. Next, the bottles were purged for 20 min with anoxic 70% nitrogen/30% carbon dioxide, and autoclaved. 100 mL of the mixed culture was transferred to each bottle under anoxic and sterile conditions. Thus, the final concentrations (expressed on a total liquid volume basis) were 1 mg/L for 1,2,3,7,8-PeCDD and Aroclors 1260, 1254 and 1242, and 4 mg/L for PCB 114, respectively.

Butyric acid was added as electron donor at a concentration of 100 µM on days 0, 11, 22, 29, 45, 61, 87, 106 and 152 for the 1,2,3,7,8-PeCDD and PCB 114 treatment groups, on days 0, 65, 108, 156 and 195 for the Aroclor 1260 treatment group and on days 0, 48, 87 and 143 for both the Aroclor 1254 and 1242 treatment groups, respectively. 50 µL of a 50 g/L fermented yeast extract solution was provided as a nutrient source and was added on days 0, 11, 22, 61 and 152 for the 1,2,3,7,8-PeCDD and PCB 114 treatment groups, on days 0, 65, 108 and 195 for the Aroclor 1260 treatment group and on days 0 and 48 for the Aroclor 1254 and 1242 treatment groups, respectively. A vitamin stock solution (Fennell et al., 2004) was added at day 0 for all treatment groups.

For each tested chemical, three sets of triplicate bottles were established at 25°C. One set of bottles received the studied chemical as the sole halogenated substrate. In a previous study, 1,2,3,4-TeCB was shown to enhance the dechlorination of 1,2,3,4,7,8-HxCDF by the mixed culture containing *D. ethenogenes* strain 195 when added as an alternate halogenated substrate (Liu & Fennell, 2008). Thus, in addition to the studied chemical, a second set of triplicate bottles also received 1,2,3,4-TeCB at a final concentration of 25 µM on days 0, 29, 61 and 152 for the 1,2,3,7,8-PeCDD and PCB 114 treatment groups, on days 0, 65, 108 and 156 for the Aroclor 1260 treatment group and on days 0 and 48 for both the Aroclor 1254 and 1242 treatment groups, respectively. The third set of bottles was autoclaved for 1 hr on each of three consecutive days to serve as negative controls. *D. ethenogenes* strain 195 was originally enriched from a mesophilic anaerobic digester and grown at a temperature of 35°C (Maymó-Gatell et al. 1997; Fennell et al., 1998). Thus, to investigate the effect of temperature on dehalogenation of PCBs and PCDDs by *D. ethenogenes* strain 195 two sets of triplicate bottles were also established at 35°C within the 1,2,3,7,8-PeCDD treatment group. One set received 1,2,3,7,8-PeCDD as sole halogenated substrate while the other received both 1,2,3,7,8-PeCDD and 25 µM 1,2,3,4-TeCB. All bottles were inverted and shaken in the dark at 120 rpm and sampled periodically over more than 200 days.

Growth assessment. To investigate the potential for growth by the culture on PCDDs or PCBs as a sole substrate, the PCE-grown mixed culture containing *D. ethenogenes strain 195* was diluted and transferred to 160 mL serum bottles with either 1,2,3,4-TeCDD or PCB 114 supplied as the sole electron acceptor. Chemicals were applied in different ways in two separate experimental trials. In the first experiment, 1,2,3,4-TeCDD was pre-coated onto 1 g sediment by adding 1 mL of 4000 mg/L stock solution in toluene. After the evaporation of toluene, 100 mL of 3% diluted mixed culture was added under sterile and anoxic conditions. . The resulting concentration of 1,2,3,4-TeCDD in the culture was 40 mg/L. In the second experiment, 1,2,3,4-TeCDD or PCB 114 was added directly into dilutions of the mixed culture from silica-based stock solutions prepared as described by Bedard et al. (2006). To make the silica-based stock solutions, Silica powder (200 mesh, Fisher Scientific, Pittsburg, PA) was wetted with 1,2,3,4-TeCDD or PCB 114 stock solutions in toluene. After the toluene was evaporated completely, the bottles were sealed and purged with sterile nitrogen gas. Next, anaerobic medium (Fennell et al., 2004) was added to the bottle and mixed with the silica powder by vortexing vigorously. The 1,2,3,4-TeCDD or PCB 114/silica stock solutions were then added incrementally into 20% diluted mixed culture in increasing amounts every ten days. The initial concentrations of 1,2,3,4-TeCDD or PCB 114 in the mixed culture were 100 µg/L, while the final concentrations were

47.8 mg/L. In addition to the treatments with 1,2,3,4-TeCDD and PCB 114, a third treatment was also included in the silica-based growth experiment with PCE supplied as the sole electron acceptor. 1 µL of neat PCE (equivalent to 9.8 µmol) was added on day 0, 18 and 40. Vitamin stock solutions were added on day 0, and butyric acid was added every two weeks in both experiments. All bottles were inverted and shaken in the dark at 25˚C and 120 rpm and sampled periodically over more than 200 days.

Chemical Analysis. Analyses of the PCDD/Fs and PCBs were carried out following previously described methods. Briefly, 1 mL of culture medium-sediment (or silica-) slurry was removed from each serum bottle using a sterile anoxic syringe with an 18-gauge needle. After centrifugation, the aqueous portion was transferred to a separate vial, the surrogate standard (2,2',5-trichlorobiphenyl for PCDD/F and PCB65-d_6 and PCB159-d_4 for PCB) was added to the solid residue. 1 mL acetone was added to rinse the solid phase and remove the water. For PCDD/Fs, the sediment was first extracted overnight with 3 mL of 2:1 vol/vol toluene/acetone solution and then with 1 mL of 2:1 vol/vol toluene/acetone solution for 4 hours and finally with 1 mL toluene for PCDD/Fs. For PCBs, the sediment was extracted in sequence with 3 mL of 1:1 hexane/acetone vol/vol solution, 1 mL of 1:1 hexane/acetone vol/vol solution and 1 mL hexane. After each step, the solvent phase was combined with the aqueous phase. The pooled solvent was back-extracted by adding several drops of saturated sodium chloride solution. The interfering compounds were then removed by passing the sample through a 2 mL glass pipette filled with Florisil® (Sigma-Aldrich, St. Louis, MO) and eluting with 3 volumes of toluene/hexane. The solvent was finally concentrated to 1 mL under a gentle nitrogen stream for GC-MS or GC-MS-MS analysis.

PCDD/Fs were analyzed using an Agilent 6890 gas chromatograph equipped with a 5973N mass selective detector (GC-MS; Agilent Technologies, Inc., Santa Clara, CA) and a HP-5MS (Agilent Technologies, Inc., Santa Clara, CA) column (30 m × 0.25 mm i.d.). The temperature program was started at 60 °C and held for 1 min, increased to 150 °C at a rate of 40 °C/min, then increased to 280 °C at a rate of 10 °C/min and finally held for 4 min. PCDDs were identified based on the retention time and selective ion monitoring (m/z: PeCDD, 356; TeCDD, 322; TrCDD, 286; and TrCB, 186). Among the nine possible dechlorination products only five standards including 1,2,7,8-TeCDD, 1,3,7,8-TeCDD, 2,3,7,8-TeCDD, 1,7,8-TrCDD and 2,3,7-TrCDD were commercially available, and these were thus used to determine the dechlorination pathway of 1,2,3,7,8-PeCDD .

Quantitative congener-specific PCB analysis was performed by using a tandem quadrupole GC/MS/MS system (Waters Quattro Micro GC). All samples were measured for a suite of 209 PCB congeners using a method similar to EPA method 1668A. The MS-MS operating parameters for the determination of PCBs are presented in **Table 4-3**. In order to achieve maximum sensitivity, the two most abundant isotopes M^+ and $[M + 2]^+$, were monitored and the total ion current (TIC) chromatogram was used for quantification with the aim of achieving maximum sensitivity. This method separated the 209 PCB congeners into around 170 peaks. A calibration standard solution with a full suite of 209 PCB congeners was prepared from five PCB congener solutions purchased from AccuStandard (New Haven, CT). Deuterated PCB congeners purchased from C D N Isotopes (Quebec, Canada) were used as surrogate (PCBs 65 and 159) and internal (PCBs 30 and 116) standards. The mole percent (mol %) of the resolved

PCB congeners was computed based on the molar concentration of each congener divided by the sum of the molar concentrations of all detected congeners. For replicate samples, an average ± one standard deviation mol % was reported.

Results and Discussion

Dechlorination pathway of 1,2,3,7,8-PeCDD. The mixed culture containing *D. ethenogenes* strain 195 dechlorinated 1,2,3,7,8-PeCDD and produced two metabolites, which were individually identified to be a TeCDD and a TrCDD based on their mass spectra. The retention time of the unknown TeCDD (14.27 min) was similar to that of the 1,3,7,8-TeCDD standard (14.28 min), while different from those of the 1,2,7,8-TeCDD (14.67 min) and 2,3,7,8-TeCDD (14.79 min) standards. Thus, the TeCDD metabolite was thought to be 1,3,7,8-TeCDD, though we could not exclude the possibilities of 1,2,3,7/8-TeCDD, which were not available as standards. Similarly, the unknown TrCDD metabolite had a retention time of 12.68 min, similar to that of the 1,3,7-TrCDD standard (12.67 min), while different from those of the 2,7,8-TrCDD (13.00 min) and 1,7,8-TrCDD (13.02 min) standards. Thus, we identified 1,3,7-TrCDD as the second metabolite of 1,2,3,7,8-PeCDD,though we could not exclude the possibility of 1,3,8-TrCDD, which was not available as standard. It was presumed that the 1,3,7-TrCDD daughter product was most likely produced by removal of a second chlorine from the first metabolite, 1,3,7,8-TeCDD. The presumed dechlorination pathway of 1,2,3,7,8-PeCDD is shown in **Figure 9-1**. This dechlorination pattern, the removal of a chlorine from a double flanked lateral position

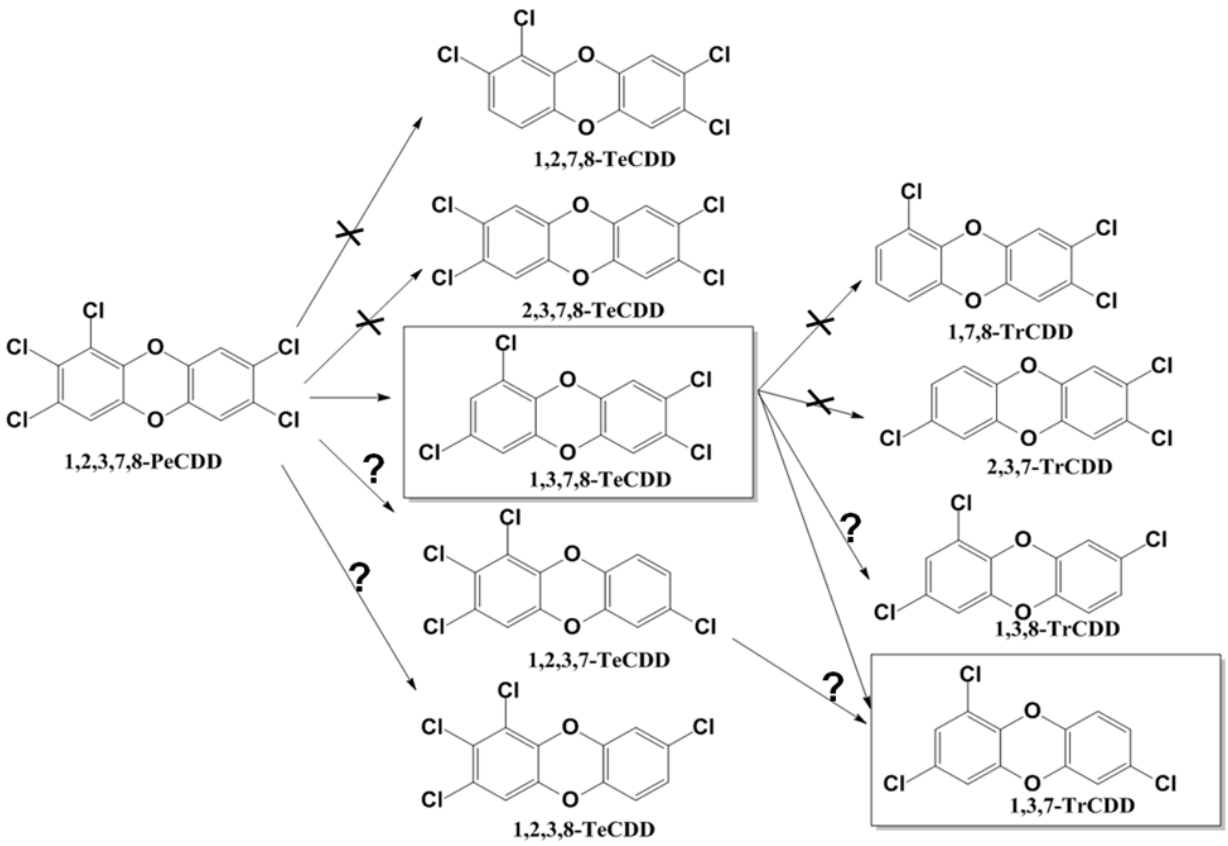


Figure 9-1. The presumed pathway of 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin dechlorination by a mixed culture containing *D. ethenogenes* strain 195.

on the first aromatic ring, followed by removal of a chlorine from a single flanked lateral position on the other ring, is consistent with the dechlorination pattern of 1,2,3,4,7,8-HxCDF by the mixed culture containing *D. ethenogenes* strain 195 (Liu & Fennell, 2008). We cannot at this time, however rule out the possibility that 1,3,7-TrCDD was produced by removal of a doubly flanked chlorine from 1,2,3,7-TeCDD. In a previous study, Bunge et al. (2003) reported that *Dehalococcoides sp.* strain CBDB1 could dechlorinate 1,2,3,7,8-PeCDD to 2,7-/2,8-DiCDD by generating the intermediate dioxin product 2,3,7,8-TeCDD. In our study, however, *D. ethenogenes* strain 195 could dechlorinate 1,2,3,7,8-PeCDD to daughter products without the 2,3,7,8-substition pattern. Thus, this pathway is a detoxification process since the congeners with 2,3,7,8-substitution are considered the most toxic of the PCDDs (van den Berg et al., 2006).

Effects of temperature and 1,2,3,4-TeCB on dechlorination of 1,2,3,7,8-PeCDD. Dechlorination of 1,2,3,7,8-PeCDD occurred continuously during the 170 day cultivation period (**Fig. 9-2**). [Note that for unknown reasons in one triplicate from the subset of cultivation at 35°C with 1,2,3,7,8-PeCDD as sole halogenated substrate, the dechlorination of 1,2,3,7,8-PeCDD stopped after 29 days when 4.1 mol % 1,2,3,7,8-PeCDD had been transformed into 1,3,7,8-TeCDD. These results are not included in the averages shown in **Figure 9-2C**.] In the autoclaved control, no dechlorination daughter products were detected during the 170 days of incubation (data not shown).

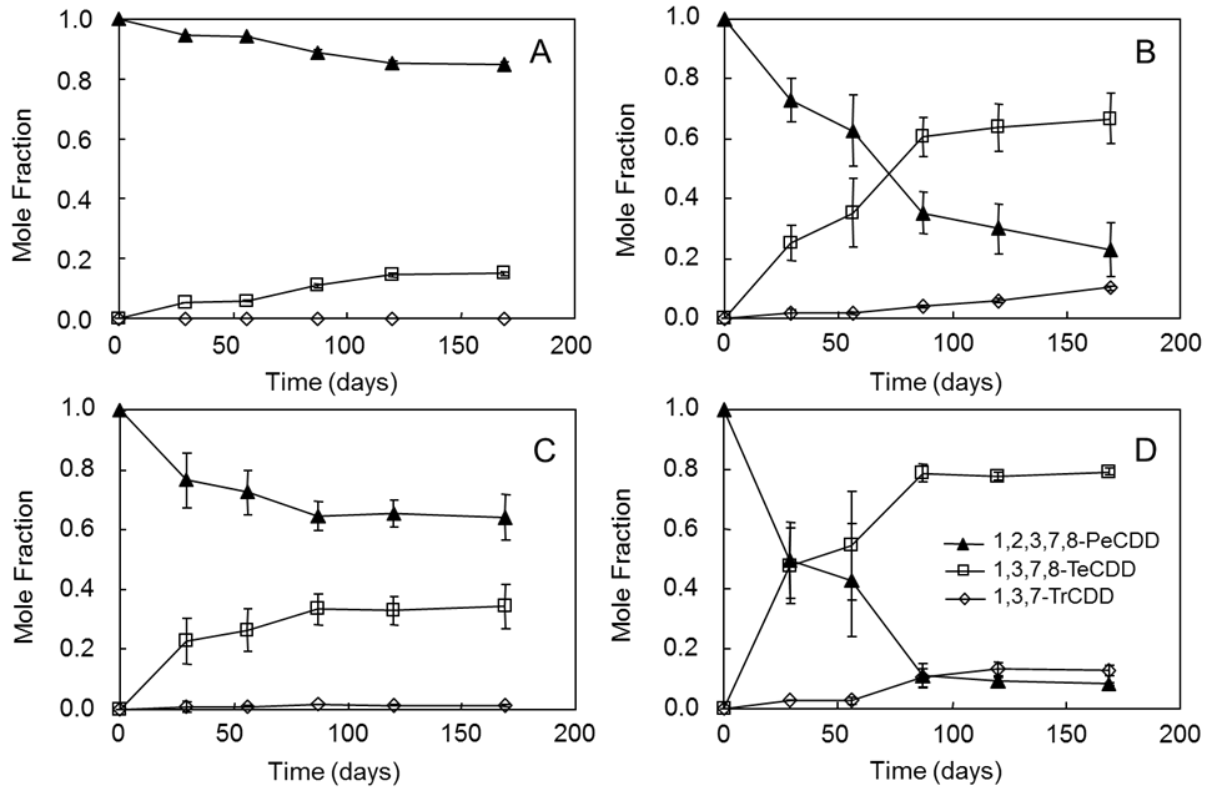


Figure 9-2. Dechlorination of 1,2,3,7,8-PeCDD in a mixed culture containing *D. ethenogenes* strain 195 (A) when added as sole halogenated substrate at 25°C , (B) when added with 1,2,3,4-TeCB as alternate halogenated substrate at 25°C, (C) when added as sole halogenated substrate at 35°C and (D) when added with 1,2,3,4-TeCB as alternate halogenated substrate at 35°C. Symbols are average of triplicates (A, B and D) or duplicate (C) and error bars are one standard deviation.

The addition of 1,2,3,4-TeCB as an additional halogenated substrate markedly increased the dechlorination rate. For example, at day 169, only 14.2 mol % 1,2,3,7,8-PeCDD was found to be transformed to 1,3,7,8-TeCDD at 25°C (**Fig. 9-2A**). However, in the bottles incubated under the same temperature but additionally amended with 1,2,3,4-TeCB, only 23.2 mol % percent of 1,2,3,7,8-PeCDD remained in the culture with the production of 68.9 mol % 1,3,7,8-TeCDD and 7.9 mol % 1,3,7-TrCDD (**Fig. 9-2B**). In addition, the increase in culture incubation temperature from 25°C to 35°C resulted in more extensive dechlorination. At 35°C, with 1,2,3,7,8-PeCDD added as a sole substrate, almost 50.0 mol % of the initial 1,2,3,7,8-PeCDD was transformed with the production of 49.4 mol % 1,3,7,8-TeCDD and 0.6 mol % 1,3,7-TrCDD at day 169. When 1,2,3,4-TeCB was added as an alternate halogenated substrate at 35°C, after 169 days of cultivation, the culture contained 8.4 mol % 1,2,3,7,8-PeCDD, 81.3 mol % 1,3,7,8-TeCDD and 10.3 mol % 1,3,7-TrCDD, respectively.

Dechlorination of Aroclors 1260, 1254 and 1242. Table 9-1 shows the PCB homolog distribution change in the autoclaved control with Aroclor 1260 and in the live strain 195 mixed culture with Aroclor 1260 alone or with 1,2,3,4-TeCB, while **Table 9-2** shows the same comparison for Aroclor 1254. After 250 days of incubation at 25°C, with Aroclor 1260, the nona-, octa- and hepta-chlorobiphenyls decreased by 31.2 mol %, 35.2 mol % and 42.2 mol %, respectively (**Table 9-1**, **Fig. 9-3**). The mole percentages of hexa-, penta- and tetra-chlorobiphenyls among total PCBs increased.

Table 9-1. PCB homolog distribution change in Aroclor 1260 after 250 days dechlorination by a mixed culture containing *Dehalococcoides ethenogenes* strain 195 at 25°C compared to the killed control.

PCB homolog	Mole percent of total PCBs			% Decrease	
	Killed Aroclor 1260	Live Aroclor 1260	Live Aroclor 1260 (with 1,2,3,4-TeCB)	Live Aroclor 1260	Live Aroclor 1260 (with 1,2,3,4-TeCB)
Tetra-CB	0.67	1.36 ± 0.11	2.64 ± 0.12		
Penta-CB	8.78	20.57 ± 2.17	27.96 ± 1.24		
Hexa-CB	47.18	48.60 ± 1.48	54.75 ± 1.24		
Hepta-CB	34.50	23.72 ± 1.00	12.96 ± 0.23	31.2	62.4
Octa-CB	7.55	4.89 ± 0.12	1.13 ± 0.03	35.2	85.0
Nona-CB	0.69	0.40 ± 0.03	0.11 ± 0.01	42.0	84.1

For the Aroclor 1254 treatment group, the hepta-and hexa-chlorobiphenyls decreased by 17.9 mol % and 3.4 mol % by day 160, respectively (**Table 9-2**). However, the homolog composition in Aroclor 1242 did not change substantially after 160 days (data not shown). These results demonstrated that the mixed culture containing *D. ethenogenes* strain 195 dechlorinated primarily highly chlorinated PCB congeners in the Aroclors.

Dechlorination patterns of Aroclors by the mixed culture containing D. ethenogenes strain 195. Table 9-3 lists all major PCB congeners that compose more than 1 mol % in Aroclor 1260. These congeners constitute 85.5% of the total PCBs in Aroclor 1260. Among the 25 major congeners, 14 of these were dechlorinated by the mixed culture containing *D. ethenogenes* strain 195, and their mol % of the total PCBs decreased from 44% to 8%. **Table 9-3** also lists the possible dechlorination pathway and dechlorination products of these major congeners. It was observed that only those 234-/345-/2345-substituted chlorobiphenyls showed substantial dechlorination. Thus, it could be concluded that primarily doubly flanked chlorine atoms from PCB rings could be removed by the mixed culture containing *D. ethenogenes* strain 195. To confirm this, we carried out a separate experiment with *D. ethenogenes* strain 195 using a single congener, PCB114 (2345-4-CB), and we found (data not shown) only two dechlorination products, 235-4-CB and 245-4-CB, to be formed, which substantiated removal of doubly flanked chlorines by this culture. This dechlorination pattern also explained our finding that the mixed culture with *D. ethenogenes* strain 195 dechlorinated Aroclor 1254 less extensively than Aroclor 1260, since it has a lower proportion of highly chlorinated congeners, and that the culture exhibited insubstantial dechlorination of the lightly chlorinated Aroclor 1242.

Table 9-2. PCB homolog distribution change in Aroclor 1254 after 160 days dechlorination by a mixed culture containing *Dehalococcoides ethenogenes* strain 195 at 25°C compared to the killed control.

PCB homolog	Mole percent of total PCBs			% Decrease	
	Killed Aroclor 1254	Live Aroclor 1254	Live Aroclor 1254 (with 1,2,3,4-TeCB)	Live Aroclor 1254	Live Aroclor 1254 (with 1,2,3,4-TeCB)
Tri-CB	0.92	0.70 ± 0.03	1.22 ± 0.09		
Tetra-CB	15.60	13.88 ± 0.43	24.15 ± 0.75		
Penta-CB	52.69	56.09 ± 0.21	56.74 ± 0.09		
Hexa-CB	28.04	27.10 ± 0.57	16.95 ± 0.85	3.4	39.6
Hepta-CB	2.52	2.07 ± 0.06	0.78 ± 0.11	17.9	69.0

Table 9-3. Major components of Aroclor 1260 and their dechlorination by a mixed culture containing *Dehalococcoides ethenogenes* strain 195.

Congener	IUPAC No.	Mole percent of total PCBs			% Decrease		Pathway and products
		Aroclor 1260	Dechlor. Aroclor 1260	Dechlor. Aroclor 1260 (with 1,2,3,4-TeCB)	Dechlor. Aroclor 1260	Dechlor. Aroclor 1260 (with 1,2,3,4-TeCB)	
245-245	153	9.95	10.14	8.79			
234-245 [a]	138(163,129)[b]	9.58	5.14	3.37	46	65	24-245
236-245	149	9.42	10.39	10.75			
2345-245	180 (193)	8.92	4.40	0.49	51	95	245-245, 235-245
2356-25/235-236	151/135 [c]	5.04	6.98	10.60			
2356-245	187	4.97	4.99	5.30			
2345-236	174	4.37	3.28	0.13	25	97	245-236, 235-236
2345-234	170	3.60	1.62	0.08	55	98	234-245, 234-235, 2345-24
2346-245/23456-25	183/185	3.12	1.49	0.06	52	98	246-245/2356-25, 2346-25
245-25	101	3.08	5.11	7.99			
2345-25	141	2.86	0.52	0.00	82	100	235-25, 245-25
234-236	132	2.85	2.95	1.22		57	24-236
234-2356	177	2.55	2.47	2.06	3	19	24-2356
236-25	95	2.39	3.24	3.09			
2356-236	179	2.03	2.06	2.07			

2345-2356	199 (198)	1.94	1.48	0.45	24	77	235-2356, 245-2356
2345-2345	194	1.75	0.93	0.10	47	95	235-2345, 2345-245
236-236	136	1.73	1.94	1.84			
236-34	110	1.66	1.75	1.48			
2346-234/23456-23	171/173	1.28	0.64	0.02	50	98	234-246, 2346-24/2356-23, 2346-23
235-245	146	1.24	5.04	9.44			
23456-245	203	1.13	0.68	0.06	40	95	2356-245, 2346-245

a) The underlined numbers represent the chlorine atom on PCB aromatic rings that could be removed by the mixed culture containing D. ethenogenes strain 195
b) The parenthesized congener is co-eluted with the major congener but existed at very low mol percentage among the total PCBs.
c) Two major congeners co-eluted and could not be separated using our Gas Chromatography method.

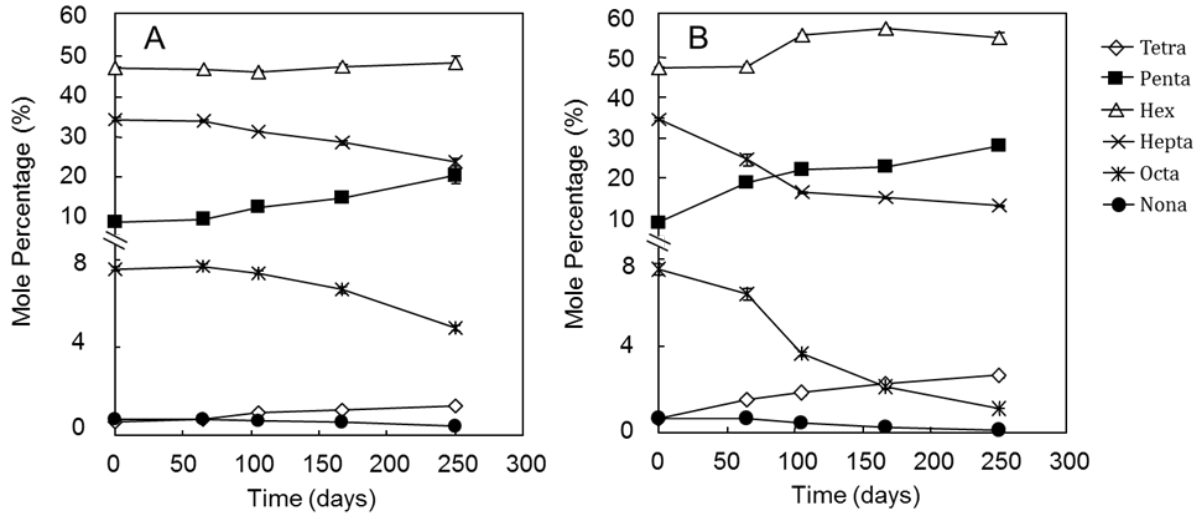


Figure 9-3. Dechlorination of Aroclor 1260 in a mixed culture containing *D. ethenogenes* strain 195 (A) when added as sole halogenated substrate at 25°C and (B) when added with 1,2,3,4-TeCB as alternate halogenated substrate at 25°C. Symbols are average of triplicates and error bars are one standard deviation.

Growth on PCBs or PCDDs could not be confirmed. Because *D. ethenogenes* strain 195 can use a wide range of halogenated compounds, including chloroethenes, chlorophenols and chlorobenzenes, as growth substrates, it is of great interest whether it could also obtain energy and grow by dechlorinating PCBs or PCDDs. In an initial experiment, 3% diluted mixed culture was supplied with 1,2,3,4-TeCDD at a nominal concentration of 40 mg/L, however, no dechlorination products were detected over 300 days. Previous studies showed that halogenated compounds such as PCBs were toxic to dehalogenating bacteria at concentrations higher than 20 mg/L (Adrian et al. 2009), thus in our second experiment, 1,2,3,4-TeCDD or PCB 114 were gradually added in an exponentially increasing amount every ten days with an initial nominal concentration of only 100 µg/L. A third treatment group was also included as a positive control with PCE, a known substrate that supports the growth of *D. ethenogenes* strain 195, supplied as the sole electron acceptor. We also increased the initial density of *D. ethenogenes* strain 195 by adjusting the dilution factor for the mixed culture from 3% to 20%. However, the second attempt to determine whether *D. ethenogenes* strain 195 could grow on PCBs or PCDDs also failed since only trace amounts (less than 0.1%) of 1,2,3,4-TeCDD or PCB 114 were dechlorinated over 120 days, while all the PCE that had been added was dechlorinated to a mixture of ethene and trace amount of vinyl chloride after 88 days (results not shown). Thus, these results suggest that PCBs or PCDDs may not support the growth of *D. ethenogenes* strain 195 in the mixed culture. This finding could partially explain our results that the addition of 1,2,3,4-TeCB, a substrate that supports the growth of *D. ethenogenes* strain 195 (Fennell et al., 2004), greatly enhanced the dechlorination of 1,2,3,7,8-PeCDD and Aroclors 1260 and 1254 by the mixed culture containing *D. ethenogenes* strain 195.

Strain 195 can dechlorinate many PCBs and PCDD/Fs of high environmental relevance, often to less toxic daughter products. However, its use as a bioaugmentation agent may be limited to cases where multiple amendments of the organism or amendment of the organism with an alternate halogenated electron acceptor such as 1,2,3,4-tetrachlorobenzene could be performed, since it does not appear to grow with selected PCDDs or PCBs.

10. Microbial Reductive Dechlorination of Weathered Polychlorinated Dibenzofurans (PCDFs).

(*Liu H, Park J-W, Ahn Y-B, Verta M, Rodenburg LA, Fennell DE, Häggblom MM. Microbial reductive dechlorination of weathered polychlorinated dibenzofurans (PCDFs) in Kymijoki sediment mesocosms. Unpublished*)

Abstract

Little is known about the potential for indigenous microorganisms to reductively dechlorinate weathered polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) in contaminated sediments. The sediments of River Kymijoki in Finland are heavily contaminated by PCDFs resulting from the manufacture of the chlorophenol-based fungicide Ky-5. In order to investigate naturally occurring microbial reductive dechlorination of weathered PCDFs and to determine the feasibility of stimulating such activities, River Kymijoki sediments were incubated in 30-L mesocosms at 18-21 °C. Treatments designed to stimulate dechlorination consisted of amendment with electron donors and a halogenated co-substrate (tetrachlorobenzene, TeCB), and bioaugmentation with a mixed culture containing *Dehalococcoides ethenogenes* strain 195. An initial onset of dechlorination of octa-, hepta- and hexa-CDFs was observed in all mesocosms in the first two years of incubation. During this initial 2-year period, the decrease in the mol% contribution of these highly chlorinated PCDFs was coupled with an increase in the mol% contribution of tetra-and penta-CDFs. The ratio of 1,2,3,4,6,7,8- to 1,2,3,4,6,8,9-hepta-CDF increased significantly between time zero and year two. Dechlorination slowed in all mesocosms after the first two years, suggesting that the increase in temperature of the mesocosms over the low temperatures typical of the Kymijoki River was responsible for the initial burst of dechlorination. Over the full seven years of the experiment, subtle differences were observed between amended and unamended mesocosms. For penta-CDFs, an decreasing mol% ratio of *peri* vs. total chlorines and decreasing mol% ratio of lateral vs. total chlorines was observed in mesocosms amended with TeCB. This may indicate that the amendments affected pathways of dechlorination, but the differences between the amended and unamended mesocosms were not statistically significant. Analysis of congener patterns using Principle Components Analysis supported the observation that dechlorination was most pronounced during the first two years, since the congener patterns of the time zero measurements fell into a different PCA cluster than the congener patterns of samples taken in years 2 through 7. PCR-DGGE analysis of 16S rRNA genes revealed a diverse Chloroflexi community. This study showed evidence for dechlorination of weathered PCDFs in Kymijoki sediment mesocosms mediated by indigenous microorganisms.

Introduction

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) are generated mainly as unwanted byproducts from chlorine utilizing industries as well as thermal and combustion processes (Esposito et al., 1980; Fiedler et al., 1990; Rappe 1993; Kulkarni et al., 2008). Because of their extremely low water solubilities, low vapor pressures, and strong absorption to particles and surfaces (high K_{ow}) (Shiu et al., 1988), aquatic sediments and soils constitute typical sinks for PCDD/Fs in the environment. Several “hot spots” of heavy PCDD/Fs contamination have been documented (Wenning et al., 1993; Bertazzi et al., 1998; Bunge et al., 2001; Schecter et al.,

2001; Kiguchi et al., 2007, Weber et al., 2008), including sediments of the Kymijoki River in Finland, which contains PCDD/Fs up to 120-193,000 µg/kg d.w. (Salo et al., 2008).

Dechlorination of freshly spiked PCDD congeners in anaerobic microcosms and enrichment cultures has been observed (Adriaens & Grbic-Galic, 1994; Beurskens et al., 1995; Barkovskii & Adriaens, 1996; Ballerstedt et al., 1997; Kao et al., 2001; Bunge et al., 2001). This dechlorination can be stimulated by amending halogenated structural analogues as co-substrates, also known as "haloprimers" (Vargas et al., 2001, Fu et al., 2005; Ahn et al., 2005, 2007, 2008), as previously shown for PCBs (Bedard et al., 1998). Amendment with electron donors also enhanced dechlorination of aged PCDD/Fs in microcosms derived from Passaic River sediment (Albrecht et al., 1999). Bacteria of the phylum *Chloroflexi*, and the genus *Dehalococcoides* in particular, contain the only isolated bacterial species that are able to dechlorinate certain PCDD congeners, namely *Dehalococcoides* sp. strain CBDB1 (Adrian et al., 2000; Bunge et al., 2003) and *Dehalococcoides ethenogenes* strain 195 (Fennell et al., 2004). Furthermore, strain CBDB1 and another highly enriched culture, "*Dehalococcoides* strain DCMB5" (Ewald et al., 2007; Bunge et al., 2008) derive energy for growth during dechlorination of PCDDs. Members of *Dehalococcoides*-related *Chloroflexi* were reported to be responsible for dechlorination of various chlorinated compounds including PCBs (Cutter et al., 2001; Wu et al., 2002a; Yan et al., 2006; Park et al., 2011), chlorobenzenes (Wu et al., 2002b) and tri-CDDs (Ballerstedt et al., 2004), indicating their important roles in transforming chlorinated pollutants in the environment.

Unlike microcosms and enrichment cultures under laboratory conditions, the potential of indigenous microorganisms to dechlorinate weathered PCDD/Fs *in situ* has rarely been investigated. Nonetheless, dechlorination of weathered PCDDs has been reported in anaerobic microcosms derived from various sources (Barkovskii et al., 1994; Barkovskii and Adriaens, 1996; Albrecht et al., 1999; Yoshida et al., 2005), but limited information is available on the effectiveness of stimulating strategies for such processes. Loss of higher chlorinated PCDD congeners was observed in the recently collected sediments when compared with archived sediment cores from Lake Ketelmeer, the Netherlands (Beurskens et al., 1993). Increasing proportions of lower chlorinated PCDDs corresponding to decreasing proportions of octa-CDD correlated with sediment ages in Queensland sediment, Australia (Gaus et al.. 2001). Polytopic vector analysis (PVC) of PCDDs profiles in Passaic River sediment, New Jersey, suggested that about 2~4% of data variance was contributed by dechlorination (Barabás et al.. 2004), which was validated by the value for the ratio of 2,3,7,8-TCDD to the sum of all 2,3,7,8-substituted PCDDs (Barkovskii and Adriaens, 1996). These studies provide important links between laboratory dechlorination results and field analysis, and they support the occurrence of natural PCDD dechlorination *in situ*. However, detailed knowledge of the transformations occurring over time and the corresponding responses of the microbial community are lacking. More importantly, knowledge of the potential for biotransformation of weathered PCDFs in contaminated sediments is limited. Only 1,2,4,6,8-penta-CDF, 1,2,3,4,6,7,8-hepta-CDF, and 1,2,3,4-tetra-CDF have been studied using either soil- or sediment-derived microcosms containing pure or mixed cultures (Adriaens & Grbic-Galic, 1994; Fennell et al., 2004; Ahn et al., 2005; Liu et al., 2008). Consequently, there is an inadequate understanding of the potential for natural attenuation of weathered PCDFs by reductive dechlorination mediated by indigenous microorganisms.

In this study we incubated historically PCDF-contaminated sediment from Kymijoki, Finland in 30-L mesocosms at 18~21ºC with different amendments provided at the start of the experiment, including 1,2,3,4-tetrachlorobenzene (TeCB) as a "haloprimer", bioaugmentation with a mixed culture containing *Dehalococcoides ethenogenes* strain 195, and lactate and propionate as electron donors. The purpose of this study was to determine the potential for microbial reductive dechlorination of weathered PCDFs in Kymijoki sediments and to investigate the feasibility of stimulating such activities *in situ*. Sediment mesocosms were monitored over 7 years of incubation for the dechlorination of PCDFs and the response of the microbial community.

Materials and Methods

Treatment/Incubations of Contaminated Sediments. The Kymijoki River in Finland was heavily contaminated with PCDD/Fs from the 1940's to the 1980's due to chlorine bleaching in the paper industry and the manufacture and use of the chlorophenol fungicide Ky-5 in the sawmill industry along the river (Salo et al., 2008; Verkasalo, 2004; Verta et al., 1999). The major chlorinated compounds are polychlorinated phenols (PCPs), PCDDs, and PCDFs; among them 1,2,3,4,6,7,8- and 1,2,3,4,6,8,9-heptaCDFs and octa-CDF are the dominant congeners (Koistinen et al., 1995; Verta et al., 1999). Contaminated sediments were collected in 2002 by dredging from downstream of Myllykoski in River Kymijoki. The dredged sediments were mixed and aliquoted in 30 L plastic fermentation buckets with a 1-3 cm surface layer of site water, sealed and fitted with an air lock. At the beginning of the experiment select mesocosms were spiked with 25 μM 1,2,3,4-TeCB as haloprimer or amended with 200 mL of a mixed culture containing *Dehalococcoides ethenogenes* strain 195 as well as 1 mM lactate and propionate as electron donors. Mesocosm controls amended with electron donors only or no additions were set up at the same time. The four types of treatments were: (1) no addition, (2) electron donor only (designed "ED" hereafter), (3) electron donor plus TeCB ("TeCB"), and (4) electron donor plus *D. ethenogenes* strain 195 ("strain 195"). All mesocosm treatments were conducted in triplicate and incubated at room temperature (18~21 °C) to sustain the dechlorination capacity. At select time points (after 0, 2, 3, 5 and 7 years of the incubation) the fermentation buckets were opened, the sediments were mixed with a rod and 30 to 50 mL samples were collected in glass jars. The sediment samples were stored at -20 °C for chemical analysis of PCDFs and at -70 °C for microbial community analysis.

Analysis of PCDFs. Liquid/solid extraction was performed to quantify PCDFs and transformation products as follows: 3 g (wet weight) of sediment was extracted with 4 mL toluene and acetone (2:1) adding 625 μg (each) PCB 14 (3,5-DiCB), PCB 23 (2,3,5-TrCB), PCB 65 (2,3,5,6-TeCB) and PCB 166 (2,3,4,4',5,6-HxCB) as recovery surrogates. Extraction tubes were capped with Teflon-lined screw caps, mixed vigorously for 1 min, and sonicated in an ultrasound bath for 30 minutes before shaking overnight on a wrist-action shaker. The extraction mixtures were centrifuged at 2,500 rpm for 3 min before transferring the solvent phase to a clean 7 mL Teflon-stoppered glass vial. The extraction procedure was repeated 3 times, the upper solvent phase was pooled and finally evaporated down to a volume of 1 mL, which was then loaded onto toluene pre-rinsed Florisil (Sigma-Aldrich, St. Louis, MO) pipette columns and eluted with 6 mL toluene. The Florisil-cleaned sample was concentrated to 1 mL under a stream of N_2 and 625 μg (each) of PCB 30 (2,4,6-TrCB) and PCB 204 (2,2',3,4,4',5,6,6'-OcCB) as added internal standards. PCDF parent and daughter compounds were analyzed by Selected Ion

Table 10-1. The most abundant molecular ions and qualifying molecular ions of different PCDF and PCB congeners.

Congener	Most abundant Molecular Ion (*m/z*)	Qualifying Molecular Ion (*m/z*)
octa-CDF	442	444
hepta-CDF	408	410
hexa-CDF	374	376
penta-CDF	340	342
tetra-CDF	306	304
tri-CDF	272	270
di-CDF	238	236
mono-CDF	204	202
PCB 14	222	224
PCB 30 & 23	256	258
PCB 65	292	290
PCB 166	360	362
PCB 204	430	428

Monitoring (SIM) mode (**Table 10-1**) on an Agilent 6890 series gas chromatograph (GC) equipped with an Agilent 5973 series Mass Selective Detector (MSD) (GC-MS, Agilent Technologies, Inc., Santa Clara, CA) using a HP-5MS capillary column (60 m × 0.25 mm, 0.25 μm film thickness, J&W Scientific, Folsom, CA). The temperature cycle program was: 60 °C, increased by 10 °C/min to 170 °C, 2 °C/min to 200 °C, 5 °C/min to 220 °C, and hold at 220 °C for 16 minutes, and increased by 5 °C/min to 235 °C, hold at 235 °C for 7 minutes, and increased by 5 °C/min to 250 °C, and finally 1 °C/min to 280 °C, and a 5 min 300 °C hold. Helium was used as the carrier gas at a constant flow rate of 1.5 mL/min. Mono-, di-, tri-, tetra-, penta-, hexa-, hepta- and octa-chlorinated dibenzofuran congeners are abbreviated as mono-CDF, di-CDF, tri-CDF, tetra-CDF, penta-CDF, hexa-CDF, hepta-CDF and octa-CDF, respectively.

Detection, Identification and Quantification of PCDFs by PCB Surrogates and Internal Standards. Standards of TeCB, octa-CDF, 1,2,3,4,7,8-hexa-CDF, 1,2,3,7,8-penta-CDF, 2,3,4,7,8-penta-CDF, 2,3,7,8-tetra-CDF, 1,2,3,4-tetra-CDF, PCB 14, 23, 65, 166, 30 and 204 were purchased from AccuStandard Inc. (New Haven, CT). These congeners were therefore identified and quantified via their standards. 1,2,4,6,8-penta-CDF, 1,2,4,7,8-penta-CDF, 2,3,4,6,8-penta-CDF, 1,2,3,4,6,8-hexa-CDF, 1,2,4,6,7,8-hexa-CDF, 1,2,4,6,8,9-hexa-CDF, 2,3,4,6,7,8-hexa-CDF, 1,2,3,4,6,7,8-hepta-CDF, 1,2,3,4,6,8,9-hepta-CDF and OCDF were identified by their relative retention times only as per Humppi and Heinola (1985), since pure standards of these congeners were not available. For each congener, the most abundant

molecular ions and a qualifying ion were monitored to assure the correct identification (**Table 10-1**). PCB 14, PCB 23, PCB 65 and PCB 166 were used as surrogates to estimate the recovery efficiencies of weathered PCDFs from sediments. PCB 30 and 204 were used as internal standards to calculate response factors (RFs) for the quantification of the parent and daughter PCDFs in sediments according to the recovery of PCB surrogates throughout the extraction, cleanup and concentration procedures. RFs were calculated by equation (1):

$$RF = \frac{C_s}{C_{is}} \times \frac{A_{is}}{A_s} \qquad \text{(Equation 1)}$$

where, RF: response factor of PCB surrogates (PCB 14, 23, 65, 166) versus PCB internal standards (PCB 30, 204) respectively; C_s: PCB surrogate concentration; C_{is}: PCB internal standards concentration; A_s: integrated chromatographic area of PCB surrogate; A_{is}: integrated chromatographic area of PCB internal standards.

Weathered PCDFs and PCB surrogates were quantified by equation (2):

$$C_p = \frac{A_p}{A_{is}} \times C_{is} \times RF \qquad \text{(Equation 2)}$$

where, C_p: concentration of weathered PCDFs or PCB surrogate; A_p: integrated chromatographic area of PCDF congener or PCB surrogate; A_{is}: integrated chromatographic area of PCB internal standards; C_{is}: PCB internal standards concentration; RF: response factors of PCB surrogates (PCB 14, 23, 65, 166) versus PCB internal standards (PCB 30, 204) respectively.

According to their retention time (rt) ranges, PCB 30 (rt, 22.02 min) was used in quantification of PCB 14 (rt, 21.11 min), PCB 23 (rt, 24.76 min), PCB 65 (rt, 28.76 min), PCB 166 (rt, 47.49) and weathered PCDFs with less than three chlorines. However, due to their extremely low concentrations in Kymijoki sediment, no weathered PCDFs with less than three chlorines were detected in our analyses. PCB 204 (rt, 52.52 min) was used in quantification of weathered PCDFs (tetra: 40-50 min, penta: 50-60 min, hexa: 60-70 min, octa-CDF: 85 min). The average recovery efficiency of PCB surrogates ranged from 80~105% for PCB 14, 23, 65 and 166 (n=60, triplicate samples from 4 treatments at 5 time points). The detection limits for PCDFs were approximately 0.5 ng/g (d.w). Since dechlorination reactions result in the loss of mass but not in total molar concentration, the congener concentrations are calculated and reported in molar percentages (and molar fractions). Each congener was calculated in mol/g d.w., and its concentration was calculated in following equation (3):

$$Mol\% = 100\% \times \frac{C_i}{\sum_{i=1}^{n} C_i} \qquad \text{(Equation 3)}$$

where C_i is the concentration of congener *i* in mol/g d.w, ΣC_i is the sum concentrations of all congeners detected in same sample in mol/g d.w, and n is the number of congeners detected in each sample for each time point.

Since PCDF contains *peri* and lateral chlorines, dechlorination is expected to change their relative molar ratio among total chlorines for each PCDF homolog as calculated in following equation (4):

$$R_l = \frac{\sum_{i=1}^{n} L_i \times M_i}{h \times \sum_{i=1}^{n} M_i}, R_p = \frac{\sum_{i=1}^{n} P_i \times M_i}{h \times \sum_{i=1}^{n} M_i} \quad \text{(Equation 4)}$$

where, R_l and R_p are molar ratio of lateral or *peri* chlorines to total chlorines in each homolog; L_i and P_i are the number of lateral and *peri* chlorines for each congener; M_i is the mol% of each congener; h is 5, 6, 7 for penta, hexa and hepta-CDFs respectively. For homologs with all congeners identified, e.g. hepta-CDFs, $R_l + R_p = 1$. However, for the penta and hexa homologs, some congeners are unidentified, so $R_l + R_p$ does not necessarily equal 1 for these homologs.

Principle Component Analysis (PCA). The dechlorination pattern of weathered PCDFs among mesocosm treatments was confirmed by the PCA. The mol% of 20 PCDFs (**Table 10-1**) in 12 mesocosms after 0, 2, 3, 5, 7 years was used as the original data (59 observations and 20 variables). Zero mol% was assumed for PCDFs under detection limit. Three uncorrelated principle components (PCs) were calculated as linear combinations of the original data. PC1 (57.84%) and PC2 (37.87%) were used to detect correlations among the original PCDF profile that were more difficult to detect by direct observation (Abollino et al., 2002).

PCR-DGGE. Bulk DNA was extracted from mesocosm sediments by using the PowerSoil DNA purification kit (MoBio, CA) according to the manufacturer's instructions. Nested PCR amplification was performed to analyze the putative dechlorinating *Chloroflexi* community using previously designed primer sets as described previously (Krumins et al., 2009; Park et al., 2011). PCR reagents were purchased from GenScript (Piscataway, NJ). Denaturing gradient gel electrophoresis (DGGE) was performed according to our established protocol (Krumins et al., 2009; Park et al., 2011) using the Bio-Rad Dcode system (Bio-Rad laboratories, Hercules, CA). After electrophoresis, the gels were stained with ethidium bromide for 20 min and photographed on a UV transilluminator. Image analyses of the DNA profiles and band intensities were conducted using Quantity one® (version 4.5.0; Bio-Rad Laboratories, Hercules, CA). Major DGGE bands were excised from the gel, eluted in PCR-grade water overnight, re-amplified with the 2nd set of primers (341F-gc/534R) under the same conditions as described above, and analyzed by DGGE to confirm the band purity.

Sequencing and Phylogenetic Analysis. Purified single bands from DGGE gels were re-amplified with primers of 341F and 534R under the same PCR conditions as above. The resulting PCR products were separated by agarose gel electrophoresis, purified and pre-added 341F primer before sending to GeneWiz for sequencing (GeneWiz, South Plainfield, NJ). Homologous sequence searches were performed using BLAST (Altschul et al., 1999) and selected sequences aligned using CLUSTAL X (Thompson et al. 1997). Phylogenetic analyses by the Neighbor-joining method were conducted using MEGA version 4 (Tamura et al., 2007).

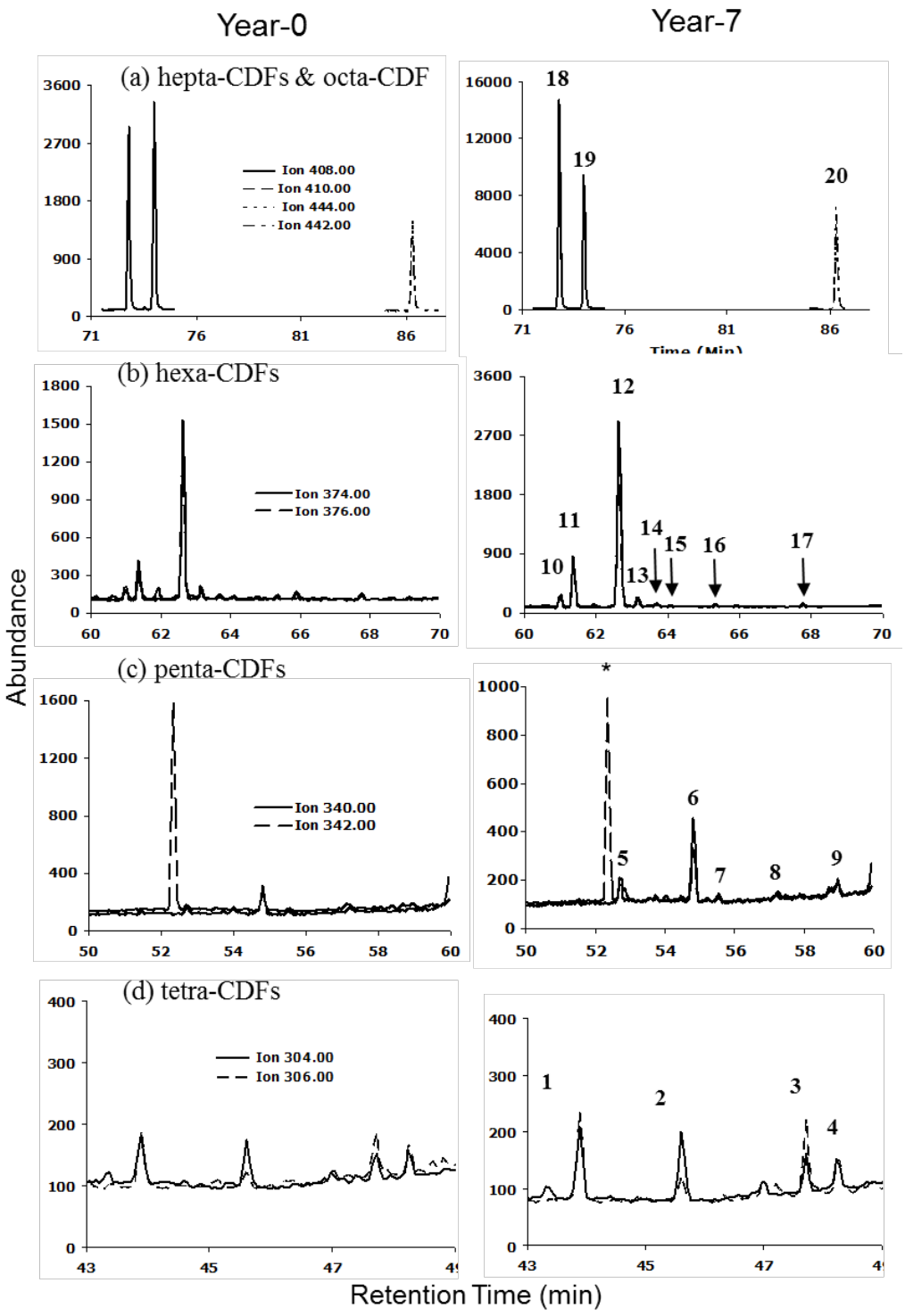


Figure 10-1. Selected ion chromatograms for the Kymijoki mesocosm amended with electron donor plus TeCB sampled at year 0 (left panels) and year 7 (right panels). Panel (a): hepta-CDFs and octa-CDF , panel (b): hexa-CDFs, panel (c): penta-CDFs , and panel (d): tetra-CDFs The peak labeled with a * is not a PnCDF congener since it does not contain the ions of 340 and 342 in the correct ratio (c – year 7).

Results

Weathered PCDF congeners in Kymijoki sediment mesocosms. The Kymijoki sediments contained a mixture of PCDF congeners. Altogether 20 peaks corresponding to PCDF congeners with four or more chlorines were detected, and among them, 14 peaks were identified. Two peaks could only be identified as hexa-CDFs and 4 peaks could only be identified as tetra-CDFs, but their exact chlorine substitution patterns were unknown (**Table 10-2**). Data for one triplicate of the TeCB-amended mesocosm is shown as an example (**Fig. 10-1**). In addition to the most

Table 10-2. Detected and identified congeners of weathered PCDFs in Kymijoki sediments at time 0.

Homolog	Peak #	Congener Name	Retention Time (min)	Conc. (ng/g d.w.)	Mol% (avg±SD)	# of *peri* Cl	# of lateral Cl
tetra-CDFs	01	tetra-CDF-1	43.84	49.0±13.3	0.53±0.12	---	---
	02	tetra-CDF-2	46.98	13.9±3.50	0.15±0.03	---	---
	03	tetra-CDF-3	47.66	35.9±7.10	0.40±0.05	---	---
	04	tetra-CDF-4	48.23	25.4±5.59	0.29±0.07	---	---
penta-CDFs	05	1,2,4,6,8-penta-CDF	52.69	32.6±15.1	0.30±0.09	3	2
	06	1,2,4,7,8-penta-CDF	54.80	104±30.9	0.99±0.28	2	3
	07	1,2,3,7,8-penta-CDF	55.49	15.3±2.09	0.16±0.04	1	4
	08	2,3,4,6,8-penta-CDF	57.20	16.2±3.17	0.18±0.06	2	3
	09	2,3,4,7,8-penta-CDF	60.57	47.7±30.5	0.48±0.22	1	4
hexa-CDFs	10	1,2,3,4,6,8-hexa-CDF	60.97	127±38.5	1.07±0.10	3	3
	11	1,2,4,6,7,8-hexa-CDF	61.37	350±109	2.82±0.27	3	3
	12	1,2,4,6,8,9-hexa-CDF	62.62	1561±345	12.4±1.82	4	2
	13	1,2,3,6,8,9-hexa-CDF	63.14	68.0±25.9	0.58±0.20	3	3
	14	hexa-CDF-5	63.65	21.1±4.51	0.21±0.03	---	---
	15	1,2,3,4,7,8-hexa-CDF*	64.05	13.1±4.61	0.12±0.05	2	4
	16	2,3,4,6,7,8-hexa-CDF	65.31	24.6±5.25	0.22±0.05	2	4
	17	hexa-CDF-8	67.71	30.3±11.9	0.27±0.11	---	---
hepta-CDFs	18	1,2,3,4,6,7,8-hepta-CDF	72.73	3281±1009	26.2±2.55	3	4
	19	1,2,3,4,6,8,9-hepta-CDF	73.93	4443±696	34.9±1.81	4	3
octa-CDF	20	octa-CDF	86.27	2491±758	17.8±2.08	4	4

abundant peaks of octa-CDF (peak 20), 1,2,3,4,6,8,9-hepta-CDF (peak 19) and 1,2,3,4,6,7,8-hepta-CDF (peak 18) (**Fig. 10-1a**), altogether eight hexa-CDF peaks were detected (**Fig. 10-1b**), including 6 known congeners identified by comparing their elution time and order based on Humppi and Heinola (1985) and two unknown isomers, peaks 14 and 16. Five peaks were detected and identified as penta-CDFs (**Fig. 10-1c**). Four peaks were detected as tetra-CDFs congeners (peaks 1 to 4) (**Fig. 10-1d**) but the chlorine substitution was not identified due to the unavailability of these tetra-CDF standards. Neither 1,2,3,4- nor 2,3,7,8-tetra-CDF were detected in Kymijoki sediment mesocosms. The identified congeners have different combinations of *peri* and lateral chlorine substitutions (**Table 10-2**).

Dechlorination of weathered PCDFs in Kymijoki sediment. Comparison of the selected ion chromatograms of all the PCDF congeners at time 0 vs. after different times of incubation (up to 7 years) showed changes in the relative abundance of different congeners over time, suggestive of reductive dechlorination. **Figure 10-1** depicts selected chromatograms for the TeCB mesocosm. Other mesocosms displayed similar trends. The chromatograms (**Fig. 10-1**) show distinct differences between year 0 and year 7, especially with respect to the abundance of 1,2,3,4,6,8,9-hepta-CDF (peak 19) and 1,2,3,4,6,7,8-hepta-CDF (peak 18). In all but the strain 195 mesocosm, the ratio of peak 18 to peak 19 changed significantly ($p < 0.05$) between year 0 and year 2, after which the changes in this ratio were not significant (**Fig. 10-2**). For example, the peak 18/peak 19 ratio increased from 0.7 ± 0.1 at time 0 to 1.0 ± 0.2 after 7 years in no-addition controls. The change in this ratio was greatest for the TeCB mesocosm, in which the peak 18/peak 19 ratio increased more than 80% from 0.7 ± 0.2 at time 0 to 1.3 ± 0.2 after 2 years and then remained stable above 1, with a slight decreasing trend over 3 to 7 years. In the strain

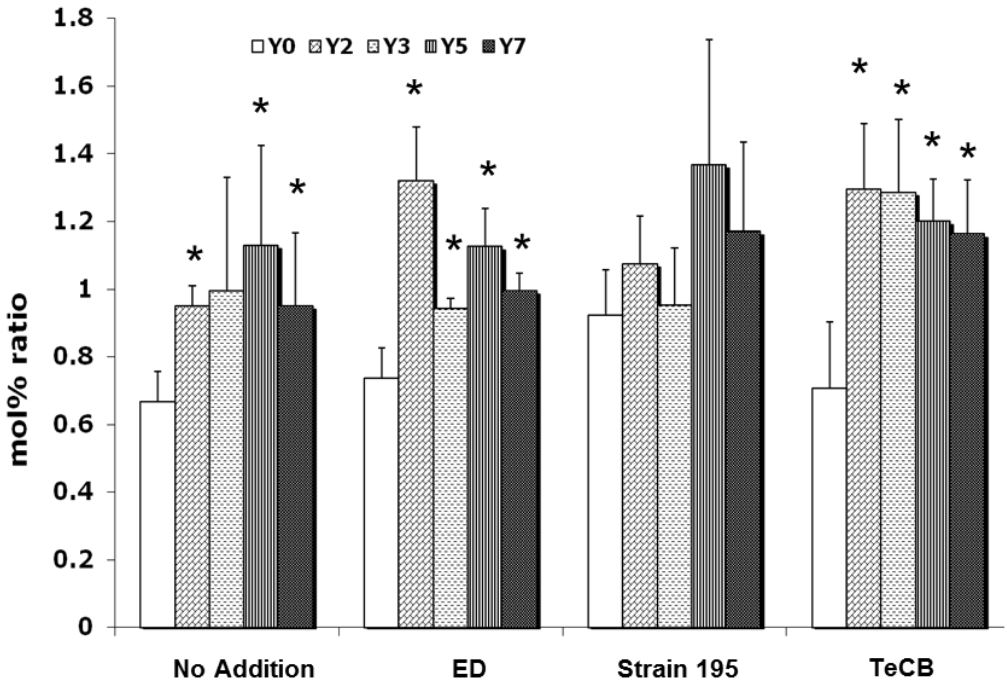


Figure 10-2. Average mol% ratio of 1,2,3,4,6,7,8-hepta-CDF (peak 18) vs. 1,2,3,4,6,8,9-hepta-CDF (peak 19) over 7 years incubation in different Kymijoki mesocosm treatments. * Indicates that the mol% ratio that is significantly different from that of year 0 by student T-test ($\alpha<0.05$).

195 mesocosm, the peak 18/19 molar ratio increased from 0.9 ± 0.1 at year 0 to 1.2 ± 0.3 at year 7, but this change was not statistically significant.

The chromatograms (**Fig. 10-1**) reveal other changes. In this particular mesocosm (the TeCB mesocosm), the relative abundance of 1,2,4,6,8,9-hexa-CDF (peak 12) decreased and 1,2,3,6,8,9-hexa-CDF (peak 13) increased over time. Also, the abundance of 2,3,4,7,8- (peak 9), 1,2,3,7,8,- (peak 7) and 1,2,4,6,8- (peak 5) penta-CDFs increased after 7 years. Finally, the abundance of tetra-CDF-3 (peak 3) and -1 (peak 1) increased after 7 years. Similar changes were observed in the ED mesocosm over the 7-year incubation period. PCA of the PCDF profiles in all mesocosms support these observations and show a clear separation of time 0 samples from all of the later time points (years 2, 3, 5, and 7) (**Fig. 10-3**).

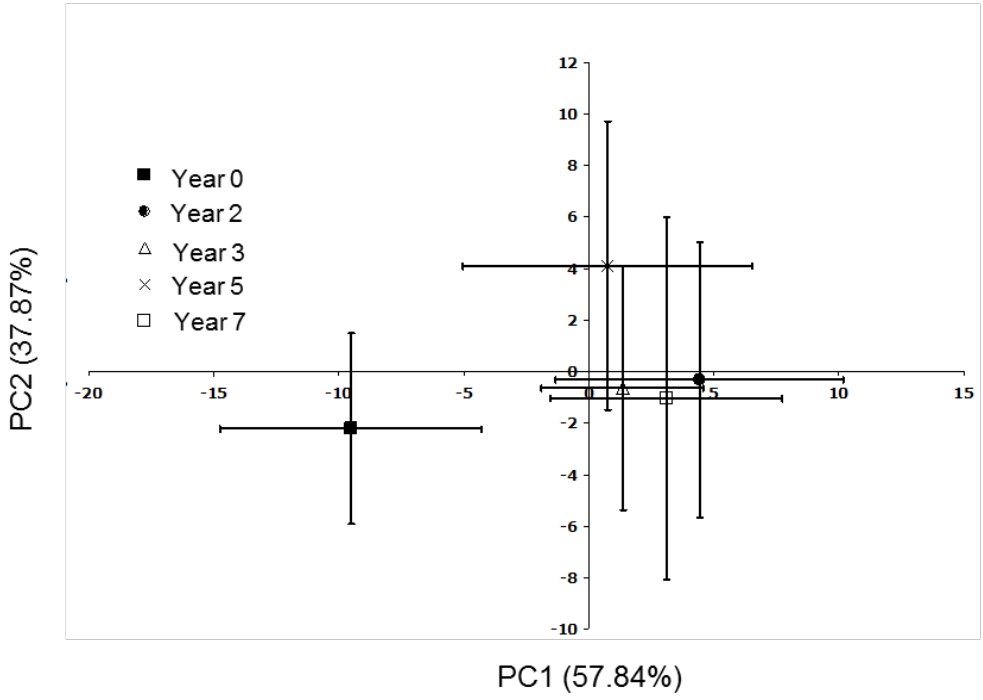


Figure 10-3. Principle Component Analysis (PCA) of PCDF congener profiles in all mesocosms over 7 years of incubation.

Effect of electron donors and TeCB amendment on the dechlorination of weathered PCDFs. All the PCDF chlorines can be classified into two groups: lateral chlorines for 2,3,7,8-substitutions or *peri* chlorines for 1,4,6,9-substitutions. Therefore, dechlorination changes their respective molar ratio among the total chlorines for congeners in each individual homolog (**Fig. 10-4**). *Peri*-dechlorination of penta-CDFs was observed in TeCB mesocosms, but not any of the other mesocosms (**Fig. 10-4b**). This *peri*-dechlorination can be demonstrated via the simultaneous changes in R_l and R_p. For hepta-CDFs, *peri* substitution decreased (i.e. R_p decreased) while lateral substitution (R_l) increased in all mesocosms over 7 years, but this trend was most pronounced in the TeCB mesocosm (**Fig. 10-4a**). For penta-CDFs (**Fig. 10-4b**), only the ED and TeCB mesocosms displayed significant changes in R_p and R_l. In both of these mesocosms, R_p increased from year 0 to year 2, but then later decreased. In the ED mesocosm, this decrease was apparent in year 3 but in subsequent years the ratio was not different from year

0. In the TeCB mesocosm, R_p was significantly less than year 0 only in year 7 (**Fig. 10-4b**). These observations suggest that *peri* dechlorination is preferred for weathered hepta-CDFs in all mesocosms and for weathered penta-CDFs in the TeCB and ED mesocosms. No significant changes in R_p and R_l were observed for hexa-CDFs in any of the treatments. Trends in R_p and R_l could not be observed for tetra-CDFs due to the presence of unknown congeners.

Comparison of the average mol% changes of weathered PCDFs in the TeCB mesocosm vs. the no-addition control after 7 years (**Fig. 10-5a & b**) showed that congeners that were less abundant in the TeCB mesocosm after 7 years are OCDF (peak 20), 1,2,3,4,6,8,9-hepta-CDF (peak 19), 1,2,4,6,8,9-hexa-CDF (peak 12), 1,2,4,6,7,8-hexa-CDF (peak 11), 1,2,3,4,6,8-hexa-CDF (peak 10), 1,2,3,7,8-peCDF (peak 7), 1,2,4,6,8-penta-CDF (peak 5), tetra-CDF-4 (peak 4). Congeners that were more abundant in the TeCB mesocosm were: 1,2,3,4,6,7,8-hepta-CDF (peak 18), 1,2,3,6,8,9-hexa-CDF (peak 13), 2,3,4,7,8-penta-CDF (peak 9), 1,2,4,7,8-penta-CDF (peak 6), tetra-CDF-3 (peak 3), tetra-CDF-1 (peak 1) (**Fig. 10-5c**). Most increasing congeners can be connected to the decreasing congeners by removing *peri* chlorines, indicating that amendment of TeCB might selectively stimulate *peri* dechlorination.

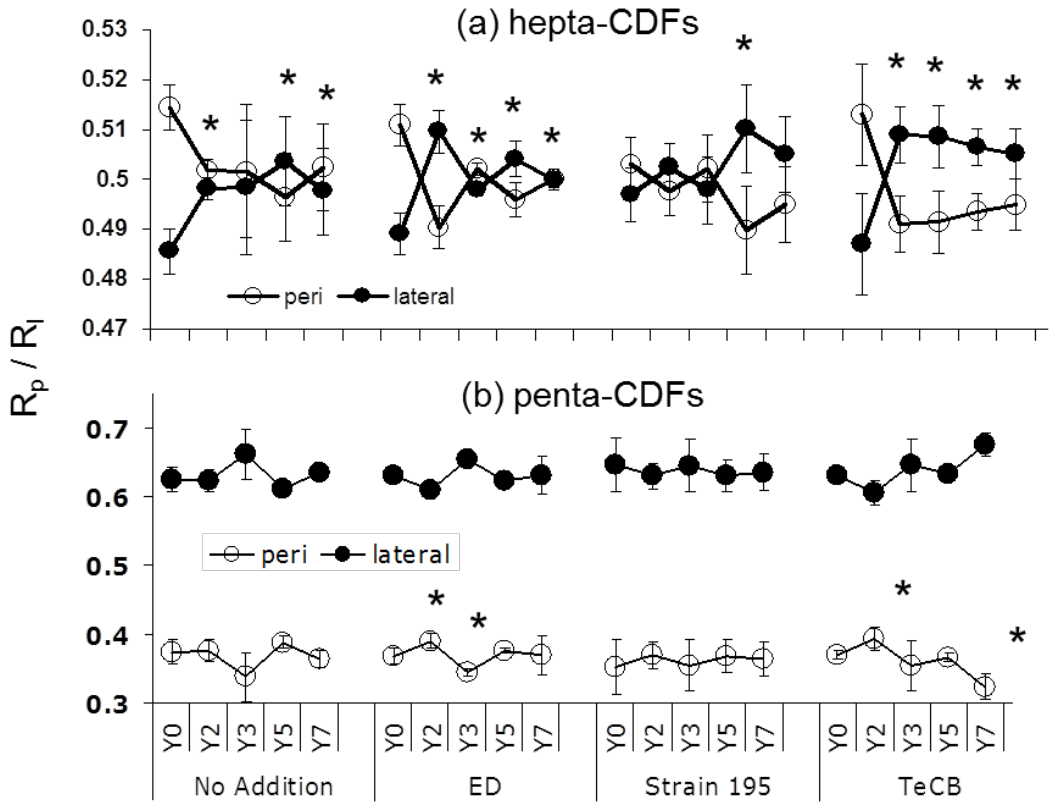


Figure 10-4. Changes in R_l and R_p over time for hepta-CDFs (a) and penta-CDFs (b) identified in Kymijoki mesocosms amended with no addition, electron donor only (ED), and electron donor plus *D. ethenogenes* strain 195 (strain 195) or plus 1,2,3,4-tetrachlorobenzene (TeCB). Y0, Y2, Y3, Y5, Y7 stands for year 0, 2, 3, 5, and 7. Value labeled with a * Indicated that was significantly different from the value at time 0 by student t-test (α<0.05). Both *peri* and lateral Cl ratio shared the same t-test value, only the t-test result of *peri* Cl was showed.

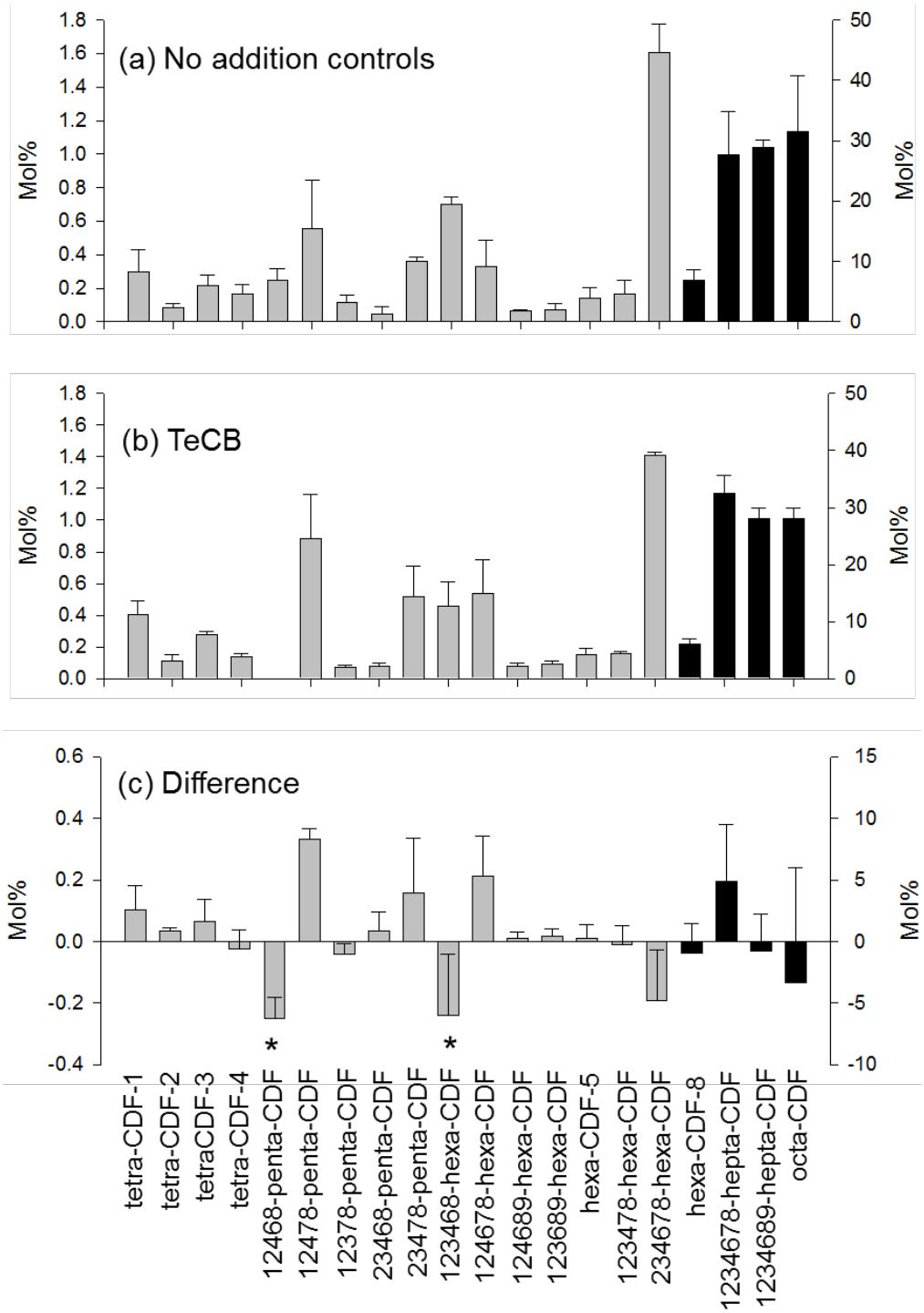
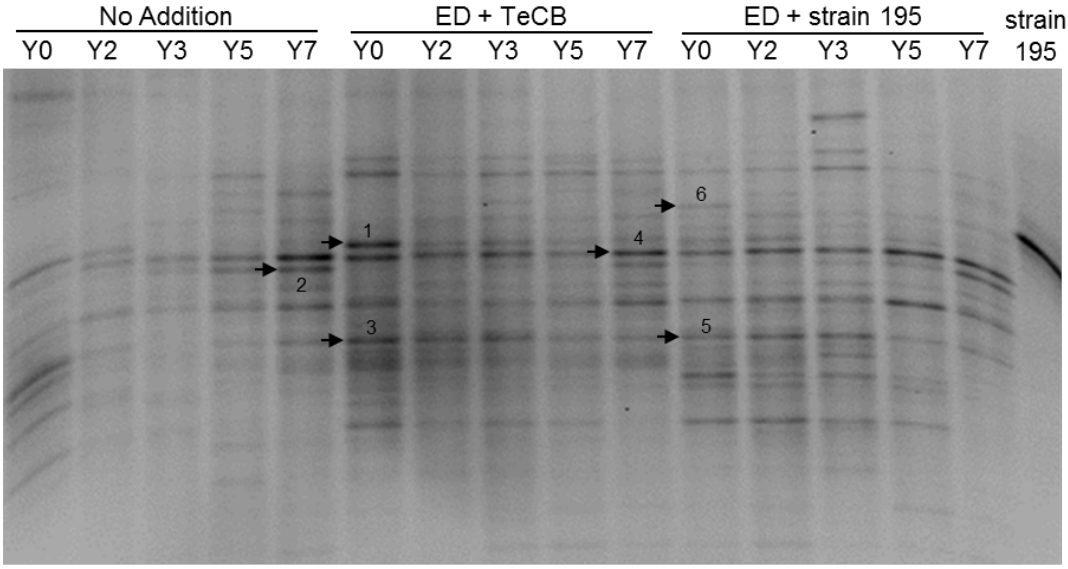


Figure 10-5. Average mol% of weathered PCDF congeners in no addition control (a), electron donor plus TeCB amendment (b), and difference of electron donor plus TeCB vs. no addition after 7 years (c). Bars are the average of triplicates ± SD. Grey bars are measured by left y axis and dark bars are measured by right y axis. * Indicates statistically significant difference between the TeCB mesocosm and the no-addition controls by student t-test (α<0.05).



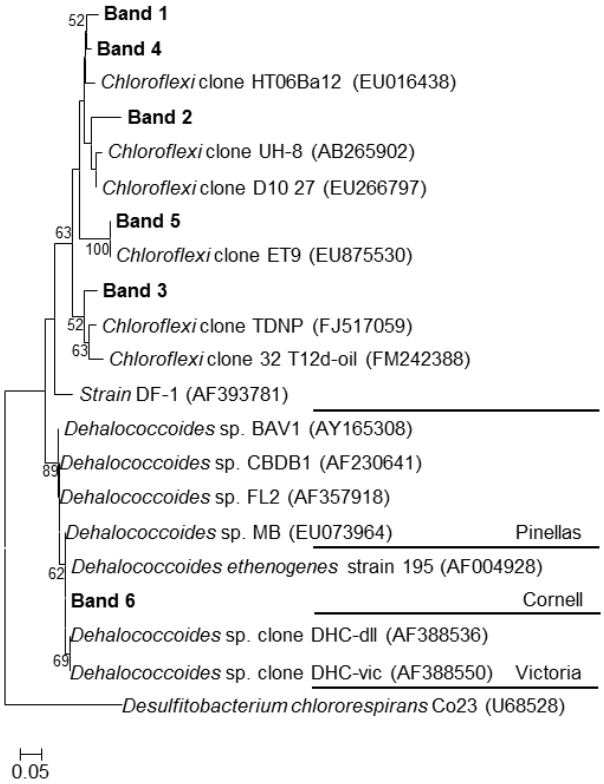


Figure 10-6. Nested PCR-DGGE analysis of putative *Chloroflexi* 16S rRNA genes in Kymijoki mesocosms over 7 years of treatment (a). 6 DGGE single bands as highlighted above were purified and sequenced for phylogenetic analysis to make a neighbor-joining tree with related *Chloroflexi* 16S rRNA genes from published sequences (b). Bootstrap values are indicated at the branch points. The tree was derived from variable region 3 of 16S rRNA genes, which was able to distinguish three subgroups of *Dehalococcoides* species.

Analysis of Putative Dechlorinating *Chloroflexi* Community. The community structure of *Chloroflexi* in each treatment was characterized in order to identify the microbial populations that may be responsible for dechlorination of weathered PCDFs in the mesocosms. The microbial succession in the mesocosms monitored by PCR-DGGE analysis of the 16S rRNA genes using *Chloroflexi*-specific primers (**Fig. 10-6**) showed a diverse *Chloroflexi* community. Six dominant DGGE bands highlighted in Figure 7 were purified and sequenced. Phylogenetic analysis (**Fig. 10-6**) showed that their sequences clustered with *Dehalococcoides* spp. and various uncultured *Chloroflexi* found previously in dechlorinating microbial consortia or methanogenic environments containing benzenes (Winderi et al., 2008; Hori et al., 2007; Kunapuli et al., 2008). Among them, Band 6 was identical to *Dehalococcoides ethenogenes* strain 195.

Discussion

This study showed three lines of evidence for dechlorination of weathered PCDFs in Kymijoki sediment mesocosms during 7 years of incubation. The first is the shifts of PCDF congener patterns after 2 years. PCA analysis of these congener patterns showed that the year 0 congener patterns clustered together and separated from those of all the other years (**Fig. 10-3**). The second line of evidence was the changing ratio of 1,2,3,4,6,7,8-hepta-CDFs to 1,2,3,4,6,8,9-hepta-CDF (i.e. the peak 18/peak 19 ratio), which increased in all mesocosms over 7 years of incubation (**Fig. 10-2**). Third, the ratio of *peri* chlorines to total chlorines for hepta-CDFs decreased in all mesocosms over 7 years of incubation (**Fig. 10-4a**). This study represents the first time that dechlorination of weathered PCDFs has been demonstrated.

Most of the PCDF dechlorination appears to have occurred during the first two years of the study. Because the extent of dechlorination in those first two years appears to be the same in all mesocosms, it is likely that this enhancement was caused by some factor in common to all mesocosms. The increase in temperature of the sediments versus conditions prevailing in the Kymijoki River may have stimulated a burst of dechlorination in the early years of this experiment. As time progressed, the sediments appeared to have reached a new steady state in which dechlorination proceeded at a slower rate. Despite this, there are some small differences apparent between the amended and unamended mesocosms that may indicate that amendment with the haloprimer TeCB enhances dechlorination of weathered PCDFs. First, the TeCB mesocosm displayed a more obvious change in the peak 18/peak19 ratio that appeared by year 2 and persisted throughout the 7 year experiment. Second, the chromatograms from the TeCB mesocosm also displayed shifts in the relative abundances of hepta-CDFs, hexa-CDFs, and penta-CDFs that were not apparent in the other mesocosms (**Fig. 10-1a**). For example, the relative abundance of four penta-CDFs (**Fig. 10-1c**) and two tetra-CDFs (**Fig. 10-1d**) increased after 7 years incubation only in the TeCB mesocosm. Third, the decrease in the prevalence of *peri* chlorines (R_p) was most pronounced in the TeCB mesocosm (**Fig. 10-4b**). Fourth, comparing the no-addition control to the TeCB mesocosm revealed clear differences. Four congeners (234678-hexa-CDF, 123468-hexa-CDF, 12378-penta-CDF, and 12468-penta-CDF) were less prevalent in the TeCB mesocosm at year 7, possibly indicating that they were dechlorinated, and three congeners (tetra-CDF-1, tetra-CDF-2, and 12478-penta-CDF) were more abundant in the TeCB mesocosm, possibly indicating that they were products of dechlorination (**Fig. 10-6c**). Moreover, these shifts in the relative abundance of congeners in the

TeCB mesocosm may be explained by invoking the pathways via *peri* dechlorination pathways. These lines of evidence suggest that haloprimer amendments may affect the rates and pathways of dechlorination, but the differences between the unamended and TeCB mesocosms are small and in some cases not significant, so further work is needed to determine the importance of haloprimers, such as TeCB, in stimulating dechlorination of weathered PCDFs.

We have proposed a preferential *peri*-dechlorination scheme that could explain the decrease of highly chlorinated congeners and the corresponding increase of less chlorinated congeners based on the PCDF congener patterns observed in the TeCB mesocosm. This possible *peri*-preferred dechlorination may have the risk of generating toxic intermediates, such as 2,3,4,7,8-penta-CDF and 2,3,7,8-tetra-CDF, both of which have higher Toxic Equivalency Factors than OCDF and the HpCDF congeners (Van den Berg et al., 2006). However, more research is needed to determine whether this should be a concern for Kymijoki sediments. Dechlorination patterns proposed for PCDDs (Fueno et al., 2002; Lynam et al., 1998) imply that lateral chlorines are more amenable for dechlorination due to the positive charges of lateral carbons and marginal preference of chlorines on them. In contrast, dechlorination mediated by microorganisms in other sediments has been shown to preferentially remove *peri* chlorines. For example, Barkovskii and Adriaens (1996) indicated that *peri*-dechlorination of 2,3,7,8-substituted hepta to penta-CDDs produced 2,3,7,8-tetra-CDD. In addition, *peri*-lateral dechlorination was shown to occur in a microbial consortium eluted from dioxin-contaminated Passaic River sediments with non-2,3,7,8-subsituted congeners as the substrates. These observations are in agreement with what we report here for the dechlorination of weathered PCDFs in Kymijoki sediments. Similarly, dechlorination of spiked 1,2,3,4-/ 2,3,7,8-tetra-CDDs and 1,2,3,7,8-penta-CDD by *Dehalococcoides* sp. strain CBDB1 proceeded via a *peri*-lateral dechlorination sequence (Bunge et al., 2003). However, in contrast, a mixed culture containing *D. ethenogenes* strain 195 mediated a lateral-*peri*-dechlorination sequence for spiked 1,2,3,4,7,8-hexa-CDF and 1,2,3,4-tetra-CDD (Liu & Fennell, 2008; Fennell et al., 2004).

Dehalococcoides spp. are widespread in the environment (Dennis et al., 2003; Duhamel et al., 2004; Fennell et al., 2001; Gu et al., 2004; He et al., 2003; Hendrickson et al., 2002; Kassenga et al., 2004; Löffler et al., 2000; Major et al., 2002; Richardson et al., 2002; Smits et al., 2004) and have been shown to dechlorinate PCDD/Fs (Bunge et al., 2001, 2003; Yoshida et al., 2005; Hiraishi et al., 2005; Ahn et al., 2007, 2008). Our previous study demonstrated the increase of a *Dehalococcoides* population along with the dechlorination of weathered PCBs in Anacostia River (Washington, DC) sediment microcosms, suggesting their potential role in PCB dechlorination (Krumins et al., 2009; Park et al., 2011). Here we show the presence of diverse *Chloroflexi* community in Kymijoki sediment mesocosms (**Fig. 10-6**); however, no noticeable community shift corresponding to the PCDF dechlorination was observed over seven years of incubation. These observations suggest a role of *Chloroflexi* in mediating dechlorination of weathered PCDFs *in situ*. Since we specifically targeted only *Chloroflexi*, it is possible that other bacteria contributed to dechlorination but were missed by this method.

In conclusion, Kymijoki sediments appear to contain active indigenous dechlorinating bacteria that reductively dechlorinate weathered PCDFs. Knowledge of the microbial community members in the contaminated sediments, especially those whose presence correlates with activity and dechlorination, can potentially be valuable in detecting, stimulating and cultivating the

relevant dechlorinating populations. The question of whether abiotic reductive dechlorination of PCDD/Fs (Adriaens et al., 1996; Lee & Batchelor, 2004) is involved here should also be clarified by further study. Understanding of the microbial attenuation of weathered PCDFs in sediments like Kymijoki will be of crucial importance for risk assessment and potential development of strategies for bioremediation of contaminated sites.

11. Enriching for Microbial Reductive Dechlorination of Polychlorinated Dibenzo-*p*-dioxins and Dibenzofurans

(*Liu H, Park J-W, Häggblom MM. Enriching for Microbial Reductive Dechlorination of Polychlorinated Dibenzo-p-dioxins and Dibenzofurans. Unpublished*)

Abstract

Anaerobic enrichment cultures derived from historically contaminated Kymijoki River sediment (Finland) dechlorinated 1234-tetrachloro-dibenzo-*p*-dioxin (1234-tetra-CDD), 1234-tetrachlorodibenzofuran (1234-tetra-CDF) and octachlorodibenzofuran (octa-CDF). The rate and extent of 1234-tetra-CDD dechlorination was enhanced by addition of pentachloronitrobenzene (PCNB) as a halogenated co-substrate. 1234-Tetra-CDD was dechlorinated via 123-tri-CDD and 124-tri-CDD as transient intermediates mainly to 23-di-CDD and 2-mono-CDD over 13 months. Dechlorination of 1234-tetra-CDF was slower than that of 1234-tetra-CDD. PCNB enhanced *peri* dechlorination of 1234-tetra-CDD, but had minimum effect on lateral dechlorination. Dechlorination of spiked octa-CDF was observed with the production of hepta-, hexa-, penta- and tetra-CDFs over 5.5 months. Analysis of the *Chloroflexi* community by denaturing gradient gel electrophoresis (DGGE) showed an increase of two major phylotypes. Phylogenetic analysis of the 16S rRNA gene sequences indicated that one of the major phylotypes was identical to the Pinellas subgroup of *Dehalococcoides*. A set of twelve putative reductive dehalogenase (*rdh*) genes increased in abundance with addition of 1234-tetra-CDF, 1234-tetra-CDD and/or PCNB. Our findings provide evidence of octa-CDF dechlorination and the stimulating of *peri* dechlorination by PCNB. This information will aid in understanding the impact of indigenous microbial communities on the fate of weathered PCDFs and ultimately in developing strategies for bioremediation of PCDD/F contaminated sediments.

Introduction

Sediments are important environmental sinks for polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) emitted from combustion sources or discharged from various industrial processes. Microbial reductive dechlorination under anaerobic conditions, typical of aquatic sediments, is the most important biological process that may transform PCDD/Fs and potentially decrease their toxicity through the removal of lateral chlorines (Ballerstedt et al. 1997; Vargas et al. 2001; Bunge et al. 2001, 2003; Gruden et al. 2003; Fennell et al. 2004; Ahn et al. 2005, 2007; Yoshida et al. 2005; Liu & Fennell 2008; Bunge et al. 2009). However, the dechlorination of PCDD/Fs is notoriously slow, which may be attributed to their limited bioavailability in the environment and to their overall chemical stability. The addition of alternate halogenated co-substrates has been shown to enhance the reductive dechlorination of PCDD/Fs (Vargas et al. 2001; Ahn et al. 2005, 2007; Buerskens et al. 1995), suggesting that the activity of PCDD/F dechlorinating microorganisms could be stimulated by appropriate co-substrates.

Different dechlorination routes of spiked 1234-tetra-CDD via removal of *peri* and/or lateral chlorines have been observed (Ballerstedt et al. 1997; Vargas et al. 2001; Bunge et al. 2001; Beurskens et al. 1995). These might be the result of differences in the microbial communities and/or diverse metabolic activities in response to specific environmental contaminants or

enrichment by co-substrates (Vargas et la. 2001; Bunge et al. 2001, 2003; Ahn et al. 2008). Two known isolates, *Dehalococcoides* sp. strain CBDB1 (Bunge et al. 2003) and *Dehalococcoides ethenogenes* strain 195 (Fennell et al. 2004; Liu & Fennell 2008; Lie et al. 2010), and one highly enriched *Dehalococcoides* sp. DCMB5 culture (Bunge et al., 2008) have been reported for their capability to dechlorinate CDD/Fs. Both strains 195 and CBDB1, as well as other *Dehalococcoides* spp., contain multiple genes predicted to encode for enzymes mediating reductive dehalogenation (*rdh* genes) (Hölscher et al. 2004; Kube et al. 2005; Seshadri et al. 2005). Therefore, analysis of *rdh* gene profiles may be a good tool to assess community changes in response to dechlorination. The detection of *Dehalococcoides*-like species in enrichment cultures dechlorinating CDD/Fs (Ahn et al. 2007; Yoshida et al. 2005; Ballerstedt et al., 2004; Liu et al. 2011) or PCBs (Yan et al. 2006; Bedard et al. 2007; Fagervold et al. 2007; Krumins et al., 2009; Park et al 2011) suggests their importance in the environmental biotransformation of these compounds. While there have been a number of reports for CDDs, dechlorination of CDFs has only been shown for 1234-tetra-CDF (Fennell et al. 2004; Ahn et al. 2005), 123478-hexa-CDF (Liu & Fennell 2008), 12468-penta-CDF, and 1234678-hepta-CDF (Adriaens & Grbic-Galic 1994; Adriaens et al. 1995).

Sediments of the River Kymijoki in Finland have some of the highest reported PCDD/Fs contamination levels in the world, up to 120-193,000 µg/kg d.w. (Salo et al. 2008). Octa-CDF, 1234678- and 1234689-hepta-CDFs are the most abundant congeners in Kymijoki sediment. Therefore, knowledge about possible dechlorination of these congeners is important for assessing the potential for remediation of these contaminated sediments. Our objectives were to assess the dechlorination of 1234-tetra-CDD/F and octa-CDF by enrichment cultures derived from historically contaminated Kymijoki sediment, to examine the "priming" effect of PCNB on dechlorination; and the microbial community response to different treatments.

Materials and Methods

Chemicals. Chlorinated dibenzo-*p*-dioxin congeners are abbreviated as mono-CDD, di-CDD, tri-CDD, and tetra-CDD, respectively. Chlorinated dibenzofuran congeners are abbreviated as mono-CDF, di-CDF, tri-CDF, tetra-CDF, penta-CDF, hexa-CDF, hepta-CDF and octa-CDF, respectively. Mono-, di-, tri-, tetra- and penta-chlorinated anilines (CAs) are abbreviated as MoCA, DiCA, TrCA, TeCA and PCA. 1234-Tetra-CDD, 123- and 124-tri-CDDs, 13- and 23-di-CDDs, 1- and 2-mono-CDDs, 1234-tetra-CDF, 123-tri-CDF, 24-di-CDF, 2- and 4-mono-CDFs, octa-CDF, 22'5-PCB and PCNB were purchased from AccuStandard Inc. (New Haven, CT). Standards of expected PCNB transformation and dechlorination products, including PCA, 2345- and 2356-TeCA, 234-, 245- and 246-TrCA, 24- and 25-DiCA, 4- and 2-MoCA, were purchased from UltraScientific (North Kingstown, RI).

Enrichment Culture Setup. Enrichment cultures were established with contaminated sediment obtained from downstream of Myllykoski in Kymijoki River, Finland, which was heavily contaminated with PCDD/Fs from production of the chlorophenolic fungicide Ky-5 production during the 1940's - 1980's (Malve et al., 2003). Sediments collected from Kymijoki River were initially incubated in 30-L drums at room temperature since 2002 (Liu et al. 2011) and subsamples used for enrichment cultures were transferred into glass jars, sealed, and stored at 4 ˚C until used. Anaerobic enrichment cultures were established as described previously (Vargas et

al., 2001; Ahn et al. 2005). Briefly, 1 g of dry sterile sediment was added to each culture flask and spiked with a stock solution of 1234-tetra-CDD, or 1234-tetra-CDF and/or PCNB in toluene. Toluene was allowed to evaporate under a sterile N_2 purge, leaving a coating of 1234-tetra-CDD, or 1234-tetra-CDF and/or PCNB on the dry sediment. Enrichment cultures were prepared by dispensing 40 mL volume of well-mixed 20% (v/v) sediment slurry to each 1234-tetra-CDD, or 1234-tetra-CDF and PCNB-spiked 50 mL serum bottle under 70%/30% N_2/CO_2. The anaerobic medium used for enrichment cultures was prepared as described previously (Fennell et al. 2004). Cultures were capped with poly(tetrafluoroethene) (PTFE)-coated butyl rubber septa and crimped with aluminum caps. The resulting nominal concentrations of 1234-tetra-CDD, 1234-tetra-CDF and PCNB (assuming no partitioning and uniform distribution throughout the enrichment culture volume) was 50 µM. Sodium lactate, sodium acetate and sodium propionate were added as electron donors to the designated treatments to a final concentration of 500 µM each. Killed controls were prepared by autoclaving at 121 ˚C for 30 min on 3 consecutive days. Five types of enrichments were established: (1) electron donor only (designed "ED" hereafter), (2) electron donor and 1234-tetra-CDF ("ED+TCDF"), (3) electron donor and 1234-tetra-CDD ("ED+TCDD"), (4) electron donor, 1234-tetra-CDD, and PCNB ("ED+TCDD+PCNB"), (5) killed control amended with electron donor, 1234-tetra-CDD, 1234-tetra-CDF, and PCNB ("killed"). All cultures were established in triplicate and incubated at room temperature and sampled over a 13-month period (time 0 samples were taken after 3 days when killed controls were ready) for both chemical and microbial community analyses.

Parallel anaerobic enrichment cultures were prepared in triplicate as described above to study octa-CDF dechlorination in the presence of PCA and 1234-tetra-CDD or 1234-tetra-CDF. Sodium lactate, sodium acetate and sodium propionate were added as electron donors to the designated treatments to a final concentration of 500 µM each, and re-spiked every 3 months. The resulting nominal concentrations of octa-CDF, PCA, 1234-tetra-CDD, and 1234-tetra-CDF (assuming no partitioning and uniform distribution throughout the microcosm volume) were 20 µM, 20 µM, and 10 µM, respectively. Killed controls were prepared as above. Three types of enrichment were established: (1) 1234-tetra-CDD, PCA, and octa-CDF ("TCDD+PCA+OCDF"), (2) 1234-tetra-CDF, PCA, and octa-CDF ("TCDD+PCA+OCDF"), (3) killed control amended with 1234-tetra-CDD, 1234-tetra-CDF, PCA, and octa-CDF ("killed"). All cultures were incubated at room temperature and sampled after 0 (time 0 samples were taken after 3 days), 3 and 5.5 months for chemical analyses.

Analytical Methods. For sampling, the cultures were shaken thoroughly and 2 mL of slurry was withdrawn with a sterile syringe flushed with oxygen-free N_2. Sediment samples were extracted with toluene:acetone (1:1, v/v) as described by Vargas et al. (2001) to analyze PCDD/Fs and PCNB/CAs. The resulting samples were analyzed by gas chromatography-mass spectrometry (GC-MS) and selected ion monitoring using an Agilent 6890 series gas chromatograph equipped with a 5973 series Mass Selective Detector (MSD) and a DB-5MS fused silica column (30 m, 0.25 mm i.d., film thickness 0.2 µm, J&W Scientific, Folsom, CA). The temperature program for mono- to tetra-CDD/Fs was as follows: 70 ˚C initial temperature, increased by 20 ˚C/min to 230 ˚C, 10 ˚C/min to 250 ˚C, and finally 15˚C/min to 300 ˚C. The temperature program for tetra- to octa-CDFs was as described in Liu et al. (2011). PCNB and its transformation products were analyzed from the same sample by GC-MS using the following temperature program: 60 ˚C initial temperature, increased by 12 ˚C/min to 180 ˚C, and then by 15 ˚C/min to 250 ˚C.

Spiked 1234-tetra-CDD, 1234-tetra-CDF, octa-CDF, PCNB, PCA and their dechlorination products, as well as the historic PCDFs were detected and identified based on the retention times of standards and their most abundant molecular ions, qualifying ions were monitored to assure the correct identification as follows (m/z): 256, 258, 22'5-PCB; 442, 444, octa-CDF; 408, 410, hepta-CDF; 374, 376, hexa-CDF; 340, 342, penta-CDF; 322, 320, tetra-CDD; 306, 304, tetra-CDF; 286, 288, tri-CDD; 270, 272, tri-CDF; 252, 254, di-CDD; 236, 238, di-CDF; 218, 220, mono-CDD; 202, 204, mono-CDF; 297, 295, PCNB; 267, 265, PCA; 231, 229, TeCA; 195, 197, TrCA; 161, 163, DiCA; 127, 129, MoCA. For PCDF congeners that are not commercially available, only the number of chlorine substitutions was determined. Of the PCNB/PCA dechlorination products, 24- and 25-DiCA co-eluted and could not be separated by GC-MS analysis.

Quantification of mono- to tetra-CDD/F, PCDFs, PCNB and CAs was performed against comparison to a 5-point curve (5 to 50 µM). For unknown historical PCDFs as well as unknown 1234-tetra-CDF and octa-CDF dechlorination products, quantification was based on the response factors of available congeners in the same homolog group. The results for each congener of PCDD/Fs or CAs as well as PCNB are presented by expressing each compound as a mole percent (mol%) of the total concentration of PCDD/Fs or PCNB plus CAs detected at each sampling point. Data are presented as the average of triplicate data points ± standard deviation (SD). The total amount of 1234-tetra-CDD, 1234-tetra-CDF and PCDFs recovered from each sample varied because of slight differences in aqueous-phase/sediment-phase volumes sampled at each time point. The extraction efficiency for 1234-tetra-CDD, 1234-tetra-CDF and octa-CDF spiked to sediment slurries in this study was approximately 80 ~ 90 % and the detection limit was approximately 2 ppt (~0.01 nM) for 1234-tetra-CDD and 1234-tetra-CDF and 10 ppt (~0.03 nM) for octa-CDF. The method of data presentation assumes that no anaerobic degradation of the dibenzo-*p*-dioxin, dibenzofuran and benzene structures occurred and that the PCDD/Fs and PCA underwent no significant reactions other than dechlorination.

PCR-DGGE Analysis. Bulk DNA was extracted by using the PowerSoil DNA isolation kit (MO BIO, Solana Beach, CA) according to the manufacturer's instructions. Nested PCR amplification was performed to analyze the putative dechlorinating *Chloroflexi* community as described previously (Krumins et al. 2009; Park et al. 2011). PCR reagents were purchased from GenScript (Piscataway, NJ). Denaturing gradient gel electrophoresis (DGGE) was performed according to our established protocol (Krumins et al. 2009; Park et al. 2011) using the Bio-Rad Dcode system (Bio-Rad laboratories, Hercules, CA). DGGE gel picture was inverted by PhotoShop for clarity. After electrophoresis, the gels were stained with ethidium bromide for 20 min and photographed on a UV transilluminator. Image analyses of the DNA profiles and band intensities were conducted using Quantity One® (version 4.5.0; Bio-Rad Laboratories, Hercules, CA). Major DGGE bands were excised from the gel, eluted in PCR grade water overnight, re-amplified with the 2nd set of primers (341F-gc/534R) under the same conditions as described above, and analyzed by DGGE to confirm band purity.

Sequencing and Phylogenetic Analysis. The eluted single DGGE bands were amplified with 341F and 534R under the same PCR conditions as described above. The resulting PCR products were purified using a PCR purification kit (MoBio Laboratories Inc., Carlsbad, CA), and used as

templates for DNA sequencing performed by GeneWiz Inc. (North Brunswick, NJ). Sequence similarity searches and alignments were performed by BLAST (Altschul et al. 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007).

PCR and intensity analysis of reductive dehalogenase genes. Twelve sets of reductive dehalogenase gene (*rdh*) were amplified and quantified based on Park et al. (2011). PCR products were loaded in one 1.5% agarose gel with same-sized wells for electrophoresis and a resulting gel image was used to measure relative band intensities by ImageJ quantification software (ver. 1.33u, National Institutes of Health, USA) according to the manufacturer's protocol (http://rsbweb.nih.gov/ij/). The band intensity of each *rdh* gene was normalized to that of enrichment culture control amended with ED.

Results and Discussion

Dechlorination of 1234-tetra-CDF and 1234-tetra-CDD. Dechlorination of 1234-tetra-CDF commenced after 9 months, with the detection of three tri-CDFs, including 123-tri-CDF, and three di-CDFs, including 24-di-CDF (**Fig. 11-1a**). The mono-CDF congener detected by the end of 11 months was neither 2- or 4-mono-CDF. After 13 months, approximately 50 mol% of 1234-tetra-CDF remained, with mono-CDF accounting for approximately 12 mol% of all CDFs.

Dechlorination of 1234-tetra-CDD without PCNB started after 8 months (**Fig. 11-1b**) with the detection of 123-tri-CDD and 13- and 23-di-CDDs as metabolites. 23-Di-CDD reached its peak amount of 14 ± 5.5 mol% after 11 months, followed by depletion to 4.5 ± 0.6 mol% by the end of 13 months. 2-Mono-CDD was observed after 9 months, and became the dominant daughter congener, approximately 58 mol%, after 13 months, when the remaining 1234-tetra-CDD was approximately 34 mol%.

With PCNB as co-substrate, dechlorination of 1234-tetra-CDD started after 5 months (**Fig. 11-1c**). At 7 months, 13- and 23-di-CDDs (14 mol% in total) and 2-mono-CDD (15 mol%) were detected, with approximately 68 mol% of 1234-tetra-CDD remaining in the culture. 23-Di-CDD reached its peak of 56 ± 8.6 mol% after 8 months, followed by depletion to 11 ± 2.8 mol% by the end of 13 months, when the remaining 1234-tetra-CDD was only 9 mol%, and 2-mono-CDD was more than 77 mol%. No dechlorination of 1234-tetra-CDD and 1234-tetra-CDF was observed in killed controls over 13 months in the presence of PCNB (data not shown).

Our results show that 1234-tetra-CDF was more resistant to dechlorination than 1234-tetra-CDD, indicated by the later onset of dechlorination and lesser extent of dechlorination over 13 months (51 mol% 1234-tetra-CDF vs. 34 mol% 1234-tetra-CDD remaining) (**Fig. 11-1a & b**). The maximum dechlorination rate of 1234-tetra-CDD (0.27 ± 0.08 mol% • $month^{-1}$) was 1.7 times higher than that of 1234-tetra-CDF (0.16 ± 0.08 mol% • $month^{-1}$). A slower dechlorination rate of 1234-tetra-CDF compared to 1234-tetra-CDD has also been observed for a mixed culture containing *D. ethenogenes* 195 (Fennell et al. 2004). Similarly, less extensive dechlorination of spiked PCDFs compared to PCDDs has been observed (Adriaens et al. 1995).

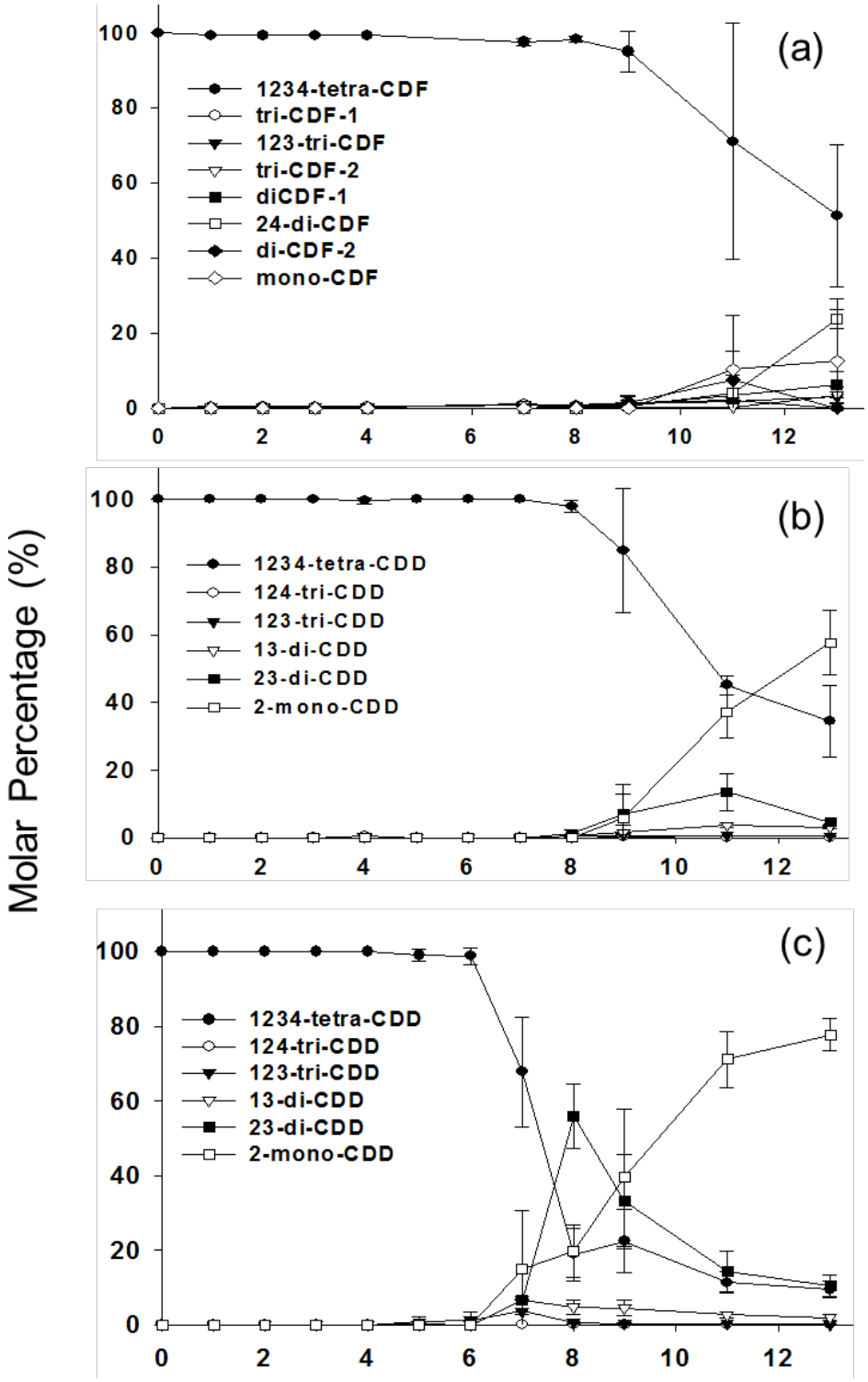


Figure 11-1. Dechlorination of 1234-tetra-CDF (a), 1234-tetra-CDD without PCNB (b) and 1234-tetra-CDD with PCNB as co-substrate (c). All cultures were supplemented with an electron donor mixture. Fresh anaerobic medium was added after 6 months to replenish the culture volume.

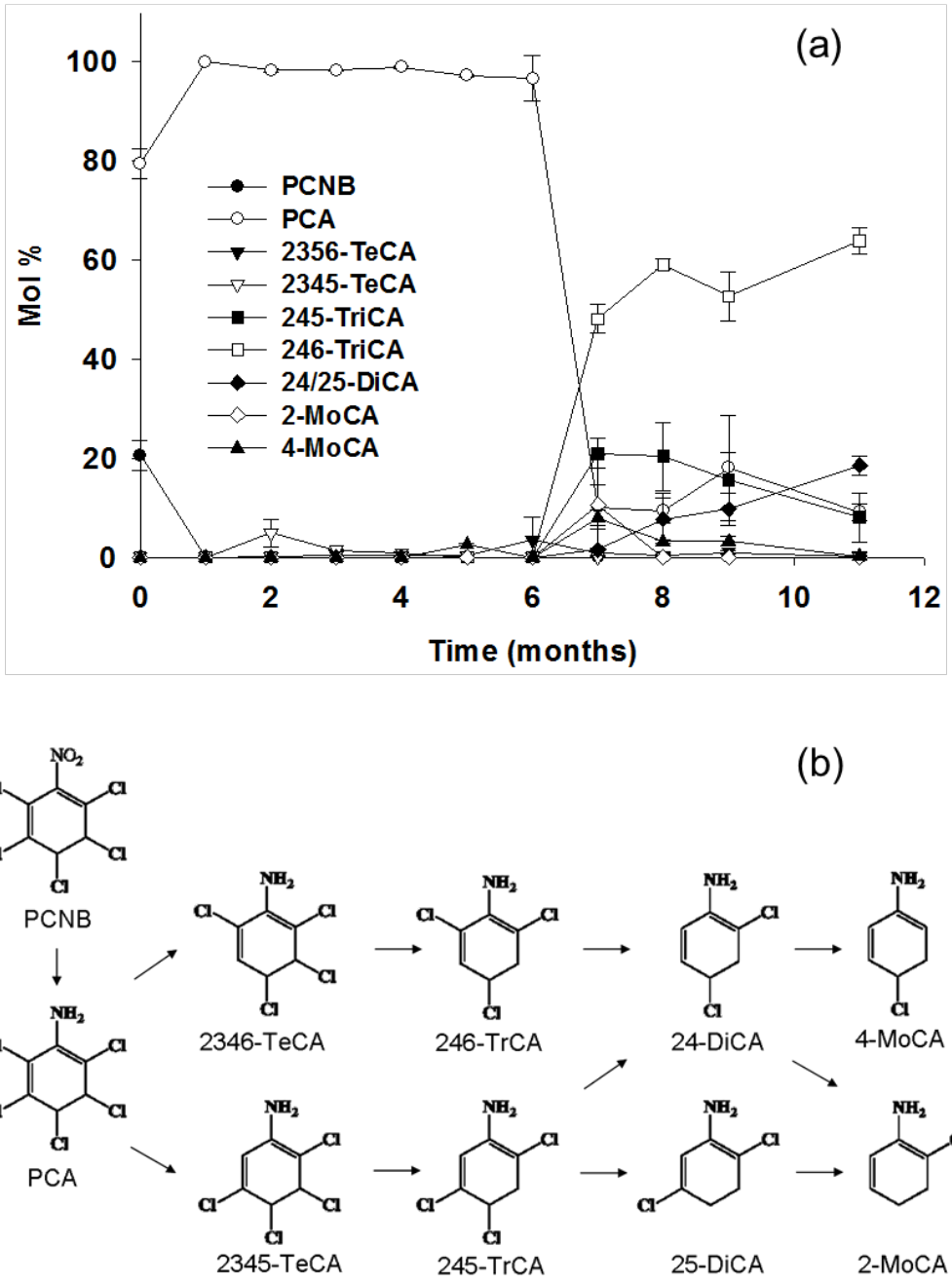


Figure 11-2. Dechlorination of PCNB in cultures spiked with 1234-tetra-CDD and PCNB (a) and the proposed dechlorination pathway based on the dechlorination products identified (b). 2346-TeCA is the intermediate postulated based on the subsequent identified TrCAs intermediates, but was not confirmed by GC-MS due to lack of an available standard.

The stimulating effect of PCNB on 1234-tetra-CDD dechlorination was evident from a shorter lag phase (5 months vs. 8 months) and greater extent of dechlorination (10 mol% 1234-tetra-CDD remaining with PCNB vs. 34 mol% without PCNB) (**Fig. 11-1b & c**). The maximum loss rate of 1234-tetra-CDD with PCNB (0.85 ± 0.18 mol% • $month^{-1}$) was >3 times higher than without PCNB (0.27 ± 0.08 mol% • $month^{-1}$). However, the accumulation rates of 2-mono-CDD with and without PCNB were nearly identical (11.9 ± 0.5 vs. 13.0 ± 0.4 mol%•$month^{-1}$). From this, it can be postulated that PCNB enhanced *peri* dechlorination of 1234-tetra-CDD to 123-tri-CDD then to 23-di-CDD, but had minimum effect on subsequent lateral dechlorination of 23-di-CDD to produce 2-mono-CDD. In addition to PCNB, TeCB, tetrachloroanisole (TeCA) and 2'3'4'-trichloroacetophenone (TrCAP) have been shown to stimulate the dechlorination of 1234-tetra-CDD (Ahn et al. 2005, 2008), however, their effects on dechlorination kinetics were not determined.

Based on the identified dechlorination products, a dominant *peri*-lateral dechlorination route of 1234-tetra-CDD via 123-tri-CDD to 23-di-CDD and 2-mono-CDD can be postulated in Kymijoki enrichment cultures, which is similar to the dechlorination pattern of 1234-tetra-CDD by *Dehalococcoides* sp. CBDB1 (Bunge et al., 2001, 2003). A similar *peri*-dominated dechlorination pathway of 124-tri-CDD by *Dehalococcoides* sp. DCMB5 was also observed (Liu et al. 2010). However, dechlorination of 1234-tetra-CDD by a mixed culture containing *D. ethenogenes* strain 195 proceeded by initial removal of lateral and then *peri* chlorines, generating 13-di-CDD (Fennell et al. 2004). A methanogenic consortium enriched on bromophenols showed lateral-preferred dechlorination of 1234-tetra-CDD as indicated by the accumulation and depletion of 124-tri-CDD with the production of 13-di-CDD over 300 days period (Vargas et al. 2001). In addition to these pure and enrichment culture studies, *peri*-preferred dechlorination (Liu et al. 2011; Adriaens & Grbic-Galic 1994; Adriaens et al. 1995) as well as *peri*-lateral combined dechlorination of spiked and historical PCDD/Fs in enrichment cultures derived from contaminated sediments (Ballerstedt et al. 1997; Beurksens et al. 1995; Barkovskii & Adriaens 1996) has been observed. These different dechlorination pathways are likely the result of different dechlorinating microbial communities that catalyze lateral vs. *peri* chlorine removal from distinct positions. These dechlorinating communities and their *rdh* genes may also be induced to different extent in the environment.

Dechlorination of PCNB. PCNB was rapidly transformed to PCA in the first month (**Fig. 11-2a**). PCA was already detected at the first 0-month time point taken 3 days after establishment of the cultures. PCA was then dechlorinated to 2356- and 2345-TeCA, which were further dechlorinated to 245- and 246-TrCA. 246-TrCA accumulated at more than 60 mol% in the cultures after 11 months. Co-eluting 24- or 25-DiCA were detected in the culture after 7 months and increased to around 18 mol% by the end of 11 months. 4-MoCA was detected after 7 months. PCNB was also transformed to PCA in killed controls after 1 month with slight amount, around 2 mol% of 2345-TeCA, 246-TrCA and 4-MoCA detected over 11 months (data not shown).

The dechlorination route of PCA in our study was slightly different from what was observed in a first generation mixed methanogenic culture of contaminated estuarine sediment (Tas & Pavlostathis 2005). Instead of 2346-TeCA and 245-TrCA as dechlorination products as shown here, 2356-TeCA and 235-TrCA were observed in addition to 2345-TeCA and 246-TrCA. 3- and

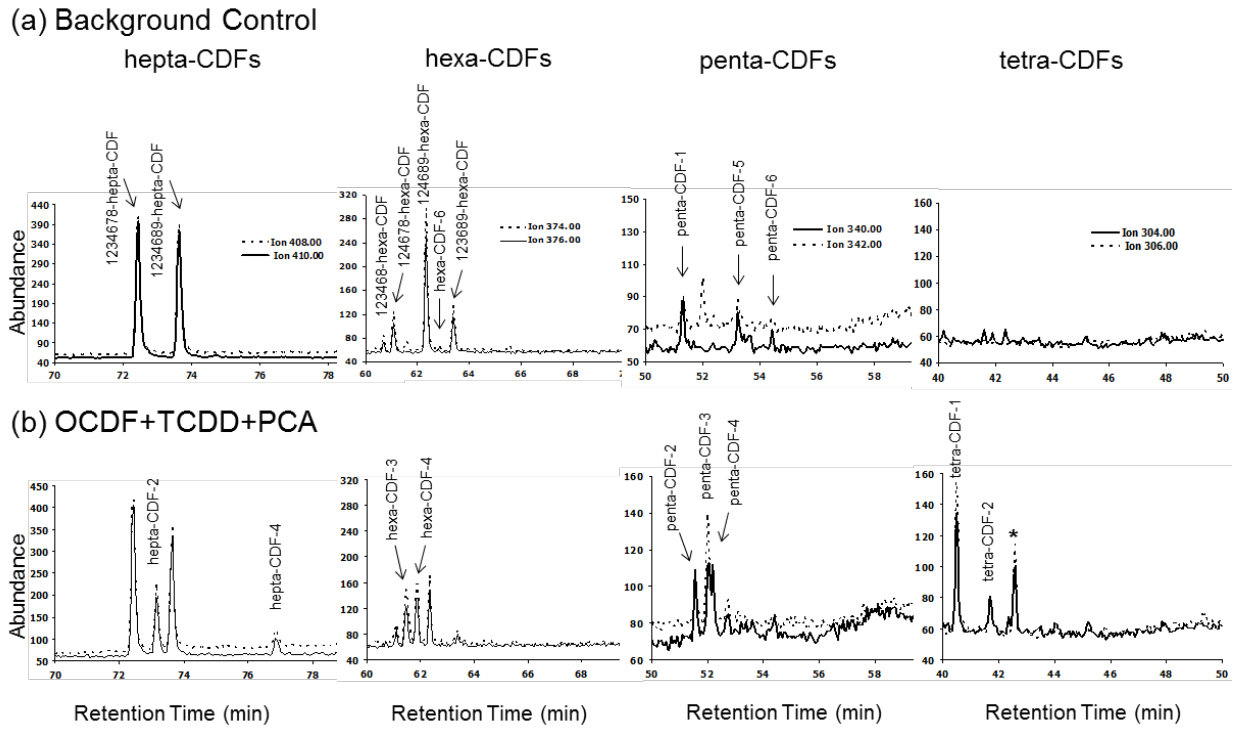


Figure 11-3. Selected ion chromatograms of hepta-, hexa-, penta- and tetra-CDFs in representative background controls and enrichment cultures amended with octa-CDF and 1234-tetra-CDD and PCA after 5.5 months. Similar results were observed in all triplicate cultures amended with octa-CDF and PCA and 1234-tetra-CDD or 1234-tetra-CDF. The peak denoted by * is not a tetra-CDF.

4-MoCA were the end dechlorination products, compared to 2- and 4-MoCA in our study. This suggests that different dechlorinating bacteria or reductive dehalogenases might be responsible for the observed differences.

Dechlorination of octa-CDF. In enrichment cultures amended with octa-CDF, two hepta-CDFs (**Fig. 11-3**; peaks hepta-CDF-2 and hepta-CDF-4) were detected at time 0 as impurities of octa-CDF. These were in addition to the historical 1234689- and 1234678-hepta-CDFs in Kymijoki River sediment, which were the dominant congeners and the only hepta-CDFs detected in non-OCDF-amended background controls (**Fig. 11-3**). The relative amount of the hepta-CDF-2 congener increased over time (**Fig. 11-4**) and since PCNB stimulated *peri* dechlorination we postulate that hepta-CDF-2 could be 1234789-hepta-CDF (however authentic standards to confirm this are not available). Two new hexa-CDFs (**Fig. 11-3**; peaks hexa-CDF-3 and -4) were detected after 3 months and increased after 5.5 months in treatments amended with PCA and 1234-tetra-CDD or 1234-tetra-CDF. In addition, three new penta-CDFs (**Fig. 11-3**; peaks penta-CDF-2, -3, and -4) were detected after 5.5 months in both treatments. One new tetra-CDF (**Fig. 11-3**; peak tetra-CDF-1) was first detected after 3 months and increased by 5.5 months, and another new tetra-CDF (**Fig. 11-3**; peak tetra-CDF-2) was first detected after 5.5 months in treatments amended with PCA and 1234-tetra-CDD. Dechlorination of spiked octa-CDF thus resulted in accumulation of one hepta-CDF, two new hexa-CDFs, three new penta-CDFs, and two new tetra-CDFs. Due to lack of standards, the chlorine substituent positions could not be

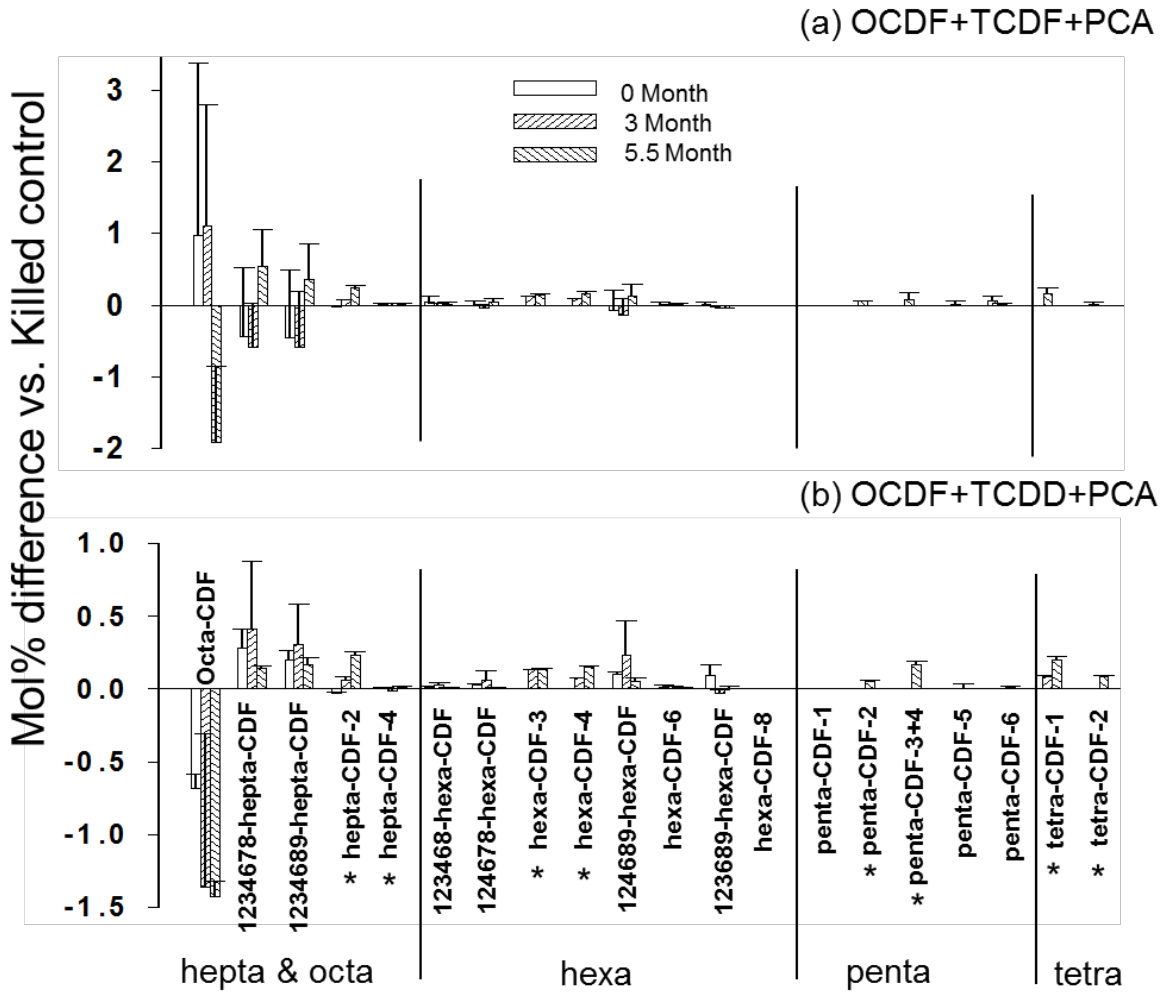


Figure 11-4. Average mol% difference of CDF congeners of triplicate enrichment cultures amended with octa-CDF + 1234-tetra-CDD + PCA (OCDF + TCDD + PCA) vs. killed control (a) and octa-CDF + 1234-tetra-CDF +PCA (OCDF + TCDF + PCA) vs. killed control (b) after 0, 3, and 5.5 months.

determined. No new CDF peaks were detected in killed or background controls over the experimental period. The average mol% of the new CDF peaks (**Fig. 11-4**) increased in both treatments compared to killed controls, including one hepta-CDF (peak hepta-CDF-2), two new hexa-CDFs (peaks hexa-CDF-3 and -4), three penta-CDFs (peaks penta-CDF-2, -3, and -4), and two new tetra-CDFs (peaks tetra-CDF-1 and -2). Spiked 1234-tetra-CDD, 1234-tetra-CDF and PCA all underwent dechlorination and produced different daughter congeners as shown earlier. Unlike octa-CDF, spiked decabromodibenzoether (BDE) and octa-CDD were not dechlorinated by a mixed culture containing *D. ethenogenes* strain 195 (Liu & Fennell 2008), indicating that the dechlorinating community in Kymijoki sediment has a different substrate range and specificity.

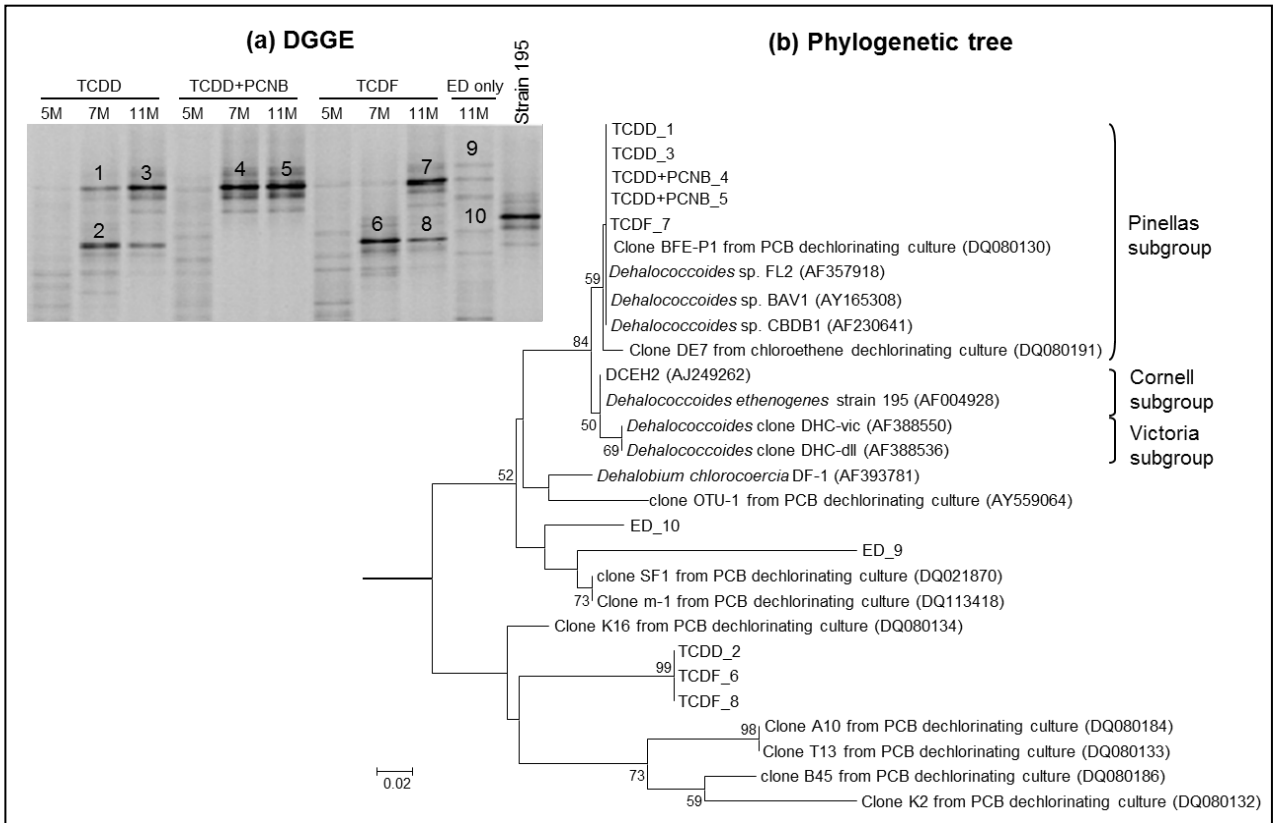


Figure 11-5. DGGE profiles of microbial community in cultures spiked 1234-tetra-CDD, 1234-tetra-CDD and PCNB, and 1234-tetra-CDF after 5, 7 and 11 months incubation (all cultures received electron donor) compared to electron donor only control after 11 months (a). Phylogenetic analysis of 16S rRNA gene DGGE band sequences compared to closely related reference sequences (b). The tree was constructed with the minimum evolution method using the MEGA version 4 program. Bootstrap values at nodes are the percentages of 1000 iterations. *Ktedobacter racemifer* strain SOSP1-21 (AM180156) in the phylum Chloroflexi was used as the outgroup. The reference bar indicates 2 nucleotide exchanges per 100 nucleotides.

Analysis of putative dechlorinating *Chloroflexi* community. In order to understand the dechlorinating microorganisms active in our enrichment cultures, *Chloroflexi*-specific DGGE analysis of Kymijoki sediment enrichment cultures was conducted. A community shift was observed after 5 months (**Fig. 11-5a**), corresponding to the onset of dechlorination of 1234-tetra-CDD and 1234-tetra-CDF. After 7 and 11 months, a noticeable increase in the intensity of two DGGE bands was observed in enrichment cultures amended with only 1234-tetra-CDD or 1234-tetra-CDF, while the intensity of one DGGE band was enhanced in cultures amended with 1234-tetra-CDD plus PCNB (**Fig. 11-5a**).

Phylogenetic analysis of the dominant DGGE bands after enrichment showed that one set of DDGE bands (TCDD_1 and _3, TCDD+PCNB_4 and _5, and TCDF_7) were 100% identical to the 16S rRNA gene of the Pinellas subgroup of group II *Dehalococcoides*, which includes strains CBDB1, BAV1, and FL2 (**Fig. 11-5b**). Enrichment of a Pinellas-subgroup community thus corresponded with dechlorination of 1234-tetra-CDD, 1234-tetra-CDF and PCNB (**Fig. 11-5a**).

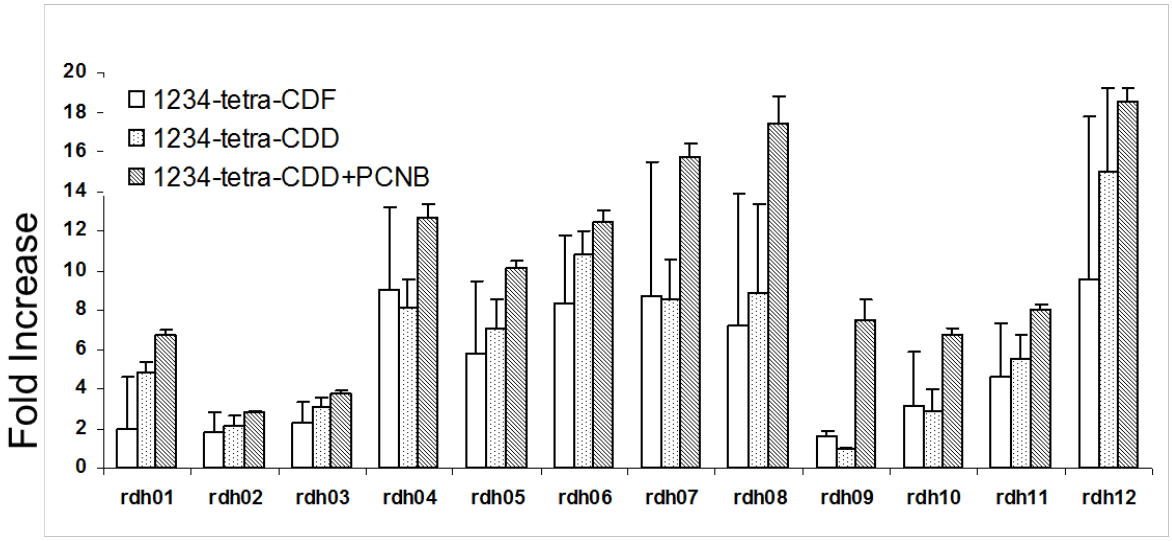


Figure 11-6. Fold increase of 12 putative *rdh* genes in Kymijoki enrichment cultures amended with 1234-tetra-CDD/F compared to control cultures amended with electron donors only after 7 months of incubation. Putative *rdh* gene amplicons correspond to the following *D. ethenogenes* strain 195 and *Dehalococcoides* sp. CBDB1 genes: *rdh* 01 (DET0180 and cbdb_A187), *rdh* 02 (DET0235 and cbdb_A243), *rdh* 03 (DET0302 and cbdb_A237), *rdh* 04 (DET0306 and cbdb_A1495), *rdh* 05 (DET0311 and cbdb_A88), *rdh* 06 (DET0318 and cbdb_A1588), *rdh* 07 (DET1171 and cbdb_A1092), *rdh* 08 (DET1519 and cbdb_A1575), *rdh* 09 (DET1522 and cbdb_A1570), *rdh* 10 (DET1535 and cbdb_A1595), *rdh* 11 (DET1538 and cbdb_A1627), and *rdh* 12 (DET1545 and cbdb_A1638). ED: electron donor, TCDD: 1234-tetra-CDD, PCNB: pentachloronitrobenzene, TCDF: 1234-tetra-CDF.

The Pinellas subgroup of group II *Dehalococcoides* includes strain CBDB1, which has the same dechlorination route of 1234-tetra-CDD (Bunge et al., 2001, 2003) as shown in this study. The second set of DGGE bands (TCDD_2, TCDF_6 and _8) were closely related to a group of *Chloroflexi* clones found in PCB contaminated soils and sediments (Yan et al., 2006). These phylotypes corresponding to upper and lower DGGE bands were not enriched in control cultures over 11 months. It remains to be determined what role these bacteria might have in CDD and CDF dechlorination. It should be noted that, since *Chloroflexi* was the specific target in DGGE analysis, it is possible that other bacteria also contributed to dechlorination but were missed by this method.

Analysis of *rdh* genes in enrichment cultures. The relative band intensity of twelve *rdh* genes in control and enrichment cultures was analyzed after 7 months (**Fig. 11-6**). The abundance of eleven *rdh* genes, excluding *rdh*09, increased in cultures amended with 1234-tetra-CDD or 1234-tetra-CDF and/or PCNB compared to electron donor-only control cultures. The *rdh*09 abundance increased exclusively in cultures amended with 1234-tetra-CDD plus PCNB. It should be noted that the relative abundance of different *rdh* genes are not directly comparable since the primers for each *rdh* gene have different PCR efficiencies.

The DGGE and phylogenetic analyses and the overall increasing intensities of eleven *rdh* genes supported the enhancement of a dechlorinating *Chloroflexi* community in Kymijoki River sediments. Park et al. (2011) showed that different *rdh* gene patterns might explain different PCB dechlorination activities in Anacostia River sediment microcosms. The "priming" effect of PCNB/PCA may be through stimulating the growth of dechlorinators and/or inducing certain *rdh* genes that are active in the dechlorination of PCDD/Fs.

In summary, we demonstrate the microbial reductive dechlorination of 1234-tetra-CDD, 1234-tetra-CDF and octa-CDF and the stimulating effect of PCNB in enrichment cultures derived from historically contaminated Kymijoki sediment. Dechlorination of octa-CDF resulted in daughter products corresponding to one hepta-CDF (peak hepta-CDF-2), two hexa-CDFs (peaks hexa-CDF-3 and -4), three penta-CDFs (peaks penta-CDF-2, -3, and -4) and two tetra-CDFs (peaks tetra-CDF-1 and -2) (**Fig. 11-3**). PCNB enhanced the dechlorination rate and extent of 1234-tetra-CDD by specifically stimulating *peri* dechlorination while having minimum effect on lateral dechlorination of 1234-tetra-CDD (**Fig. 11-1b & 1c**). This study provides strong evidence for dechlorination of PCDFs in Kymijoki sediment although the dechlorination pathways of octa-CDF are not confirmed. This work will aid in future attempts to enrich and stimulate the activity of PCDD/F-dechlorinating bacteria at contaminated sites. Further characterization of the selective and synergistic activities of PCDD/F dechlorinating microorganisms with different contaminant mixtures is essential for generating models to predict the dechlorination potentials at impacted sites and design effective *in situ* treatment strategies.

12. Enhanced reductive dechlorination of polychlorinated biphenyl impacted sediment by bioaugmentation with a dehalorespiring bacterium

(Rayford B Payne RB, Chun C, May HD, Sowers KR. 2011. Enhanced reductive dechlorination of polychlorinated biphenyl impacted sediment by bioaugmentation with a dehalorespiring bacterium. Env. Sci. Tech. 45: 8772–8779)

Abstract

Anaerobic reductive dehalogenation of commercial PCBs such as Aroclor 1260 has a critical role of transforming highly chlorinated congeners to less chlorinated congeners that are then susceptible to aerobic degradation. The efficacy of bioaugmentation with the dehalorespiring bacterium "*Dehalobium chlorocoercia*" DF1 was tested in 2-liter laboratory mesocosms containing sediment contaminated with weathered Aroclor 1260 (1.3 ppm) from Baltimore Harbor, MD. Total penta- and higher chlorinated PCBs decreased by approximately 56% (by mass) in bioaugmented mesocosms after 120 days compared with no activity observed in unamended controls. Bioaugmentation with DF-1 enhanced the dechlorination of doubly flanked chlorines and stimulated the dechlorination of single flanked chlorines as a result of an apparent synergistic effect on the indigenous population. Addition of granulated activated carbon had a slight stimulatory effect indicating that anaerobic reductive dechlorination of PCBs at low concentrations was not inhibited by a high background of organic carbon that could affect bioavailability. The total number of dehalorespiring bacteria was reduced by approximately half after 60 days. However, a steady state level was maintained that was greater than the non-bioaugmented indigenous population in untreated sediments and DF1 was maintained within the indigenous population after 120 days. The results of this study demonstrate that bioaugmentation with dehalorespiring bacteria has a stimulatory effect on the dechlorination of weathered PCBs and supports the feasibility of using *in situ* bioaugmentation as an environmentally less invasive and lower cost alternate to dredging for treatment of PCB impacted sediments.

Introduction

Polychlorinated biphenyls (PCBs) are persistent organic pollutants that are globally dispersed as a result of cycling between air, water, and soil (Alder et al., 1993; Quensen et al., 1990; Versar, 1976). PCBs are hydrophobic and bioaccumulate throughout the food chain by absorption in the fatty tissue of animals and humans where they have been reported to act as endocrine disrupters (Crisp et al., 1998) and possible carcinogens (Safe, 1993). The most widely applied technologies for treating PCB-impacted sites are removal by dredging and subsequent transfer to hazardous waste landfills or *in situ* containment in subaqueous sites by capping (EPA, 2005). In addition to being cost prohibitive for treating large areas of contamination in rivers, lakes and coastal sediments, these technologies are disruptive to environmentally sensitive areas such as marshes and wetlands. Development of an *in situ* treatment system that utilizes microbial activity, bioaugmentation, could potentially reduce high costs and environmental impacts associated with dredging and capping.

There have been reports on the potential of aerobic bioaugmentation with bacteria, fungi and plants, but these processes have limited capacity to attack highly chlorinated congeners often found in PCB impacted sites (Abraham et al., 2002). Microbial reductive dechlorination of higher chlorinated PCB congeners has the potential to complement these processes, but there have been very few studies to date describing the use of anaerobic bioaugmentation to stimulate *in situ* treatment of PCB impacted sediments. Wu and Wiegel (1997) and Bedard *et al*. (1997) observed only slight or no stimulation of weathered Aroclor 1260 in Housatonic River sediments bioaugmented with PCB enriched soil and sediment slurries. Natarajan *at al.* (1997) reported dechlorination of weathered Aroclor 1242 and 1248 in microcosms inoculated with microbial granules from a UASB digestor. However, the observed activity likely resulted from stimulation of indigenous PCB dechlorinating bacteria by hydrogen generated from fermentation of wood powder added an electron donor rather than the result of bioaugmentation with dehalorespiring bacteria. More recently *Dehalococcoides* spp. and related species within the Chloroflexi have been identified that are capable of reductively dechlorinating PCBs by utilizing PCBs as terminal electron acceptors, a process termed dehalorespiration, and several strains with specific dechlorinating capabilities have been reported (Bedard et al., 2007; Cutter et al., 2001; Fagervold et al., 2005; Wu et al., 2002). The commercial PCB mixture Aroclor 1260 was reported to be significantly dechlorinated by a consortium consisting of one or more phylotypes enriched from sediment microcosms (Fagervold et al., 2007), sediment-free microcosms (Bedard et al., 2007) and by an individual species, "*Dehalococcoides*" sp. CBDB1 (Adrian et al. 2009). A recent report showed that "*Dehalobium chlorocoercia"* DF-1, bacterium *o*-17, phylotypes SF1 and DEH10, each with different PCB congener specificities for dechlorination, would alter the overall pathway for dechlorination of spiked Aroclor 1260 differently depending on which microorganisms were added to sediment microcosms (Fagervold et al., 2011). Bedard *et al*. (2007) observed in a sediment-free enrichment culture that a critical mass of cells was required before reductive dechlorination of spiked Aroclor 1260 was detected and proposed that low indigenous numbers of dehalorespiring bacteria might explain why substantial attenuation of PCBs is rarely observed in the environment.

Although these combined reports suggest that bioaugmentation could be a potential strategy for treatment of PCB-impacted sediments, several questions need to be addressed to determine if *in situ* treatment of weathered PCBs by bioaugmentation is possible. Bedard et al. (1997) demonstrated that bioaugmentation with sediment slurries enriched for PCB dechlorinating bacteria combined with PCB 116 as a primer stimulated dechlorination of relatively high levels (50 ppm) of weathered Aroclor 1260 from Housatonic River sediment. However, the effectiveness of bioaugmentation for dechlorinating low levels of PCBs most commonly associated with weathered PCB contaminated sites where both kinetic and availability issues might affect the effectiveness of *in situ* PCB transformation is currently unknown (Fish et al., 2001). A recent report by May *et al*. (2008) showed that bioaugmentation with DF-1 stimulated the reductive dechlorination of weathered Aroclor 1260 (4.6 ppm) in contaminated soil microcosms, and Krumins et al. (2009) found that the addition of "*D. ethenogenes"* and pentachloronitrobenzene stimulated the dechlorination of weathered Aroclors 1248, 1254 and 1260 (2.1 ppm) in sediment microcosms. Combined, these results suggest that using bioaugmentation for low levels of weathered PCBs is feasible. Another unknown is whether dehalorespiring microorganisms used for *in situ* bioaugmentation can successfully compete with

indigenous populations and be sufficiently sustainable to have a significant impact on reduction of higher chlorinated PCB congeners.

In this report we tested the efficacy of using bioaugmentation with the dehalorespiring bacterium *D. chlorocoercia* DF-1 to stimulate the reductive dechlorination of weathered Aroclor 1260 in sediment mesocosms that simulate *in situ* conditions. Specifically we examined the effects of bioaugmentation on Aroclor transformation over the course of 120 days in the presence of activated carbon to assess any potential effects of bioavailability and monitored the microbial population to assess the sustainability of DF-1 within the background of the indigenous bacterial community.

Materials and Methods

Media and growth conditions. ***"****Dehalobium chlorocoercia"* DF1 was grown anaerobically in estuarine mineral medium (ECl) as described previously (Berkaw et al., 1996; May et al., 2008). Sodium formate (10 mM) was added as the electron donor and PCB 61 (2,3,4,5-PCB) was added in acetone (0.1% v/v) at a final concentration of 173 μM. *Desulfovibrio* sp. extract (1% v/v) was added as a growth factor and titanium(III) nitrilotriacetate (0.5 mM) was added as a chemical reductant (May et al. 2008; Moench & Zeikus, 1983). *D. chlorocoercia* was routinely grown in 50 ml of medium in 160-ml serum bottles sealed with 20-mm Teflon-coated butyl stoppers (West Pharmaceutical, Inc.). All cultures were incubated statically at 30°C in the dark. Growth was monitored by gas chromatographic analysis of PCB 61 dechlorination to PCB 23 (2,3,5-PCB) and by quantitative RT-PCR of 16S rRNA gene copies (described below).

Mesocosm experiments. Mesocosms were prepared in glass 2 liter TLC tanks (Fisher Scientific). PCB impacted sediments were sampled on 14 May 2009 from the Northwest Branch of Baltimore Harbor (BH) with a petite Ponar grab sampler at 39°16.8_N, 76°36.2_W and stored in the dark under nitrogen for 19 days at 4 °C prior to use. Sediment was pooled and homogenized anaerobically by stirring in an anaerobic glove bag. Two liters of sediment were added to each mesocosm tank with 2 cm of indigenous water above the sediment surface. A glass plate covered each mesocosm to minimize evaporation with a 1 cm gap on one end for air exchange. Water lost due to evaporation was periodically replenished with deionized water to maintain the osmolarity of the harbor water.

The bioaugmentation inoculum was prepared using ten 50 ml cultures of DF1 grown until 50% of PCB 61 was dechlorinated. The cultures were transferred into 250 ml Oak Ridge bottles in an anaerobic glove box and sealed under nitrogen-carbon dioxide (4:1). The bottles were centrifuged at 22,000 x g for 30 min, decanted, and the pellets were pooled in 50 ml of sterile ECl medium. The concentration of pooled DF1 was approximately 5×10^7 16S rRNA gene copies per ml. Spent medium supernatant was prepared by passing DF-1 culture supernatant through a 0.22 micron filter (Millipore, www.millipore.com) to remove residual cells. Mesocosms were amended with one of four treatments in an anaerobic glove box: (1) 20 ml of concentrated DF1 (5×10^5 cells $gram^{-1}$ sediment); (2) 20 ml of spent cell-free growth medium; (3) 20 ml of concentrated DF1 adsorbed to 25 g granulated activated carbon for 1 hour; or (4) 20 ml of spent cell-free growth medium adsorbed to 25 g activated carbon (CAS# 7440-44-0, type TOG-NDS 80×325, Calgon Carbon Corp.) for 1 hour. The total GAC added in an individual

mesocosm (4.4 % sediment dry weight) was similar to concentrations (3-5%) used *in situ* to sequester PCBs from benthic organisms (Ghosh et al., 2011). No exogenous electron donor was added. Mesocosms were homogenized after addition of the amendments by stirring with a Teflon spoon, then removed from the anaerobic glove box and incubated at 23^{o}C in the dark. Mesocosms were sampled by taking six cm deep cores using a five ml syringe barrel with the end cut off. Triplicate cores were sampled for each time point using a random sampling grid and homogenized prior to analysis for PCBs and DNA as described below.

DNA extraction. DNA was extracted by adding 0.25 g of sediment from each sample core to a PowerBead microfuge tube of a Power Soil DNA Isolation Kit (MOBIO Laboratories, Inc.). The PowerBead tubes were mixed by hand prior to 30 s of bead beating at speed "4.5" using a FastPrep120 (Q-Biogene, 8 CA). Total DNA was then isolated from the PowerBead tubes according to the manufacturer's directions. DNA was eluted in 100 µl of TE buffer and quantified with a NanoDrop 1000 Spectrophotometer (ThermoScientific). Extracted DNA samples had an A260/280 ratio of ≥ 1.6 and an A260/230 ratio of ≥ 2.0. All DNA samples were diluted to 2 ng/µl in TE buffer.

Enumeration of PCB dehalorespiring bacteria by quantitative PCR. The quantification of putative dechlorinating Chloroflexi in each subcore was performed by quantitative PCR (qPCR) using iQ SYBR green supermix (Bio-Rad Laboratories) and primers specific for the 16S rRNA gene of a deep branching, putative dechlorinating clade within the Chloroflexi (348F/884R) (Fagervold et al., 2005). Each 25-µl reaction volume contained 1x iQ SYBR green supermix, 500 nM forward and reverse primers and 1 µl of sample DNA. PCR amplification and detection were conducted in an iCycler (Bio-Rad Laboratories). qPCR conditions were as follows: initial denaturation for 15 min at 95°C followed by 35 cycles of 30 s at 95°C, then 30 s at 61°C, then 30 s at 72°C. One copy of the gene per genome was assumed based on the genomes of *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp., strain CBDB1(Kube et al., 2005; Seshadri et al., 2005). qPCR data were analyzed with MJ Opticon Monitor Analysis Software v3.1 and compared to a standard curve of purified DF1 348F/884R 16S rRNA gene product. The standard curve consisted of duplicate dilutions over 6 orders of magnitude. The specificity of qPCR amplification was verified by melting curve analysis followed by gel electrophoresis. Amplification efficiencies of dilutions of gel purified DF1 16S rRNA gene PCR product used as standards were 87% ± 7% (r^2 = 0.99), and amplification efficiencies of DF1 cells and environmental samples were almost identical to those of the standard curve (86 ± 7%). The observed departure of amplification efficiencies from 100% was possibly due to the consensus nature of primer 884R, which has 1 mismatch with the DF-1 16S rRNA gene combined with the stringency of the PCR reaction conditions.

Community analysis of PCB dechlorinating bacteria by denaturing HPLC. Denaturing HPLC (DHPLC) analyses were performed using a WAVE 3500 HT system (Transgenomic, Inc.) as described previously (Kjellerup et al., 2008) except that the instrument was equipped with a florescence detector (excitation 490 nm, emission 520 nm). The primer set 348F/884R was used for PCR amplification of 16S rRNA genes from bacteria within the Chloroflexi (Fagervold et al., 2005). DHPLC fractions were sequenced as described previously (Kjellerup, Sun, Ghosh, May and Sowers, 2008). A phylogenetic tree was created with Tree Builder (http://rdp.cme.msu.edu/index.jsp).

PCB extraction. Sediment samples were extracted using an Accelerated Solvent Extractor (Dionex) following EPA method 3545. Approximately 5 grams wet weight sediment was dried with pelletized diatomaceous earth (Dionex) at room temperature in a desiccator containing $CaCl_2 \cdot 2H_2O$. The dried sediment (1 g) was transferred to an 11 ml stainless steel extraction cell containing 0.6 g Cu and 2.4 g fluorisil between two cellulose filters on the bottom of the cell and the remaining cell volume was filled with anhydrous Na_2SO_4. To correct for extraction efficiency, 10 μl of a 400 $\mu g\ l^{-1}$ solution of PCB 166 in hexane was pipetted on top of the Na_2SO_4. The sample containing the surrogate was extracted with approximately 20 ml of pesticide grade hexane (Acros Organics) at 100°C and purged with 1 MPa nitrogen. The sample was evaporated to a final volume of 1 ml at 30°C under nitrogen using a N-EVAP 111 nitrogen evaporator (Organomation Associates). Before PCB analysis, 10 μl of PCB 30 and PCB 204 (400 $\mu g\ l^{-1}$ each in acetone) was added to the sample as internal standards.

PCB analysis. PCB congeners were analyzed using a Hewlett-Packard 6890 series II gas chromatograph (GC) with a DB-1 capillary column (60 m by 0.25 mm by 0.25 μm; JW Scientific) and a ^{63}Ni electron capture detector by a modified method of EPA 8082. PCB congeners in a mixture containing 250 $\mu g\ l^{-1}$ Aroclor 1232, 180 $\mu g\ l^{-1}$ Aroclor 1248 and 180 $\mu g\ l^{-1}$ Aroclor 1262 were quantified with a 10-point calibration curve using PCB 30 and PCB 204 as internal standards. Individual congeners and respective concentrations were obtained from Mullins et al (Mullin et al., 1984). Fifty-five additional congeners not present in the Aroclor mixture that were potential dechlorination products were added to the calibration table containing the Aroclor congeners. The additional congeners were quantified with 10-point calibration curves at concentrations of 2, 5, 10, 20, and 40 $\mu g\ l^{-1}$ (in duplicate) for the low range calibration and 40, 100, 200, 400, and 800 $\mu g\ l^{-1}$ (in duplicate) for the high range calibration. Using this protocol 173 congeners were resolved in 130 individual peaks (excluding internal standards PCB 30 and PCB 204 and surrogate PCB166). The final concentration of individual congeners in samples was corrected for the recovery efficiency of the surrogate (82 ± 6%), which was reduced below 100% by the high content of carbon black in Baltimore Harbor sediments (Grossman and Ghosh, 2009) and the activated carbon amendment. Co-eluting peaks were indicated as multiple congeners. Total chlorines per biphenyl was calculated as the product of the average number of chlorines and molar concentration of each congener divided by the sum of the total molar concentration of all congeners (Cho et al. 2003). When co-eluting peaks were different homologs, equal amounts of each homolog were assumed (Cho et al. 2001). All PCBs (99% purity) were purchased from AccuStandard.

Results

Characteristics of mesocosm sediment. Sediment used in mesocosms was black in color with a slightly gelatinous texture. Relatively high amounts of PAHs, trans-nonachlor and heavy metals have been reported near this site as recently as 1997 (Baker et al., 1997). Total PCB concentration in the pooled sediment samples was 1.3 ± 0.15 ppm with a mean of 4.76 chlorines per biphenyl. The congener profile was consistent with the first reported survey of PCBs in BH by Morgan and Sommer (Morgan & Sommer, 1979) showing that Aroclor 1260 occurred predominantly with smaller amounts of Aroclor 1254. However, less chlorinated congeners were detected that are potential weathered products of Aroclor 1260.

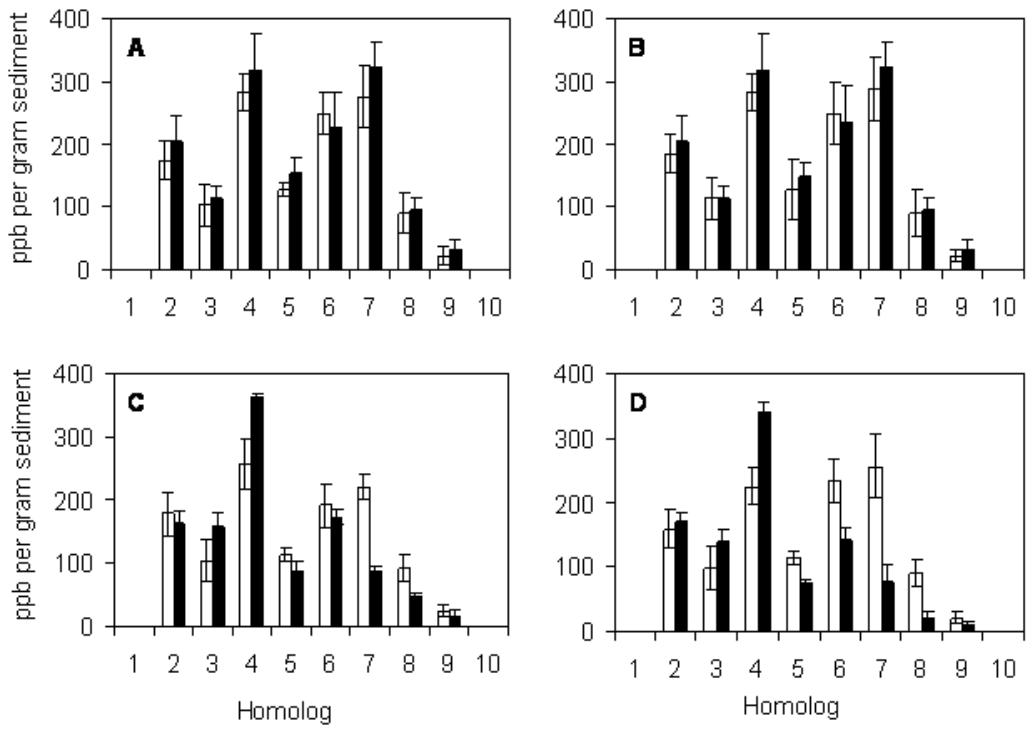


Figure 12-1. PCB analysis by homolog at day 0 (*white bars*) and day 120 (*black bars*) after treatment with filter sterilized spent growth medium (*A*), sterilized spent growth medium and GAC (*B*), concentrated DF1 in growth medium inoculated directly into the sediment (*C*) and concentrated DF1 adsorbed onto GAC (*D*). Each bar represents the mean and standard deviation of three replicates samples.

After bioaugmentation the sediments appeared to be anaerobic 1-2 cm below the surface based on black coloration and the distinct odor of sulfide. In mesocosms without GAC the sediment surface developed a grey coloration; with GAC the sediment surface developed a distinct orange-brown coloration. The surface of the mesocosm was disrupted within 1 week by holes (about 0.2 cm in diameter) caused by the activity of benthic worms but the activity was less apparent by day 60.

Effects of treatments on reductive dechlorination of weathered PCBs. BH sediment mesocosms were bioaugmented with dehalorespiring *D. chlorocoercia* DF-1 to a final concentration of approximately 5×10^5 cells g^{-1} sediment and PCBs were monitored over 120 days. In mesocosms bioaugmented directly with DF1, there was a net decrease in the total amounts of septa- and octa-PCBs (**Figure 12-1C**). In mesocosms bioaugmented with DF-1 with GAC, which reduces the bioavaiability of PCBs (Ghosh, Luthy, Cornelissen, Werner and Manzie), there was a net decrease in the total amounts of penta- through octa- PCBs and a net increase in the total amount of tetra- and tri-PCBs over the course of 120 days (**Figure 12-1D**). In contrast, there was no obvious change in homolog distribution in non-bioaugmented mesocosms treated with spent cell-free medium with or without GAC. Bioaugmentation both directly and with GAC as an adsorption substrate resulted in 0.6 to 0.7 mol Cl per biphenyl dechlorinated, respectively, after 120 days (**Figure 12-2**). A greater rate of dechlorination

(0.0067 Cl/biphenyl/day) was observed after bioaugmentation with GAC compared with direct injection (0.0041 Cl/biphenyl/day). A t-Test (two-sample assuming unequal variances, α=0.05) showed a significant difference in dechlorination rates between the bioaugmentation treatments with a P-value of 0.041 (df = 4).

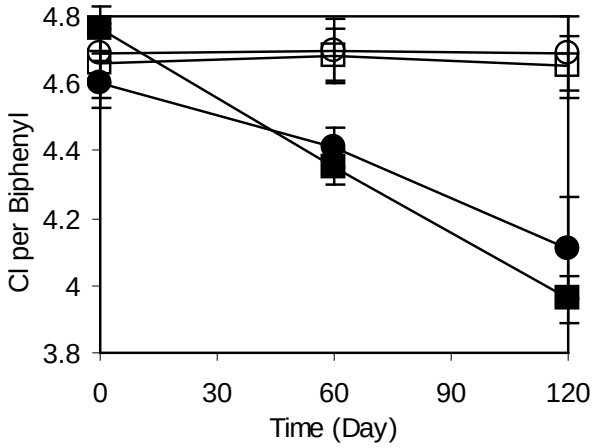


Figure 12-2. Changes in Cl per biphenyl in mesocosms over time after treatment with sterilized spent growth medium (○), sterilized spent growth medium and GAC (□), DF1 inoculated directly into sediment (●), and DF1 adsorbed onto GAC inoculated into sediment (■). Each datum point represents the mean and standard deviation of three replicate samples.

Single congener analysis of the mesocosm bioaugmented with DF1 adsorbed to GAC resulted in a significant decrease in higher chlorinated congeners and corresponding increase in lesser chlorinated congeners (**Figure 12-3**). The total sum of predicted substrates and products (**Figure 12-3**) was 1.26 ± 0.307 $\times 10^{-9}$ mol per gram sediment at day 0 and 1.02 ± 0.639 $\times 10^{-9}$ mol g^{-1} sediment at day 120. Predicted substrate congeners decreased from 1.00 ± 0.228 to 0.291 ± 0.282 $\times 10^{-9}$ mol per gram sediment and product congeners increased from 0.256 ± 0.0786 to 0.729 ± 0.356 $\times 10^{-9}$ mol per gram sediment. The inability to obtain a zero mass balance might have resulted from the absence of possible dechlorination products in the GC method (*e.g.*, PCBs 187, 109, 92, 96, and 84 among others), volatilization of less chlorinated congeners or aerobic degradation by indigenous microorganisms. Strikingly, PCB 194 decreased from about 23 to 8

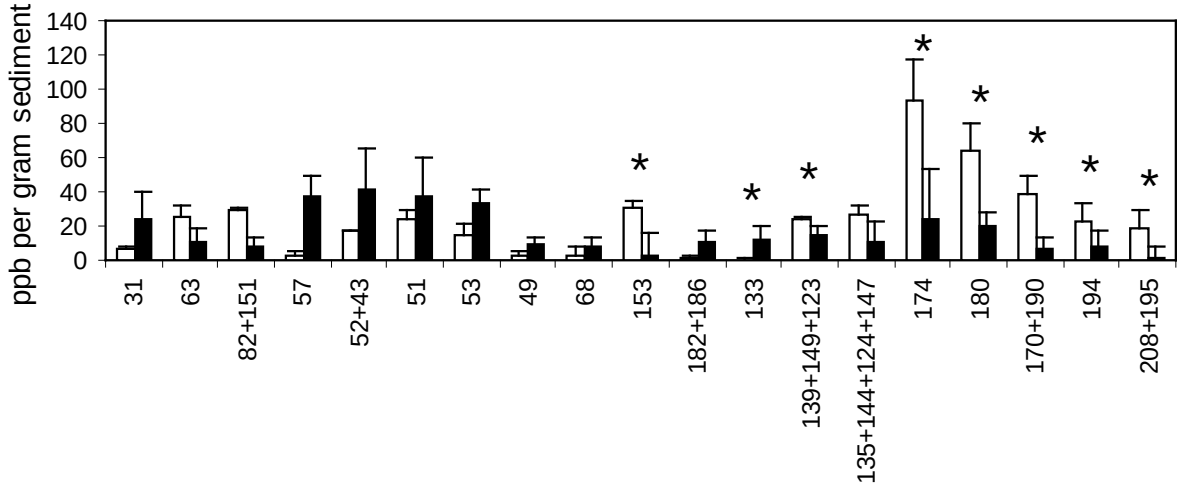


Figure 12-3. Congeners showing greatest change in mesocosms receiving both GAC and DF1 at day 0 (□) and day 120 (■). An asterisk (*) indicates congeners that could be dechlorination substrates of DF1, or products from double flanked dechlorination by DF-1. Each bar represents the mean and standard deviation of three replicates samples.

ppb and PCB 133, the predicted product of sequential double flanked *para* reductive dechlorination of PCB 194 (via PCB 172) was found to increase from about 1 to 12 ppb over 120 days (**Figure 12-3**). Potential dechlorination products of double flanked reductive dechlorination of PCB 208 (PCB 179 or PCB 136), PCB 195 (PCB 187), PCB 170 (PCB 87), PCB 190 (PCB 163), PCB 180 (PCB153 or PCB 141) and PCB 174 (PCB135 or PCB 149) were detected after 120 days; however, these products did not accumulate to substantial amounts. Furthermore, the less chlorinated congeners that did accumulate were not products resulting from dechlorination of double flanked chlorines (PCB 51, PCB 53, PCB 52, PCB 49, PCB 32, and PCB 57, Table S2). Since DF-1 is reported to reductively dechlorinate only doubly flanked chlorines (May et al., 2008) the dechlorination products likely resulted from enhanced activity by the indigenous population of dehalorespiring bacteria. Addition of cell-free medium used to grow DF-1 did not stimulate PCB dechlorination, indicating that the enhanced activity by indigenous microorganisms did not result from "priming" by residual PCBs or biostimulation by the medium.

Sustainability of *D. chlorocoercia* DF1 after bioaugmentation. To determine whether DF-1 was sustainable in the presence of relatively low PCB concentrations and the indigenous microbial community, putative dehalorespiring microorganisms were enumerated during the experiment based on the number of 16S rRNA gene copies g^{-1} dry sediment. In both bioaugmented mesocosms the numbers of putative dehalorespiring bacteria was initially about 2-fold higher than in untreated mesocosms (1.3×10^6 compared to about 6.0×10^5 copies per gram, respectively) indicating that added DF-1 accounted for approximately half the total population of putative dehalorespiring bacteria in those mesocosms. A Student's t-Test (α=0.05) showed a significant difference between initial 16S rRNA gene copy numbers between treatments with a two-tail P-value of 0.01 (df=3). The total 16S rRNA copy numbers in treated mesocosms decreased by 60 days before reaching an apparent steady state for the 120 day incubation period (**Figure 12-4**). However, for bioaugmentation treatments by both direct injection and on GAC substrate, the total number of putative dehalorespiring microorganisms remained nearly 2-fold higher in the bioaugmented mesocosms compared with the untreated mesocosms (8.0×10^5 compared to about 4.5×10^5 copies per gram at day 120, respectively). Detection of putative dechlorinating bacteria in untreated inactive mesocosms suggests that only a small proportion of the indigenous bacteria within this clade of the Chloroflexi were capable of

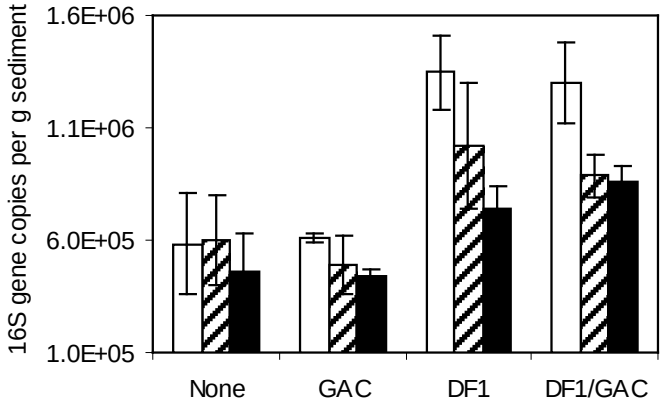


Figure 12-4. Enumeration of putative dechlorinating Chloroflexi normalized to 16S rRNA gene copies/gram sediment at day 0 (□), day 60 (▨) and day 120 (■). Treatments included spent medium only, spent medium and GAC, amendment with DF-1 by direct injection, and amendment with DF-1 adsorbed to GAC. Each bar represents the mean and standard deviation of three replicates samples.

dechlorinating PCBs. The results indicate that greater numbers of dehalorespiring microorganisms were maintained in bioaugmented mesocosms during active dechlorination of the weathered Aroclor compared with non-bioaugmented controls.

Seven predominant phylotypes were detected by DHPLC in the BH sediment mesocosms. The community phylotypes were generally similar between mesocosms over 120 days, with the exception of DF1, which was only detected in bioaugmented mesocosms (**Figure 12-5**). The putative DF1 fraction was collected, sequenced, and found to be 100% identical to DF1. One phylotype present at time zero (BH 4) was 100% identical to phylotype DEH10, a PCB dechlorinating bacterium previously detected BH sediment microcosms (Fagervold et al., 2007; Fagervold et al., 2005), but no other previously reported BH phylotypes were detected.

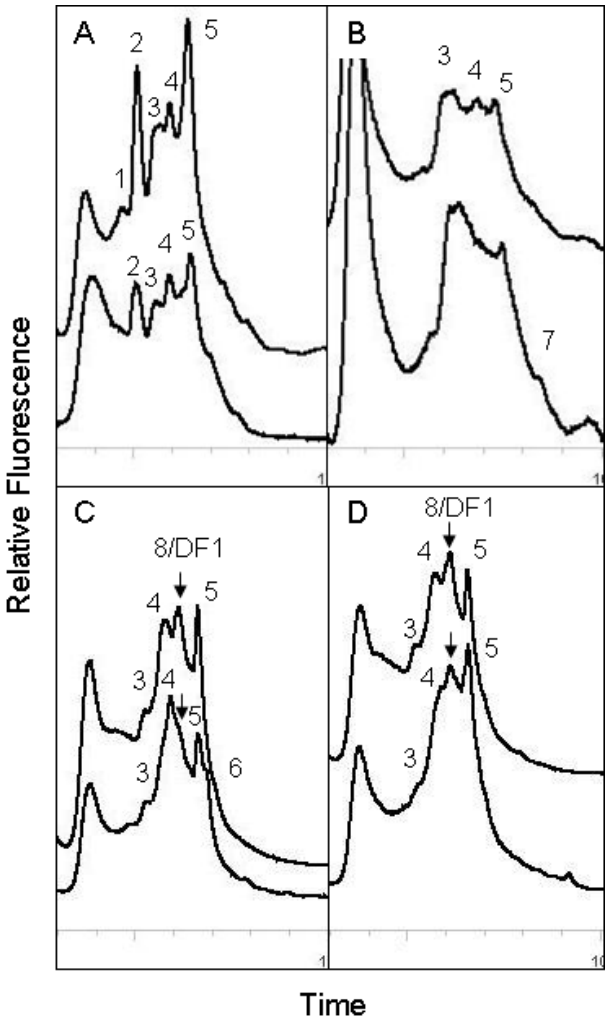


Figure 12-5. DHPLC community analysis of putative dechlorinating Chloroflexi 16S rRNA genes in mesocosms at day 0 (*bottom trace*) and day 120 (*top trace*). *A*, addition of spent growth media alone; *B*, addition of spent growth media and GAC; *C*, addition of DF1; *D*, addition of DF1and GAC. Peak eluting at about 6.3 minutes (labeled 8/DF1 with *arrow*) in *C* and *D* confirmed DF1 16S rRNA gene by sequencing. Other phylotypes of putative dechlorinating Chloroflexi are labeled *1*, *2*, *3*, *4*, *5*, *6*, *or 7*.

Discussion

There have been several reports on aerobic bioremediation by microbes and plants, but these approaches are only effective for treatment of less chlorinated congeners (Abraham et al., 2002). Although highly chlorinated congeners commonly associated with commercial Aroclors such as Aroclor 1254, 1258, 1260 and 1262 can be reductively dechlorinated to less chlorinated congeners that serve as substrates for complete degradation by aerobic bacteria, concerns over several factors have called into question whether bioaugmentation by dehalorespiring bacteria is an effective strategy for *in situ* treatment of PCB impacted sediments.

Bioavailability. Previous studies on the kinetics of reductive dechlorination in sediment microcosms spiked with Aroclor 1242 (Fish 1996) or 1248 (Rhee et al., 2001) reported that activity was limited at concentrations below 37-40 ppm. However, Magar et al. (2005) reported evidence of reductive dechlorination at PCB concentrations as low as 1–2 ppm in Lake

Hartwell over a period of 11 years, which suggested that dehalorespiring bacteria slowly dechlorinated low levels of weathered Aroclor over time. In the current report we show unequivocally that bioaugmentation with the dehalorespiring bacterium DF-1 successfully stimulates the reductive dechlorination of sediment containing 1.3 ppm of weathered Aroclor 1260. Furthermore, addition of GAC, which increases the partition coefficient of PCBs between the sediment matrix and aqueous phase, had no inhibitory effect on dechlorination activity in bioaugmented mesocosms. GAC has been reported to reduce the bioavailability of PCBs to macroorganisms that ingest the particles (Sun & Ghosh, 2008), however, the slight stimulatory effect of GAC observed in the bioaugmented mesocosm suggests that PCB dehalorespiring bacteria have mechanisms that enable them to utilize weathered PCBs sequestered in the inorganic carbon fraction. The true threshold of bioavailability for PCB dechlorinating bacteria is not currently known, however, the results clearly demonstrate that bioaugmentation can stimulate reductive dechlorination of low levels of weathered PCBs in the 1-2 ppm range even with activated carbon present to sequester PCBs from any rapid desorption pool of sediment-associated PCB. Although the less chlorinated PCBs generated by bioaugmentation are more bioavailable to benthic organisms, they are generally less toxic because they are non-coplanar or have at least two adjacent unsubstituted carbon atoms that would make them subject to rapid metabolic degradation (Safe, 1989).

Sustainability. One factor that is critical for successful bioaugmentation of weathered PCB-impacted sediments is the ability of the inoculated biocatalyst to compete with the indigenous community of non-dehalorespiring bacteria for electron donors and nutrients. In the current study DF-1 was detected in bioaugmented sediment mesocosms after 120 days indicating that non-native DF1 could coexist with the indigenous sediment community. The total numbers of putative dehalorespiring bacteria decreased by approximately half after 90 days to a steady state of 7-8 x 10^5 cells per gram of sediment, but community analysis showed that DF-1 was sustained as a predominant member of the putative dehalorespiring community. Although the total numbers of putative dehalorespiring phylotypes was 1-2 orders of magnitude lower than observed in prior reports of Aroclor 1260 dechlorinating microcosms (Bedard et al., 2007; Fagervold et al., 2007) neither exogenous electron donors nor electron acceptors were added in our study; therefore the lower steady state numbers likely reflect lowers concentrations of indigenous electron donors or acceptors available in the sediment. The observation that dehalorespiring bacteria were sustained in the sediment mesocosms without an exogenous electron donor is consistent with the ability of dehalorespiring bacteria to outcompete hydrogenotrophic sulfate reducers, acetogens, and methanogens in the presence of limited hydrogen concentration (Fennell & Gossett, 1998; Loffler et al., 1999). Interestingly, the indigenous phylotypes of putative dehalorespiring bacteria, with the exception of phylotype DEH10 had not been reported previously in Baltimore Harbor sediment Aroclor 1260 enrichment microcosms (Fagervold et al., 2007). One possible explanation is that a different population of dehalorespiring bacteria was enriched with high concentrations of spiked Aroclor 1260 in the prior studies and the population in the current study is more relevant at *in situ* concentrations. The results show that DF-1 was able to coexist successfully with the indigenous microbial population even with the lower background concentrations of electron donor.

Another significant observation was the detection of PCB congeners that did not result from dechlorination of doubly flanked chlorines. These dechlorination reactions were not observed in

control mesocosms with spent cell-free medium indicating that the activity observed was neither due to a "priming" effect from residual PCB 61 or 23 in the inoculum nor a nutrient effect from carryover of formate or cell byproducts in the medium. This phenomenon has been reported previously. May et al. (2008) observed enhanced dechlorination of congeners without flanked chorines in Aroclor 1260 impacted soil microcosms inoculated with DF-1. Krumins et al. (2009) observed in PCB-impacted Anacostia River sediments bioaugmented with *D. ethenogenes* strain 195 stimulated PCB dechlorination and although this species was not sustained enhanced activity continued, which was attributed to the indigenous dehalorespiring community. One possible explanation for this effect is "priming" by the accumulation of dechlorination products from the initial activity of high numbers of DF-1 inoculated into the sediments. Addition of PCB congeners and analogs is known to stimulate the reductive dechlorination of PCBs in lab studies (Bedard et al., 1998; Deweerd & Bedard, 1999; Krumins et al., 2009) and in field tests (Bedard & Quensen 1995). Overall the results indicate that in addition to initiating and directly dechlorinating weathered PCBs, bioaugmentation with DF-1 had a synergistic effect on the indigenous dehalorespiring community by an as yet unknown mechanism that contributed further to the dechlorination process.

Bioaugmentation. There have been recent attempts to test the effects of bioaugmentation with pure cultures of dehalorespiring bacteria. Krumins et al. (2009) reported enhanced reductive dechlorination of weathered PCBs (*ca*. 2 ppm) in sediment microcosms after bioaugmentation with *D. ethenogenes* strain 195 and May et al. (2008) reported enhanced dechlorination of Aroclor impacted soil (4.6 ppm) after bioaugmentation with DF-1. In contrast to the prior studies where bioaugmentation stimulated the reductive dechlorination of PCB by 0.2 Cl/biphenyl after 415 days and 0.35 Cl/biphenyl after 145 days, respectively, bioaugmentation in the current study stimulated the reductive dechlorination by 0.7 Cl/biphenyl after only 120 days. Furthermore, bioaugmentation results in the current study stimulated 56% by mass reduction of penta- through nona-chlorobiphenyls to lesser-chlorinated congeners (primarily tetrachlorobiphenyls), which are susceptible to aerobic degradation, with no detectable activity in untreated controls. The discrepancies in the rates and extent of dechlorination could possibly occur due to a number of factors including available nutrients, presence of inhibitory co-contaminants, the dehalorespiring strain used and the growth state and numbers of cells used for bioaugmentation. Distribution of cells in the current study was effective either by direct injection or on GAC particles. The ability to use a solid substrate such as GAC for inoculation of cells offers a possible solution for dispersing cells in the field.

The results of this study support the potential feasibility of using bioaugmentation for treatment of PCB impacted sediments. We showed that bioavailability does not prevent bioaugmentation from treating low levels of weathered PCBs in sediment mesocosms and that GAC actually enhanced the overall process. Furthermore, DF-1, a non-indigenous species, was sustained throughout the dechlorination process and had a positive synergistic effect on the indigenous dehalorespiring population that contributed to the process. Although DF-1 was used successfully to bioaugment reductive dechlorination of weathered Aroclor 1260 in Baltimore Harbor sediments, discrepancies between the current and prior studies highlight the importance of testing inoculum with sediments from each site. All PCB bioaugmentation studies including the current study have been conducted on a laboratory scale and field studies will be required ultimately to validate the approach for *in situ* treatment of contaminated sites. However, the

overall results of this mesocosm-based study provide compelling evidence to support further testing and development of bioaugmentation with dehalorespiring bacteria as an environmentally less invasive and lower cost alternative for *in situ* treatment of PCB impacted sediments.

Conclusions and Implications for Future Research/Implementation

One of the most challenging scenarios for the application of bioremediation is the treatment of aquatic sediments. Sediments contain a variety of contaminants which generally enter the aquatic system in association with the particles. One of the most common classes of sediment contaminants are the organohalides, including intentionally produced chemicals such as the polychlorinated biphenyls (PCBs) and polybrominated flame retardants and the inadvertently produced polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/Fs) Contaminated aquatic sediments are of concern because they may re-release of contaminants back into the water column thus endangering human health and the environment. Further, sediments may need to be dredged from the body of water to accommodate navigational needs and the removed sediments may need to be landfilled or undergo treatment to avoid re-release of contaminants back into the environment. To date, treatment of contaminated sediments is generally limited to natural attenuation, capping, or dredging to sequester or remove the pollutants.

To utilize dehalogenation as a bioremediation approach for contaminated sediment, dehalogenating bacteria and biostimulation agents such as electron donors or haloprimers could be added to contaminated sediment and dehalogenation end-products would be produced. This approach has not been realized yet because of the lack of knowledge about the organisms and because the technologies needed to incorporate the amendments have also not been proven. Further, the end-products of dehalogenation should be less toxic than the parent compounds and/or amenable to other fate processes such as aerobic degradation. Bioaugmented bacteria—either specific strains or activated indigenous bacteria could also be injected into sediment. The injection could be followed by or preceded by capping to contain the organisms in the sediment, to maintain or shift redox processes, and to prevent migration of biostimulants or daughter products.

The micro- and mesocosm studies described here with different contaminated sediments demonstrate that addition of appropriate amendments can enhance microbial dehalogenation of historic organohalide contaminant mixtures, including PCBs and PCDD/Fs. We have developed the molecular tools to quantify and monitor microbial activity during dehalogenation and identify key populations mediating the processes. Furthermore, the different chemical analytical methods (GC-MS, GC-MS-MS, GC-ECD) for detection and quantification of organohalide mixtures have been validated. Our data shows that various contaminated sediments (Anacostia River, Washington D.C.; Kearny Marsh, NJ; Kymijoki River, Finland; Grasse River, NY; Passaic River, NJ) and soils (Mechanicsburg, PA) tested contain active populations of native dechlorinating bacteria with potential for biodegrading the historical PCDD/F and PCB contaminants. Active dechlorinating bacterial populations are present in these sediments and biostimulation may enhance the activity of both native *Dehalococcoides* spp. and the bioaugmented dehalogenating bacteria, such as *D. ethenogenes* strain 195. Our findings on the identity of species and genes involved in anaerobic PCB dechlorination might be used to evaluate environmental PCB dechlorination potential and to monitor the PCB dechlorination process under various conditions. Eventually it will help to understand the anaerobic PCB dechlorination mechanism in detail and to accelerate the detoxification process of environmentally contaminated PCBs. For example, dehalogenating bacteria of the Pinellas

subgroup of *Dehalococcoides* spp. are indigenous to Anacostia River sediments. Biostimulation with pentachloronitrobenzene and/or bioaugmentation with *D. ethenogenes* strain 195 resulted in increased dechlorination of weathered PCBs. Biostimulation with either tetrachlorobenzene or pentachloronitrobenzene increased the abundance of putative reductive dehalogenase (*rdh*) genes. This detailed monitoring will provide needed guidance for evaluating the effects of biostimulation and/or bioaugmentation used for *in situ* bioremediation of PCB contaminated sediments.

In conclusion, dechlorination of historical PCDD/F and PCB sediment contaminants can be stimulated by addition of amendments and/or bioaugmentation with dechlorinating bacteria. The enhanced dechlorination correlates with increased numbers of dehalorespirer populations and reductive dehalogenase genes, supporting our hypothesis that the halogenated co-substrates enhance dechlorination of historic pollutants by supporting growth and activity of dehalogenating bacteria. Although biostimulation and bioaugmentation were used successfully to stimulate reductive dechlorination of weathered Aroclors in sediments, observed differences between sites highlight the importance of testing inoculum with sediments from each site. Recent advances have examined addition of particles such as coke or activated carbon to sediments for physical sequestration of pollutants via sorption. Incorporation of dehalogenating bacteria into or on these particles could lend a bioactive element to the treatment. What remains is to grow sufficient quantities of these difficult to grow organisms and perform field testing to further refine the process. All PCB biostimulation and bioaugmentation studies including the current study have been conducted on a laboratory scale and field studies will be required ultimately to validate the approach for in situ treatment of contaminated sites. However, the overall results of this microcosm-based study provide compelling evidence to support further testing and development of biostimulation and bioaugmentation with dehalorespiring bacteria as environmentally less invasive and lower cost alternatives for in situ treatment of PCB impacted sediments.

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