MICROBIAL REDUCTIVE DEHALOGENATION OF PERSISTENT HALOGENATED AROMATIC CONTAMINANTS IN SEDIMENTS OF THE HACKENSACK RIVER IN NEW JERSEY

By

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ABSTRACT OF THE DISSERTATION

Microbial Reductive Dehalogenation of Persistent Halogenated Aromatic Contaminants in Sediments of the Hackensack River in New Jersey

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Microbial reductive dechlorination of problematic halogenated aromatics was observed in aquatic sediments. Laboratory experiments demonstrated reductive dehalogenation of hexachlorobenzene (HCB) and pentachloroaniline (PCA) in anaerobic microcosms set up with sediment samples originating from different sites (H1 ~ H5) along the Hackensack River, NJ. The dehalogenation products and intermediates were detected by gas chromatography-mass spectrometry. HCB was dechlorinated via pentachlorobenzene (PeCB) and 1,3,5-trichlorobenzene (TriCB) to 1,3- and 1,4-dichlorobenzene (DCB), while PCA was dechlorinated via 2,3,5,6-tetrachloroaniline, 2,4,5- and 2,4,6-trichloroaniline, and 2,4-, 2,5-, 2,6-, and 3,5-dichloroaniline, to monochloroaniline. Debromination of hexabromobenzene (HBB) was not observed after up to 12 months incubation. However, when HCB was added as a co-substrate, reductive debromination of HBB occurred with 1,2,3,5-tetrabromobenzene (TeBB) and 1,3,5-tribromobenzene (TriBB) detected at low concentration. *Chloroflexi* specific 16S rRNA gene PCR-DGGE

followed by sequence analysis detected members of the "Pinellas subgroup" of *Dehalococcoides mccartyi* in H1 sediment. H1 and H5 cultures had different reductive dehalogenase (*rdh*) gene profiles based on the analysis of a set of 12 *rdh* genes. Generally, more *rdh* genes were detected in H1 cultures, corresponding with higher dehalogenating activity and greater abundance of *Dehalococcoides* species in H1 sediment. Cultures amended with both HBB and HCB had all 12 tested *rdh* genes.

Carbon compound specific isotope analysis (CSIA) was conducted to obtain isotope fractionation data for dehalogenation of HCB, PeCB, 1,2,3,5-TeCB, 1,2,3,5-TeBB, and 1,3,5-TriBB. Strong evidence of isotope fractionation coupled to dehalogenation was not observed, possibly due to the low solubility of the halobenzene substrates and a dilution of the isotope signal. However, we could detect a depletion of the $\delta^{13}C$ value in the DCB product from dechlorination of HCB; isotope fractionation of TeCB and the sequential depletion and enrichment of $\delta^{13}C$ value for TriCB in TeCB dechlorinating cultures; and the enrichment of $\delta^{13}C$ during debromination of TriBB.

From this study, we conclude that indigenous anaerobic microorganisms in the Hackensack River, NJ are capable of dehalogenating chloro- and bromobenzenes. Molecular community analyses demonstrated that there are different responsible microbial communities corresponding to the locations of the Hackensack River. A "priming" effect of HCB on HBB dehalogenation was observed. CSIA data for highly halogenated benzenes suggest that it may have application for assessing *in situ* microbial reductive dehalogenation.

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CHAPTER 1

Introduction - Microbial Reductive Dehalogenation of Halogenated Aromatics

Characteristics of Organohalides

Organohalides are compounds that have one or more covalent bond between a carbon and a halogen. The strongly electronegative halogen group contains five elements: fluorine, chlorine, bromine, iodine and astatine. The strength of the carbon-halogen bond is determined by both the molecular weight and ionic radius of the halogen atom. The increase in molecular weight decreases the strength of the carbon-halogen bond; that is, a carbon-fluorine bond is stronger than a carbon-bromide bond. Hence, the smaller atom makes the bond harder to break. However, the increase in ionic radius can make the compound more difficult to be attacked by microorganisms because of its bulkiness even though the bond strength is decreased.

Origins of Organohalides

Organohalide compounds are widely dispersed in the environment. Historically, halogenated organics were regarded as xenobiotic compounds introduced into the environment through anthropogenic activities. However, environmental data are supportive that halogenated organic compounds originate from natural sources as well as anthropogenic sources (Häggblom and Bossert, 2003). In many cases, these natural organohalides are predominant to their anthropogenic counterparts (Gribble, 1999). Either biogenic and geogenic processes are involved in the formation of organohalides naturally. As biogenic sources, organohalides are produced by a variety of organisms in

the ecosystem, such as bacteria, fungi, plants, sponges, insects and mammals (for review, see Häggblom and Bossert, 2003). These organisms and their organohalide products are observed in both terrestrial and marine environments. Marine systems contain rich nutrients to support diverse activities of organisms, and notably, several biogenic organohalides are derived from the marine environment. For example, a high concentration of dibromophenol (9.9 μmol/g fresh weight) was found in hemichordates inhabiting marine sediments (King, 1986). Another example is sponge species, in the phylum *Porifera*, which have been studied for their ability to produce a variety of brominated metabolites such as bromoindoles and bromophenols (Ebel et al., 1997; Gribble, 1999; Turon et al., 2000). These brominated metabolites may also be biosynthesized by bacteria or microalgae associated with the sponge (Ebel et al., 1997; Gribble, 1999). Higher animals, even humans, are observed to metabolize organohalides, as well. For example, chlorinated dioxins and dibenzofurans are converted from chlorophenols by enzyme myeloperoxidase isolated from human leucocytes (Wittsiepe et al., 2000).

Geogenic sources of organohalides include eruption of volcanoes, forest fires and geothermal processes. These activities can cause formation of chlorinated organics such as chlorinated dioxins, vinyl chloride, and chloroform. Alcock et al. (1998) detected polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs) in historic soil samples from before 1900 in the UK. PCDD/Fs have been generated through natural incineration processes. In the USA, 2,3,7,8-dibenzo-*p*-dioxin was detected in mined ball clays (Ferrario and Byrne, 2000). These studies suggest a natural origin of dioxins. Significant

amounts of vinyl chloride can also be produced in terrestrial environments by soil processing (Keppler et al., 2002). Forests have recently been identified to release chloroform into the environment (Laturnus et al., 2002). Even though estimated amounts of chloroform from northern temperate forests is less than 1% to the annual global atmospheric input, forest sources could be significantly greater when the large tropical forests are considered. Another study suggested that up to 10 % of the analyzed aromatic structures in freshwater fulvic acid samples were mono- or dichlorinated (Dahlman et al., 1993).

Human activities also cause a formation of halogenated organic compounds. For example, chlorinated compounds are released by combustion of natural materials such as wood. Ahling and Lindskog (1982) showed that chlorobenzenes and chlorophenols were released from combustion of fresh wood. Combustion of waste containing chlorinated compounds also releases chlorinated compounds into the environment. Chlorinated compounds, including PCDD/Fs and chlorobenzenes, are produced during municipal waste incineration as well (Milligan and Altwicker, 1993).

Organohalides had been made in large amounts for industrial purposes and personal uses before their regulation or bans. Chlorinated ethenes, chlorophenols, chlorinated and brominated benzenes, brominated flame retardants (BFRs), and polychlorinated biphenyls (PCBs) are representatives of the many man-made organohalides. They are widely used in diverse fields. For example, tetrachloroethene (PCE) is used as a

degreasing and cleaning agent. Chlorinated benzenes and chlorinated phenolics and their derivatives, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA), are used as pesticides. Brominated compounds such as tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), and polybrominated diphenyl ethers (PBDEs) are frequently used flame retardants (Birnbaum and Staskal, 2003). PCBs were used in electrical applications as coolants and lubricants. They were also used for manufacturing of paint, dyes and carbonless paper. As a result of their diverse usages, organohalides have become prominent pollutants in soil, sediments and aquifers.

Halogenated Aromatics

Halogenated aromatics are compounds with one or more benzene rings and one or more halogen substituent(s). Some important examples are: chlorinated dioxins, PCBs, chlorobenzenes, tetrabromobisphenol A (TBBPA), bromobenzenes and chlorophenols. They have been manufactured for several purposes in industry and as household chemicals. They are frequently persistent and cause harmful effects on environment. A short description for their structures and usages are given in Table 1-1. In 2002, the Stockholm Convention announced the proposed ban on twelve persistent organic pollutants. All of them were chlorinated compounds (Table 1-2).

Dioxins

Dioxins encompass the polyhalogenated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), in which halogen atoms are bonded to two aromatic rings joined by two or one

ether bond, respectively. PCDDs and PCDFs are the most relevant persistent pollutants (Weber et al., 2008). They are hydrophobic and they can adsorb strongly to organic matter. Dioxins are persistent in the environment. A calculated half-life for 2,3,7,8-PCDD in sediments of the Baltic Sea was more than a hundred years (Kjeller and Rappe, 1995).

Dioxins are formed and released as byproducts from the production of other chlorinated chemicals, such as pentachlorophenol and PCBs (Faengmark et al., 1994). They have not been intentionally synthesized in large quantities. They are found as complex mixtures in the environment. Generally, less chlorinated congeners have higher mobility in the water phase and higher volatility than highly chlorinated congeners. Hence, the products of dehalogenation become more bioavailable and biodegradable.

Dioxins are problematic because of the acute toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) and other 2,3,7,8-substituted congeners. The toxicity of dioxins is related to a specific chlorine substitution pattern, not to the number of chlorines. The lateral position, 2,3,7,8 is regarded as a responsible combination for the toxicity of PCDD/Fs (Wittsiepe et al., 2007). Dioxins have harmful health effects on humans and animals, causing several health problems including chloracne, severe wasting syndrome, immunotoxicity, reproductive toxicity, neurotoxicity and carcinogenicity (WHO, 2010). By binding to the cytosolic aryl hydrocarbon receptor, dioxins initiate the major toxic effect in organisms and trigger a series of transcriptional events.

Notwithstanding their toxicity, dioxins can be used as carbon and energy sources by aerobic bacteria and as electron acceptors for respiration by anaerobic bacteria (for review, see Bunge and Lechner, 2009). The total number of chlorines on the dioxin congeners determines how they are degraded. Non-chlorinated and mono-chlorinated dioxins can serve as the sole carbon and energy sources for aerobic bacteria. Steric hindrance caused by the bulky chlorine substitutes prevents the activity of ring-cleavage dioxygenases of aerobic bacteria (Wittich, 1998). Generally, chlorinated dioxins with more than four chlorines cannot undergo aerobic degradation. Instead, highly chlorinated dioxins are subject to reductive dechlorination by anaerobic bacteria. Recently, Liu et al. (2014) and Kuokka et al. (2014) demonstrated that indigenous anaerobic bacteria in Kymijoki River sediments dechlorinate a series of PCDDs and PCDFs. There are some isolated strains known for dioxin dehalogenation. They are *Dehalococcoides mccartyi* strains 195 and CBDB1, and *Dehalococcoides* strain DCMB5 (Bunge et al., 2003, 2008; Fennell et al., 2004). In addition, PCDD/Fs are subject to fungal degradation. For example, Takada et al. (1996) reported that white-rot fungi can degrade chlorinated dioxin co-metabolically regardless of their number of chlorine substitutes.

***Polychlorinated biphenyls* (*PCBs*)**

Polychlorinated biphenyls are man-made compounds with no natural sources. They were introduced into the environment for industrial usage. PCBs had been used in electronic equipment such as transformers and capacitors due to their flame retardant and insulating character. They were also used as dyes, inks, and carbonless papers between the 1950s and 1970s. Because of their accumulation in the environment and harmful health effects

on humans, their production in the USA was stopped in 1977 (ATSDR, 2000). However, a significant amount of PCBs are still detected in environmental samples (Abramowicz, 1993; Domingo and Bocio, 2007; Srogi, 2008; Weaver, 1984).

Commercially used PCBs are complex mixtures of chlorinated biphenyls of different congeners. As a mixture of 209 congeners, PCBs can exist in various forms such as oily liquids, solids, or vapor. Reductive dechlorination of PCBs occurs in the environment and it can change the composition of the mixture of PCB congeners. Microbial PCB dechlorination was firstly reported in the 1980s (Brown et al., 1987; Quensen III et al., 1988). Later, it was found that there was a threshold concentration of PCBs to make microbial reductive dechlorination amenable. Microbial reductive dechlorination did not occur when PCBs concentration was less than 40 ppm (Cho et al., 2003).

Dehalococcoides-like bacteria are involved in PCB dechlorination (e.g., Bedard et al., 2007; Krumins et al., 2009; Wu et al., 2002b; Zanaroli et al., 2012). There are some known identified bacteria, such as *o*-17, DF-1 and *Dehalococcoides mccartyi* strain 195, that are capable of dehalorespiration of PCBs. These three bacteria belong to the *Chloroflexi*. Bacterium *o*-17 and DF-1 have not been isolated in pure culture. Bacterium *o*-17 was identified from DGGE profiles of 16S rRNA genes amplified from a PCB dechlorinating culture (Cutter et al., 1998). This bacterium dechlorinates 2,3,5,6-tetraCB (Cutter et al., 2001; May et al. 2006). Bacterium DF-1 was identified from PCR/DGGE analysis of 16S rRNA genes from sediment free PCB dechlorinating culture. DF-1 dechlorinates PCBs with doubly flanked chlorines when formate or mixture of hydrogen

and carbon dioxide was in the culture (Wu et al., 2002b). *Dehalococcoides mccartyi* strain 195 (formerly *D. ethenogenes*), well known for its ability to dechlorinate PCE completely to ethane, is also capable of dechlorinating a variety of PCBs (Fennell et al., 2004).

Brominated flame retardants

Brominated flame retardants (BFRs) are chemicals used to prevent/suppress fire in different products. Typically, they are used in polyurethane foam, plastics, various textiles such as curtains and carpets. Most BFRs are mixed directly into the product during manufacture as additives. They are not reactive with the materials, thus they may be released into the environment by migration from the materials (De Wit et al., 2010). Some examples of BRFs are: polybrominated diphenyl ethers (PBDEs); polybrominated biphenyls (PBBs); tetrabromobisphenol A (TBBPA); and hexabromobenzene (HBB; Alaee, 2003). However, they are also of concern because of their properties: they are persistent, potentially toxic, and they tend to bioaccumulate (Birnbaum and Staskal, 2004). For their toxicity, generally, they cause negative effects on neurological function, thyroid hormone, and reproduction system (for review, see Letcher et al., 2010).

Halogenated benzenes

Brominated benzenes and chlorinated benzenes are mostly well known and widely used halogenated benzenes (Birnbaum and Staskal, 2004). They are persistent and toxic. They can be adsorbed onto particles in soils and sediments. The hydrophobicity makes them easily sorb onto particulate matters and cause accumulation in sediments, hence their

transport in aquatic system is limited. Interestingly, two fully halogenated benzenes, hexachlorobenzene (HCB) and hexabromobenzene (HBB), are even found in the Arctic, an indication of their atmospheric transport (Su et al., 2006**;** Wit et al., 2010).

One BFR chemical, hexabromobenzene (HBB) was heavily used as an additive flame retardant in China and Japan. It was used in the manufacturing of paper, wood, textile, electronics, and plastic goods (Covaci et al., 2011). In fact, HBB is detected almost everywhere, including in air, sea water, freshwater, sediment, marine mammals, eggs of birds, breastmilk, placenta, human blood serum adipose tissue, polar bear adipose tissue (Wit et al., 2010). Bromobenzenes also have natural origins, for example they have been detected in the volatiles of oakmoss (Gribble, 1999).

Brominated benzenes have acute toxicity and cause liver impairment. Asymmetrical bromobenzenes cause hepatotoxic necrosis, but symmetrical bromobenzenes such as 1,4-dibromobenzene (DBB) and HBB do not (Szymanska, 1997). Even though HBB has a symmetrical structure, it is still of concern as a problematic compound because it can go through reductive debromination pathway and asymmetrical bromobenzenes can be formed as intermediates.

Chlorinated benzenes have been used as pesticides, solvents, and additives in other organic chemicals. Historic emissions of hexachlorobenzene peaked in the 1960s. Even though commercial hexachlorobenzene production was banned in 1982 in the United States, it continued to be released as a byproduct of production of chlorinated solvents

and from incomplete combustion (ATSDR, 2013). Hexachlorobenzene is a well-studied chlorinated benzene because it is on the original list of the "dirty dozen", persistent organic pollutants (POPs) of the 2011 Stockholm convention. Pentachlorobenzene was later added to the list of POPs. Because of their low water solubility and volatility (vapor pressure for HCB at 20 °C: 1.09×10^{-5} mm Hg), highly chlorinated benzene congeners accumulate in aquatic sediments (Beurskens et al., 1994; Prytula and Pavlostathis, 1996). Chlorobenzenes have been detected even in vegetable soil near industrial plants in China (Song et al., 2012). Thus, it may not safe to grow crops near industrial plants.

Chlorobenzenes are subject to both aerobic and anaerobic metabolism. There are several aerobic bacterial strains known for growing on chlorinated benzenes as a sole source of carbon and energy (for reviews, see Häggblom, 1992; Field and Sierra-Alvarez, 2008). Most of them can grow on monochlorobenzene or dichlorobenzenes. Only a few aerobic bacterial strains can grow on tri- or tetrachlorobenzenes as a sole source of carbon and energy. Chlorinated benzenes are subject to oxidation by aerobic bacteria. The process begins with dioxygenase-mediated attack with chlorocatechols formed as intermediates (see e.g. Häggblom 1992). Finally, CO_2 and chloride are released.

Higher chlorinated benzene congeners are subject to reductive dechlorination to lower chlorinated benzenes by anaerobic bacteria. Some reductive dechlorination processes of chlorinated benzenes are listed in Table 1-3. There are some strains capable of reductive dechlorination of chlorinated benzenes. Among them, *Dehalococcoides mccartyi* strain CBDB1 was isolated from a bioreactor for its ability to halorespire trichlorobenzene

(TriCB) and tetrachlorobenzene (TeCB; Adrian et al., 1998, 2000b). Later, it was shown that strain CBDB1 is also capable of reductive dechlorination of hexachlorobenzene (HCB) to TCB and dichlorobenzene (DCB; Jayachandran et al., 2003). The rate of complete dechlorination of TCB to DCB was faster when strain CBDB1 was cultivated in mixed culture than when CBDB1 was alone. Other bacteria in the mixed culture may support dechlorination activity of CBDB1 through production of needed cofactors (Yan et al., 2013). Chlorobenzene reductive dehalogenase gene *cbrA* was identified in CBDB1. It is the first identified chlorobenzene reductive dehalogenase (Adrian et al., 2007).

Dehalococcoides mccartyi strain 195, known for PCE dechlorination, is also capable of dechlorinating hexachlorobenzene to 1,2,3,5-TeCB and 1,3,5-TCB (Fennell et al., 2004). "*Dehalobium chlorocoercia*" strain DF-1, closely related to *Dehalococcoides* spp., is capable of reductive dechlorination of HCB to 1,3,5-TCB as well (Wu et al., 2002a). Recently, two *Dehalobacter* strains were isolated for their ability to utilize DCBs for reductive dehalogenation. They are *Dehalobacter* sp. 12DCB1 and 13DCB1 and their strain designation represents which DCB isomer was used for isolation (Nelson et al., 2014).

Chlorophenols

Chlorophenols include pentachlorophenol, tetrachlorophenol, trichlorophenol, dichlorophenol, and chlorophenol. Chlorophenols have been introduced into the environment through their use as biocides and as by-products of chlorine bleaching in the pulp and paper industry (ATSDR, 1999). Chlorinated phenols are formed naturally as

well. For example, 2,6-dichlorophenol is produced by a dozen species of ticks for the insect pheromone (for review, see Sonenshine, 2006).

Since the production of pentachlorophenol as an antiseptic in 1936, the total production of chlorophenols increased rapidly before bans of some of them, including 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) by the 1990s. Their solubility in organic solvents and in water as sodium salts made them versatile. Chlorophenols have been used for wood preservation by dip treatment or spraying over several decades. Consequently, it caused serious local contamination by chlorophenols (for review, see Häggblom and Valo, 1995).

Introduction of chlorine substituents to phenol increases its toxicity, with the degree of toxicity related to the number of chlorines (for review, see Henschler, 1994). 2,4,6-Trichlorophenol and pentachlorophenol can be carcinogenic to mice and rats. The carcinogenic effect of pentachlorophenol is more obvious than trichlorophenol. In addition, mono-, di-, and trichlorophenol have been shown a tumor-promoting activity in mouse skin tests.

Chlorophenols are subject to both of anaerobic and aerobic metabolism (for review, see Häggblom and Valo, 1995; Field and Sierra-Alvarez, 2008). Reductive dechlorination is a predominant pathway of chlorophenol metabolism under anaerobic conditions. Chlorinated phenols can be used for electron acceptors regardless of their number of chlorines by dehalorespiring bacteria. Generally, dehalorespiring bacteria remove *ortho*-

chlorines. However, *Desulfitobacterium hafniense* (formerly *D. frappieri*) can remove *para*- and *meta*-chlorines as well (Bouchard et al., 1996; Villemur et al., 2006).

Under aerobic conditions, chlorinated phenols can be used as an electron and carbon source by aerobic bacteria to support growth (see Field and Sierra-Alvarez, 2008). When chlorophenols are used as the electron and carbon source by aerobic bacteria, different strategies apply according to their number of chlorines. Chlorinated phenols with lower number of chlorines are generally attacked initially by monooxygenase and yielding chlorocatechols. On the other hand, highly chlorinated phenols are converted to chlorohydroquinones as the initial intermediates (Häggblom and Valo, 1995). Also, there are some bacteria and fungi reported for their capability to degrade chlorophenols. For example, two wood degrading fungi, brown rot fungi and white rot fungi, can degrade chlorophenols.

The Environmental Fate of Organohalides

Organohalides are problematic contaminants because of their persistence and toxicity. Thus, the fate of halogenated organic compounds is of concern to human and environmental health. They have carcinogenic and lethal effects on biota (Henschler, 1994). As a result of their introduction in the environment, organohalides are deposited in soil, groundwater aquifers and sediments. They can be volatilized into the atmosphere and adsorbed onto particles based on their Henry's law constants. Less halogenated compounds tend to be more readily volatilized. Their hydrophobic characteristics lead them to attach on soil and sediment particles. Since they are hydrophobic, they can be

dissolved in lipids, resulting in their bioaccumulation and biomagnification in the food chain and cause harmful effects on living organisms including humans.

Abiotic and biotic processes contribute to the overall fate of organohalides in the halogen cycle. Abiotic dechlorination of halogenated compounds usually shows complete dechlorination reaction even though it is usually slower than microbe-dependent dechlorination. Lee and Batchelor (2002) showed that trichloroethene (TCE) was subject to reductive dechlorination by iron-bearing soil minerals. Its main product was acetylene. Generally, *in situ* abiotic dechlorination processes can be enhanced by the addition of zero-valent metals. Zero-valent iron (Fe^0) and zero-valent zinc (Zn^0) have been used in engineering to cause abiotic reductive degradation. The advantages of Fe^0 are that it is versatile in dechlorinating a wide concentration range of chlorinated organics at a low cost, while Zn^0 has greater reducing power than Fe^0. Experiments showed that the presence of Zn^0 made dechlorination of tetrachloroethene (PCE) three times shorter than the presence of Fe^0 (for review, see Tobiszewsky and Namieśnik, 2012).

Even though abiotic dehalogenating processes is one factor that determines the fate of organohalides, natural processes of abiotic dehalogenation are slow and occasionally will need other substances to trigger the process. Input of substances can cause additional cost and contamination. For example, reductive dehalogenation by zero-valent iron could form iron oxides and other corrosion by-products on the surface of iron to cause further prevention of dechlorination reaction (Ma and Wu, 2008).

Microbial degradation is one of the key mechanisms that determine the ultimate fate of organohalides. Microorganisms play a major role in the "halogen cycle". In aerobic environments, halogenated compounds are used as carbon and energy sources by aerobic bacteria. In anaerobic environments, reductive dehalogenation is a predominant pathway. In reductive dehalogenation, organohalides function as an electron acceptor for respiration.

Microbial Dehalogenation

Cleavage of the carbon-halogen bond is one of the critical steps in dehalogenation. As key players in the halogen cycle, microorganisms have evolved several known mechanisms for dehalogenation (Häggblom and Bossert, 2003). Firstly, under aerobic conditions, organohalides can serve as electron donors and provide the organism carbon and energy source. Aerobic microorganisms typically attack the carbon backbone initially and dehalogenation follows. Secondly, for anaerobic microorganisms, reductive dehalogenation is considered to be the predominant process in degradation of organohalides. Organohalides can serve as electron acceptors for anaerobic respiration, in the process termed respiratory reductive dehalogenation (also halorespiration or dehalorespiration). Halorespiring bacteria can use organohalides as terminal electron acceptors to gain energy through proton motive force ATP generation.

Two processes of reductive dehalogenation have been identified (Mohn and Tiedje, 1992). They are hydrogenolysis and vicinal reduction/dihaloelimination. In hydrogenolysis, a halogen substituent is replaced with a hydrogen atom. In vicinal

reduction, two halogen substituents are removed from a molecule and the additional bond is formed between the carbon atoms. Alkyl halides are transformed by both of hydrogenolysis and dihaloelimination, but aryl halides are subject only to hydrogenolysis. Lastly, dehalogenation reactions also undergo detoxification reactions or fortuitous reactions that do not provide any benefit to the organism.

Removal of a halogen substituent from an organic compound usually makes the compound less or nontoxic (Borja et al., 2005). In addition, it makes the compound amenable to further degradation by aerobic microorganisms by eliminating steric hindrance. Oxidation of the carbon backbone is unfavorable when it is highly halogenated because the halogen atom is electronegative (Wohlfarth and Diekert, 1997). In fact, reductive dehalogenation is the only biodegradation mechanism for highly halogenated compounds. Reductive dehalogenation is energetically favorable in anoxic environments such as river sediments and groundwater aquifers (Dolfing and Harrison, 1992). Estimated values of Gibbs free energy of formation for various halogenated aromatics were suggestive that hydrogen dependent dehalogenation are energetically more favorable than either sulfate reduction or methanogenesis.

Reductive dehalogenation is a means for microorganisms to gain energy for living when it is coupled to a respiratory electron transport chain (Holliger et al., 1999). Dolfing and co-workers first demonstrated that the anaerobic bacterium strain DCB-1 (*Desulfomonile tiedjei*) reductively dechlorinates 3-chlorobenzoate coupled to ATP production (Dolfing and Tiedje, 1987; Dolfing, 1990; Mohn and Tiedje, 1991). In respiratory dehalogenation,

the halogenated substrate acts as a terminal electron acceptor. This process also requires electron donors. Hydrogen and small chain organic acids such as pyruvate, butyrate, propionate and acetate are frequently used electron donors for reductive dehalogenation.

For bioremediation applications microbial reductive dehalogenation has some advantages over abiotic reductive dehalogenation. It is a much faster and safer mechanism than abiotic dehalogenation. Furthermore, it is suitable for oxygen depleted organohalide-contaminated sites such as sediments and aquifers. Also, it is potentially less expensive since indigenous microorganisms can be utilized to remediate organohalides-contaminated sites. They can be stimulated to facilitate microbial remediation.

Factors Which Affect Microbial Degradation of Organohalides

The effectiveness of microbial degradation of organohalides depends on many environmental and chemical factors. These factors include the presence of alternate electron acceptors and donors, hydrogen concentration, temperature, water solubility of the pollutant, the concentration of the pollutant and the presence of co-contaminants or co-substrates.

Different competing electron acceptors and electron donors

In any given environment, available organic carbon and electron acceptors as well as the thermodynamics of redox reactions of alternate electron acceptors determine the dominant respiratory process. The most important electron acceptors in terms of microbial respiration and carbon flow in anoxic environment are nitrate, sulfate, Fe(III),

Mn(IV), and carbonate. They are essential to the processes of denitrification, sulfidogenesis, iron reduction, manganese reduction, and methanogenesis, respectively. In freshwater sediments and anoxic soils, the main respiratory processes are denitrification, iron/manganese reduction and methanogenesis. In marine sediments, sulfate reduction is the dominant process for carbon metabolism. The sulfate concentration is about 20 to 30 mM in seawater compared to less than 200 μM in most freshwater systems. Methanogenesis is less significant in marine systems due to the inhibition by sulfate (for review, see Häggblom and Milligan, 2000).

Halogenated aromatic compounds are biodegradable by diverse anaerobic microbial communities under a variety of anaerobic conditions. To make reductive dehalogenation occur, organohalides should serve as more available electron acceptors than alternate electron acceptors. Alternative electron acceptors could compete for electrons and inhibit reductive dehalogenation (Mohn and Tiedje, 1992). However, in terms of degradation, these alternate electron acceptors can also support anaerobic degradation (oxidation of the carbon structure) of halogenated aromatic compounds. These compounds can be biodegraded by diverse anaerobic communities under a variety of anaerobic conditions. For example, halobenzoic acids can be utilized by diverse bacteria in environmental samples from different environments and geographical locations under denitrifying condition (for review, see Häggblom and Milligan, 2000). There are two possible degradation mechanisms of halobenzoate by denitrifying bacteria. Halobenzoate can be degraded by initial reductive or hydrolytic dehalogenation, or dehalogenation after ring reduction and cleavage. Mineralization of halogenated aromatic compounds to CO_2 may

be coupled to either sulfate, iron (III) or nitrate reduction. For example, 2-bromophenol can be reductively debrominated to phenol and its mineralization is coupled to sulfate reduction, iron reduction or denitrification.

Electron donors can influence reductive dehalogenation. For example, Fennell and Gossett (1997) tested several electron donors affecting in reductive dehalogenation of tetrachloroethene. They tested butyric acid, ethanol, lactic acid, and propionic acid. All of them facilitated dechlorination to vinyl chloride and ethane in comparable amounts. However, in short-term, butyric acid and propionic acid resulted in predominance of dechlorination. As low H_2-producing electron donors, they would support dechlorination by resulting in low levels of H_2 that is less available to methanogens. When there was a depletion of the electron donors and hydrogen, dechlorination slowed significantly and remaining PCE was only degraded slowly.

Effect of sulfate on reductive dechlorination

Generally, sulfate has a negative effect on reductive dehalogenation. High sulfate levels may inhibit the enrichment of a dehalogenating population. In these systems, sulfate-reducing bacteria may outcompete dehalogenating microorganisms for hydrogen, but the interactions may be more complex (Townsend et al., 1997).

Sulfate inhibition effects on degradation of halogenated organic compounds were studied in mixed cultures as well as in pure cultures. In a sulfate inhibition study with mixed cultures, Gibson and Suflita (1986) suggested that sulfate might compete more effectively

for electrons than chlorinated aromatic compounds in sulfate-reducing conditions (29 mM of sodium sulfate in cultures). Their experiments explained that the dechlorination of chloroaromatics was inhibited under sulfate-reducing conditions while dechlorination was observed under methanogenic conditions. Alder et al. (1993) suggested that the continuous input of sulfate is adverse to the microbial dechlorination of PCBs. The fact that the dechlorination of preexisting PCBs in samples collected from harbor sediment occurred in the laboratory under methanogenic conditions suggested that the high *in situ* sulfate level was inhibitory for reductive dehalogenation. Under sulfidogenic conditions, PCBs were not dechlorinated regardless of the origins of sediment samples: lake, harbor, or river. Kuhn et al. (1990) observed retardation of tetrachloroaniline degradation with the amendment of sulfate (less than 0.1 mM). The delayed initial dehalogenation step also prevented further metabolism of intermediates. Heimann et al. (2005) reported that sulfate (2.5 mM in cultures) had a negative effect on reductive dehalogenation of trichloroethene when electron donor was limited. The presence of sulfate in the culture reduced the activity of the reductive dehalogenase in *Desulfomonile tiedjei*, dehalogenating, sulfate reducing organism (Townsend and Suflita, 1997). On the other hand, the presence of sulfate did not inhibit dehalogenation in resting cell suspensions of *Desulfomonile tiedjei* (DeWeerd et al., 1991). For *Dehalococcoides* sp. strain FL2, sulfate (10 mM) does not have inhibitory effect on dechlorination of trichloroethene (He et al., 2005).

Even though it has been observed in many cases that sulfate inhibits reductive dehalogenation, there are other studies that show that sulfate is not inhibitory. Kohring et

al. (1989) reported that reductive dechlorination of 2,4-dichlorophenol and 4-chlorophenol occurred in samples from freshwater sediments amended with sulfate. Sulfate reduction was also observed concomitant with reductive dechlorination. Häggblom and Young (1990) reported that 4-chlorophenol was reductively dechlorinated to phenol by a sulfate-reducing culture and complete mineralization of the phenol to carbon dioxide can be coupled to sulfate reduction. Townsend et al. (1997) observed that 3-chlorobenzoate was degraded in the presence of sulfate. In their enrichment cultures, both sulfate reduction and reductive dehalogenation occurred concurrently.

Hydrogen concentration

The level of hydrogen in dehalogenating cultures plays a key role in influencing reductive dehalogenation. Hydrogen is known as the ultimate electron donor for dehalogenation by *Dehalococcoides* species (Maymo-Gatell et al., 1997; He et al., 2005). As the best electron donor for *Dehalococcoides* species for reductive dehalogenation, hydrogen is a preferable electron donor for diverse microbial populations, as well. Therefore, microorganisms compete for hydrogen. Several microcosm studies showed that the hydrogen concentration should be controlled at a low level to make reductive dehalogenation the prevalent process in a mixed culture. Fennell et al. (1997) suggested propionate and butyrate as competent electron donors to stimulate reductive dehalogenation specifically in the mixed culture containing methanogens and sulfidogenic bacteria. Dehalogenators can be competitive in mixed cultures containing propionate and butyrate because these small chain fatty acids release hydrogen slowly. Yang and McCarty (1998) demonstrated that dehalogenators competed against

methanogens and homoacetogens in a mixed culture when the hydrogen level was maintained between 2 and 11 nM. He et al. (2002) observed that acetate treated microcosms dechlorinating tetrachloroethene (PCE) and trichloroethene (TCE) reached ethene, a final dehalogenating product, faster than hydrogen treated one. It was surprising because an increase of hydrogen was regarded as a means to enhance reductive dehalogenation. This suggested that low concentrations of hydrogen are more supportive of reductive dehalogenation. Sufficient hydrogen could make microorganisms other than dehalogenating populations predominant. In conclusion, it is important to keep low hydrogen level consistent to support reductive dehalogenation.

Temperature

Temperature is a key factor that influences the rates of microbial metabolism, including reductive dehalogenation processes. Holliger et al. (1992) reported an optimum temperature range between 25 °C and 30 °C for trichlorobenzene (TriCB) dechlorination. Beurskens et al. (1994) reported that there was an optimum temperature around 30 °C for hexachlorobenzene (HCB) dechlorination. Nonetheless, dechlorination of HCB still occurred at lower temperature, 3 °C. Tas et al. (2011) also reported that highest HCB transformation rates were observed at 25 °C and 30 °C, but the dechlorination rate decreased below 20 °C and above 30 °C. Wu et al. (1997) showed that there was a temperature range that allowed for reductive dehalogenation of PCBs to occur. Based on their results, reductive dehalogenation of Aroclor 1260 only occurred at 8 °C to 34 °C and 50 °C to 60 °C. Their results also demonstrated that the pattern of dehalogenation changes depending on temperature. Kuokka et al. (2014) showed that 1,2,3,4-TeCDF had

a high dechlorination rate at the higher incubation temperature (21 °C) and low dechlorination rate at the lower temperature (4 °C). Temperature also caused differences in the dechlorination pattern. At higher temperature, TeCDF tended to be dechlorinated at the lateral position and hence, it promoted to form less toxic products. However, at lower temperature, dechlorination occurred more equally at all chlorines positions. In conclusion, higher temperature was more effective for detoxification of dioxins.

Fletcher et al. (2011) demonstrated that elevated temperature decreased dechlorination activity even though biomarker gene transcript abundance was greater at higher temperature. At 30°C, a PCE-dechlorinating culture had higher dechlorination activity than at 35 °C and 40 °C. The abundance of several reductive dehalogenase (RDase) genes was in agreement with the result of dechlorination activity. However, RDase gene transcript abundances were greater at higher temperature as well. The authors suggested that RDase genes are up-regulated in response to stress such as elevated temperatures and exposure to oxygen. Therefore, biomarker gene transcripts abundances are not always agreeable with dechlorination rates and it can cause error in prediction (Tas et al., 2009).

Water solubility

The water solubility of halogenated compounds affects their bioavailability to anaerobic dehalogenating bacteria. Microorganisms can readily access compounds with high aqueous solubility (Borja et al., 2005). Generally, an increase in chlorine substituents decreases water solubility. The hydrophobicity of organohalides decreases their bioavailability and makes them transfer to environmental interfaces and partition and

accumulate there (Eisenreich et al., 1989; Larsson, 1985; McFarland and Clarke, 1989; McKinney et al., 1985).

To increase bioavailability of halogenated aromatics in liquid cultures, various carriers were introduced (for review, see Mohn and Tiedje, 1992). They included a liquid organic phase, sediments, bark chips and granular activated carbon.

Concentration of organohalide contaminants

A low concentration of contaminants may be insufficient to obtain growth of responsible organisms or to induce responsible enzymes for reductive dehalogenation. A high concentration can also be an obstacle to organisms to conduct biodegradation since it could be toxic to the organisms. There are some organohalides that can be degraded at low concentration rapidly, but are persistent at higher concentration because of their toxic effect on responsible organisms (Madsen and Aamand, 1992; Mohn and Kennedy, 1992). In particular, for PCB dechlorination, the optimum rate of dechlorination is usually detected for concentrations between several hundred to 1000 ppm (w/w) of sediment (Quensen III et al., 1988).

Presence of co-contaminants/co-substrates

Toxic organic co-contaminants and toxic heavy metals may inhibit dehalogenating bacteria and other community members. Heavy metal toxicity can be reduced if the site geochemistry supports the formation of sulfides that precipitate free metals. However, precipitated heavy metals can be mobilized with lowering pH. There are other co-

contaminants providing beneficial effects for reductive dehalogenation. They include petroleum hydrocarbons or organic solvents such as acetone and methanol. Their degradation products may also act as electron donors to support reductive dehalogenation (for review, see Fennell and Gossett, 2003).

Since the discovery of the priming effect of organohalides on reductive dechlorination of PCBs (Wu et al., 1997), co-substrates have been introduced to anaerobic cultures to stimulate dehalogenation. Several studies showed that usage of halogenated co-substrates ("haloprimers") enhances a rate of reductive dehalogenation. Wu et al. (1997) showed that dechlorination of Aroclor residue only occurred when 2,3,4,6-tetrachlorobiphenyl was added as a priming agent. Bedard et al. (1998) reported that polybrominated biphenyl stimulated reductive dechlorination of PCBs in sediments. Ahn et al. (2005) tested several haloprimers to enhance dehalogenation activity of indigenous bacteria in sediment samples. 1,2,3,4-TeCDD and TCDF dechlorination was enhanced most effectively by adding haloprimers such as 1,2,3,4-tetrachlorobenzene, 2,3,4,5-tetrachloroanisole, 2,3,4,5-tetrachlorophenol and 2',3',4'-trichloroacetophenone. Krumins et al. (2009) reported that tetrachlorobenzene and pentachloronitrobenzene enhanced PCB dechlorination. Kuokka et al. (2014) showed that reductive dehalogenation of 1,2,3,4- TeCDF was improved with 2,3,4,6-tetrachlorophenol as a haloprimer. Liu et al. (2014) added pentachloronitrobenzene for halopriming effect and observed the enhancement of the rate and extent of 1,2,3,4-TeCDD dechlorination.

Dehalogenating Bacteria

Organohalide respiring bacteria have been identified from diverse bacterial phyla, including the *Proteobacteria*, *Firmicutes* and *Chloroflexi* (for review, see Hug et al., 2013). They have different characteristics of metabolism. Proteobacterial organohalide respirers have versatile metabolisms encoded on relatively large genomes (Maphosa et al., 2010). In contrast, organohalide respiring *Chloroflexi* are all obligate organohalide respirers with a very restricted metabolism. In between, *Firmicutes* contains both characteristics of them, versatile or restricted.

The isolation and characterization of dehalogenating bacteria from environmental samples is important in several aspects. Firstly, it could enhance biodegradation of halogenated pollutants by allowing investigation of dehalogenating bacteria. Once we have knowledge that certain bacteria are responsible for degradation of a specific contaminant, we can use that species to remediate the contaminated site. We can stimulate bacterial dehalogenating activity by adding stimulants such as co-substrates (haloprimers) based on their characteristics (Bedard et al., 1998; Liu et al., 2014). Secondly, we can block unwanted biotransformation pathway of the contaminants that can release toxic compounds as intermediates based on knowledge about their capabilities. Lastly, we could develop tools like molecular probes to monitor or study *in situ* degradation (Cutter et al., 2001).

Table 1-4 shows examples of identified or isolated dehalogenating bacteria. The very first anaerobic dehalogenating bacterium isolated in pure culture was *Desulfomonile tiedjei*

strain DCB-1. It was isolated from sewage sludge for dehalogenation of 3-chlorobenzoate. However, dehalogenating bacteria are difficult to isolate in the laboratory due to toxic substrates, various growth requirements, and difficulty to obtain a colony of an active obligate anaerobic bacterium (Adrian et al., 2000a). For example, in the case of *Dehalococcoides mccartyi* strain CBDB1, traditional isolation steps for aerobic bacteria did not work. However, the researchers were successful in isolating a colony of strain CBDB1 by cultivation in anaerobic agarose shake cultures. Even though it is difficult to isolate anaerobic dehalogenating bacteria by traditional isolation procedures, they can be identified by PCR without isolation and studied while they exist in consortia. For example, "strains" *o*-17 and DF-1 were detected from Baltimore Harbor sediment microcosms that dechlorinated PCBs (Cutter et al., 2001; Wu et al., 2002b). Bacterium *o*-17 is the first microorganism that was identified for its ability to reductively dechlorinate PCBs (Cutter et al., 2001). *o*-17 is responsible for ortho dechlorination of 2,3,5,6-tetrachlorobiphenyl. Bacterium DF-1 is capable of PCB dechlorination restricted to double flanked chlorines on the biphenyl (Wu et al., 2002b). Even though it is tricky, many scientists are trying to isolate more dehalogenating bacteria from enriched cultures to gain a better understanding.

Proteobacterial organohalide respiring bacteria include *Geobacter*, *Desulfuromonas*, *Anaeromyxobacter* and *Sulfurospirillum* species. All of them are non-obligate with versatile metabolisms including organohalide respiration. One example of a halorespiring *Proteobacteria*, *Geobacter lovleyi* strain SZ, in *Deltaproteobacteria* was isolated from non-contaminated freshwater sediment in South Korea (Sung et al., 2006). It is the first

chlorinated ethane dechlorinating organism utilizing both of hydrogen and acetate as electron donors.

The Firmicutes contain non-obligate organohalide-respiring *Desulfitobacterium* spp. as well as metabolically restricted *Dehalobacter* organohalide respiring bacteria (Maphosa et al., 2010). The first isolate of *Desulfitobacterium* was *Desulfitobacterium dehalogenans*. It was isolated from freshwater sediment. It is capable of dehalogenation of chlorinated phenolics, hydroxylated PCBs and chloroalkenes (Utkin et al., 1994; Wiegel et al., 1999). Another isolate, *Desulfitobacterium hafniense* Y51 was isolated from soil contaminated with tetrachloroethene (PCE) and can dehalogenate PCE and polychloroethanes (Suyama et al., 2001). It has a relatively large genome compared to *Dehalococcoides mccartyi* strain 195, which suggest that *Desulfitobacterium* has a broader range of substrate utilization than *Dehalococcoides* (Nonaka et al., 2006). Strain PER-K23 was the first isolate of *Dehalobacter* species isolated from tetrachloroethene dechlorinating, packed-bed column filled with Rhine River sediment and ground anaerobic granular sludge from a sugar refinery. It is a strict anaerobe dechlorinating PCE and TCE to 1,2-DCE (*cis*-DCE; Holliger et al., 1998). The gram positive *Dehalobacter* is restricted in its metabolism to dehalorespiration similar to *Dehalococcoides* spp. (Smidt and de Vos, 2004). Recently, two additional *Dehalobacter* strains were isolated based on their characteristics capable of using diverse halogenated compounds such as chlorobenzenes, dichlorotoluenes, and tetrachloroethene (Nelson et al., 2014).

Dehalococcoides species are well studied dehalogenating *Chloroflexi*. *Dehalococcoides* species are strictly anaerobic and highly specialized in respiratory dehalogenation. They have very diverse range of chlorinated compounds for reductive dehalogenation. They can dechlorinate chlorinated organics with three or more chlorines such as PCBs, dioxins, PCE, TCE and chlorinated benzenes (Adrian et al., 1998; Fennell et al., 2004; Wu et al., 2002a). They prefer to remove doubly flanked chlorines. Their genomes are relatively small, but have a large number of *rdh* genes. Ten to 36 *rdh* genes are found in the individual strains (Tas et al., 2010a). They are mesophilic and their optimum pH is neutral. They use hydrogen as the electron donor and a halogenated compound as an electron acceptor (Freedman and Gossett, 1989; Loffler et al., 1999; Maymo-Gatell et al., 1997; Middeldorp et al., 1997; Zhang and Wiegel, 1990). Since they require hydrogen as the electron donor, they are more easily maintained in a microbial community with fermentative partners.

So far, eight *Dehalococcoides* strains, including strain 195, CBDB1, BAV1, VS, FL2, GT, DCMB5, and MB have been cultivated even though it is difficult to maintain them as a pure culture (Adrian et al., 2000b; Bunge et al., 2008; Cheng and He, 2009; Cupples et al., 2003; He et al., 2003, 2005; Maymo-Gatell et al., 1997; Sung et al., 2006). The first isolate of *Dehalococcoides* was *Dehalococcoides mccartyi* strain 195 (formerly, *Dehalococcoides ethenogenes* strain 195; Maymo-Gatell et al., 1997). It was isolated from methanol-PCE methanogenic culture developed of digested sludge from Ithaca wastewater treatment plant (Freedman and Gossett, 1989). Following studies revealed

that strain 195 could dechlorinate a variety of highly chlorinated compounds as well such as chlorobenzenes, PCBs, dioxins and dibenzofurans (Fennell et al., 2004). *Dehalococcoides mccartyi* strain CBDB1 was initially isolated for reductive dehalogenation of tetra- and trichlorobenzenes from a fluidized bed bioreactor (Adrian et al., 1998). It was shown later that it also dehalogenates hexachlorobenzene, pentachlorobenzene and chlorinated dioxins (Bunge et al., 2003; Jayachandran et al., 2003). Isolated *Dehalococcoides* strains share 98% nucleotide identity of their 16S rRNA genes.

As presented earlier in this chapter, organohalides have many natural origins. Therefore, it is not unexpected that diverse dehalogenating microbial communities are observed in pristine as well as polluted environments. *Dehalococcoides*-like microorganisms in the *Chloroflexi* were observed to dechlorinate PCE in uncontaminated environments, such as sediments (Kettelmann and Friedrich, 2008). In addition, Krzmarzick et al. (2011) detected *Chloroflexi* 16S rRNA genes in uncontaminated forest soils. Furthermore, they reported that there was no lag period in growth of *Dehalococcoides*-like *Chloroflexi* from uncontaminated soil in the presence of organochlorines.

There are a group of studies that elucidate the geographical distribution of dehalogenating bacteria by PCR targeting 16S rRNA gene of *Dehalococcoides* and *Desulfitobacterium* (Lanthier et al., 2001; Hendrickson et al., 2002; Tas et al., 2009). These studies showed that *Dehalococcoides* and *Desulfitobacterium* were ubiquitous. Lanthier et al. (2001) suggested why *Desulfitobacterium* species are ubiquitous. According to them,

Desulfitobacterium could be related to the sulfur cycle since it uses sulfite, thiosulfate and sulfur as electron acceptors.

Reductive dehalogenation by aerobic bacteria

Even though reductive dechlorination is a predominant dehalogenating mechanism in anaerobic environments, several studies suggest that it can occur in aerobic environments as well. Halogenated compounds even with highly chlorinated aromatic ring can be subject to aerobic reductive dehalogenation (for reviews, see Häggblom, 1992; Mohn and Tiedje, 1992). For example, pentachlorophenol (PCP) can be a target of aerobic mineralization that involves reductive dehalogenation steps (Apajalahti and Salkinoja-Salonen, 1987). An enzyme catalyzing reductive dechlorination step in PCP degradation in *Mycobacterium chlorophenolicum* (formerly, *Rhodococcus chlorophenolicus*) was oxygen tolerant, but had a requirement for reducing power. Another aerobic bacterium, *Alcaligenes denitrificans* NTB-1 was reported to be capable of reductive dechlorination in the initial step in the mechanism of 2,4-dichlorobenzoate to 4-chlorobenzoate (Van Den Tweel et al., 1987).

Reductive Dehalogenases

Traditionally, 16S rRNA sequence analysis has been used for phylogenetic analyses. However, sometimes dehalogenating bacteria of the same species have diverse dehalogenating activities, thus it is not an appropriate method to delineate the functions of dehalogenating bacteria. For example, *Dehalococcoides* strain BAV1 and FL2 share 100% sequence identity of 16S rRNA gene even though they have different

dehalogenating abilities. Strain BAV1 can respire VC to support growth, but strain FL2 cannot. Thus, it is confusing to characterize dehalogenating ability of bacteria with their 16S rRNA sequences alone (He et al., 2003, 2005). To overcome this situation and provide more accurate information, reductive dehalogenase genes are used instead of 16S rRNA genes for analysis and elucidate bacterial dehalogenating capability (Cupples, 2008; Park et al., 2011).

The respiratory reductive dehalogenase gene is composed of two subunits, *rdhA* and *rdhB*. *RdhA* encodes the catalytically active enzyme and *rdhB* is a gene encoding a putative membrane-anchoring protein. With few exceptions, *rdhA* has two iron-sulfur cluster-binding motifs and a twin-arginine signal motif as conserved features (Magnuson et al., 2000). Their association with the cytoplasmic membrane reinforces its role in membrane-associated electron transport-coupled phosphorylation (Smidt and de Vos, 2004).

The presence of cofactors is essential for reductive dehalogenation. For example, *Dehalococcoides* species require vitamin B_{12} to conserve energy via organohalide respiration. A study with isolated *Dehalococcoides* including strain BAV1, strain GT and strain FL2 let us know that they require corrinoid cofactor produced by their co-cultures for reductive dehalogenation (Yan et al., 2013).

Table 1-5 lists purified reductive dehalogenases so far. The first reductive dehalogenase that was biochemically characterized was the 3-chlorobenzoate reductive dehalogenase of

Desulfomonile tiedjei strain DCB-1 (Ni et al., 1995). This enzyme has a haem cofactor. It is unique since most of the following reductive dehalogenases identified have a corrinoid cofactor. A purification of reductive dehalogenases is tricky because the enzyme is oxygen sensitive and it is difficult to culture dehalogenating bacteria in sufficient mass for biochemical analyses. Therefore, scientists have developed procedures to identify more reductive dehalogenases by sequence analysis, cloning, and transcription and activity test without complete purification. A few examples of reductive dehalogenases are *dcaA*, *cbrA*, *vcrA*, *bvcA* and *tceA* (Adrian et al., 2007; Krajmalnik-Brown et al., 2004; Magnuson et al., 2000; Marzoratie et al., 2007; Müller et al., 2004).

The genomes of *Desulfitobacterium*, *Dehalococcoides*, *Dehalogenimonas* and *Dehalobacter* strains contain multiple *rdhA* genes (for review, see Hug et al., 2013). Sequence studies on *Dehalococcoides mccartyi* strains have shown that their genomes bear 10-36 homologous *rdhA* genes (McMurdie et al., 2009). Smidt et al. (1999) found that closely related species have very different numbers and kinds of dehalogenases. These factors suggest that horizontal gene transfer has occurred in dehalogenating bacterial species. Additional support for horizontal gene transfer in the distribution and evolution of *rdh* genes comes from analysis of genetic data of *Dehalococcoides* spp. showing that transposable elements are highly involved in the evolution and distribution of genes responsible for dechlorination (Futagami et al., 2007). These transposable elements are also located in the vicinity to *rdh* genes in the genomes.

A study of trichloroethene reductive dehalogenase (*tceA*) genes by Krajmalnik-Brown et al. (2007) also supports horizontal gene transfer for distribution of reductive dehalogenase genes. They studied the distribution of *tceA* of *Dehalococcoides* spp. in ethane producing enrichment cultures from diverse geographical locations in the USA. Their investigation showed that *tceA* genes have high sequence similarity intergenomically; in addition, the intergenic spacer regions between *tceA* and *tceB* were 100% identical. This suggests that the dissemination of the *tceAB* region occurred recently. Moreover, remnants of transposable elements were observed surrounding *tceA* and *tceB*. All these factors suggest horizontal gene transfer occurred in the dissemination *tceA*, and more broadly, probably other RDase genes among *Dehalococcoides* species.

A structure of reductive dehalogenase was recently reported by the research group of Diekert in Germany (Bommer et al., 2014). They studied PceA from *Sulfurospirillum multivorans*, a bacterium able to couple reductive dehalogenation of tetrachloroethene (PCE), trichloroethene (TCE), and dibromoethene (DBE) to growth. They showed that PceA from *S. multivorans* consisted of five compartments including an N-terminal unit, a norpseudo-B_{12} binding core, an insertion unit, an iron-sulfur cluster binding unit and a C-terminal unit. Conformation of the enzyme changed when substrate was present.

Under stressful conditions such as elevated temperature, *Rdh* genes are up-regulated (Fletcher et al., 2011). Since reductive dehalogenation is the only known energy generation procedure in *Dehalococcoides* (Loffler and Edwards, 2006), it is not surprising that genes involved in energy metabolism are up-regulated in response to

stressors. For example, up-regulation of the TCA cycle in response to elevating temperature was observed in a certain strain in *Escherichia coli* strain and in *Staphylococcus aureus* (Lüders et al., 2009; Fleury et al., 2009).

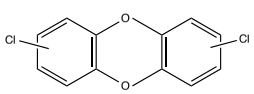
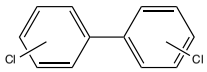
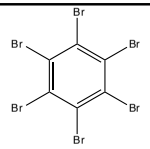
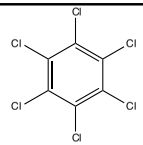
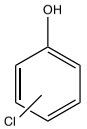
Hypothesis and Objectives

Halogenated aromatics are of concern because of their toxicity and persistence. Microbial dehalogenation is one of the main processes affecting the fate of halogenated compounds in the environment. Dehalogenation can result in reduction of the toxicity of halogenated compounds. Problematic halogenated aromatic compounds containing one benzene ring such as hexachlorobenzene (HCB), hexabromobenzene (HBB) and pentachloroaniline (PCA) can be used as model compounds to study *in situ* dehalogenation. The hypothesis of this study is that indigenous microorganisms are responsible for dehalogenation of persistent halogenated compounds in aquatic sediments.

The objective of my work was to determine the activity of indigenous anaerobic dehalogenating bacteria in aquatic sediments and to evaluate the potential for *in situ* biodegradation. The Hackensack River was selected as a model system because the river is located in a metropolitan area and has been historically exposed to diverse contaminants, including many organohalides. Samples were collected from five different locations along the river to compare reductive dehalogenation patterns and microbial communities for each location.

To pursue the first objective, several research goals were introduced. First, dehalogenation of HCB, HBB and PCA in Hackensack River sediment was studied by monitoring dehalogenation rates and intermediates with a reconstruction of dehalogenation pathways based on detected intermediates. Second, the responsible microorganisms in reductive dehalogenation in Hackensack River sediments was investigated by PCR-DGGE and sequencing. The third aim was to understand different induction patterns of reductive dehalogenases with each electron acceptor used. Finally, to develop tools for evaluating *in situ* biodegradation, carbon compound specific isotope analysis was conducted for halogenated benzenes including hexachlorobenzene (HCB), pentachlorobenzene (PeCB), tetrachlorobenzene (TeCB), tetrabromobenzene (TeBB), and tribromobenzene (TriBB).

Table 1-1. Examples of halogenated organic pollutants.

	Dioxins	**PCBs**	**HBB**	**HCB**	**CPs**
Structure					
Natural sources	Forest fires	Volvanic activity	Oakmoss	Volcanic activity, minerals	Sponges
Anthropogenic sources	Waste incineration, bleaching	Insulating fluid, dye, carbonless paper	Flame retardants	Pesticide, dye, waste incineration	Pesticides, wood treatment
Health effects	Cancer, endocrine disruption	Skin and liver damage, possibly cancer	Indirect liver damage	Immune system damage, liver damage, cancer	Cancer

Abbreviations: PCBs, polychlorinated biphenyl; HBB, hexabromobenzene; HCB, hexachlorobenzene; and CPs, chlorophenols.

Table 1-2. Persistent organic pollutants (POPs; Stockholm Convention, 2001).

Name	Use
Aldrin	Insecticide
Chlordane	Insecticide
Dieldrin	Pesticide
Dioxins (PCDDs)	Unintentional by-product
DDT	Insecticide
Endrin	Insecticide
Furans (PCDFs)	By-product
Heptachlor	Pesticide
Hexachlorobenzene	Industrial and technical chemical
Mirex	Insecticide
Polychlorinated biphenyls (PCBs)	Industrial and technical chemical
Toxaphene	Insecticide

Table 1-3. Studies demonstrating reductive dechlorination of chlorinated benzenes.

Substrate	Products	Source	reference
HCB	1,3,5-TCB	Sediment slurry	Chang et al., 1997
	1,3,5-TCB	Sediment	Chen et al., 2000
	1,3,5-TCB	Sludge and sediment	Chen et al., 2001
	1,3,5-TCB	PCB-enriched culture with DF-1	Wu et al., 2002
	TriCB	*Dehalococcoides mccartyi* strain CBDB1	Jayachandran et al., 2003
	DCB	*Dehalococcoides mccartyi* strain CBDB1	Jayachandran et al., 2003
	DCB	*Dehalococcoides mccartyi* strain 195	Fennell et al., 2004
	1,3-DCB	Sediment	Pavlostathis and Prytula, 2000
	1,3-DCB	Sludge and sediment	Chen et al., 2001
	1,3-DCB	Soil	Brahushi et al., 2004
	CB	Soil slurries	Ramanand et al., 1993
	MCB	Sediment	Susarla et al., 1996
PeCB	1,3,5-TCB	Sediment	Chen et al., 2000
	TriCB, DCB	*Dehalococcoides mccartyi* strain CBDB1	Jayachandran et al., 2003
	CB	Soil slurries	Ramanand et al., 1993
TeCB, TriCB	TCB, DCB	*Dehalococcoides mccartyi* strain CBDB1	Adrian et al., 2000
1,2,4-TriCB	CB	Soil slurries	Ramanand et al., 1993
	MCB	Sludge	Hölscher et al., 2010
	CB, lactate	Sediment	Middeldorp et al., 1997
1,2,3-TriCB	MCB	Sludge	Hölscher et al., 2010
	1,3-DCB	Sediment	Holliger et al., 1992
DCBs	MCB, benzene	Sediment	Fung et al., 2009
1,2-DCB	MCB, benzene	*Dehalobacter* sp. 12DCB1	Nelson et al., 2014
1,3-DCB	MCB	*Dehalobacter* sp. 13DCB1	Nelson et al., 2014
1,4-DCB	MCB	*Dehalobacter* sp. 14DCB1	Nelson et al., 2014
MCB	CH4, CO2	Sediment	Liang et al., 2013
CBs		Sediment	Masunaga et al., 1996

Abbreviations: HCB, hexachlorobenzene; PeCB, pentachlorobenzene; TeCB, tetrachlorobenzene; TriCB, trichlorobenzene; DCB, dichlorobenzene; and MCB, monochlorobenzene.

Table 1-4. Identified/isolated anaerobic dehalogenating bacteria.

Name	Substrate	Source	Reference
Dehalococcoides mccartyi strain 195	PCE	Groundwater	Maymo-Gatell et al., 1997
Dehalococcoides mccartyi strain CBDB1	Chlorobenzenes	River sediment	Adrian et al., 2000
Dehalococcoides sp. strain MB	PCE	Sediment	Cheng and He, 2009
Dehalococcoides sp. strain GT	TCE	Aquifer material	Sung et al., 2006
Dehalococcoides sp. strain FL2	TCE/1,2-DCE	River sediment	He et al., 2005
Strain MS1	PCE	Aquifer	Sharma and Mccarty, 1996
Dehalobium chlorocoercia DF-1	PCB	Estuarine sediment	Wu et al., 2002
Desulfitobacterium chlororespirans strain Co23	2,3-DCP	Compost soil	Sanford et al., 1996
Desulfitobacterium hafniense (DCB-2T)	TCP	Municipal sludge	Christiansen and Ahring, 1996
Desulfitobacterium frappieri (strain PCP-1T)	PCP	Sewage sludge/soil	Bouchard et al., 1996
Desulfitobacterium dehalogenans	2,4-DCP/3-Cl-4-OHPA	Lake sediment	Utkin et al., 1994
Desulfitobacterium sp. strain PCE1	PCE	Soil	Gerritse et al., 1995
Desulfitobacterium sp. strain Y51	PCE/polychloroethanes	Contaminated soil	Suyama et al., 2001
Dehalobacter restrictus strain PER-K23	PCE/TCE	Sediment	Holliger et al., 1998
Strain TEA related to *Dehalobacter restrictus*	PCE/TCE	Anaerobic charcoal reactor	Wild et al., 1996
Dehalospirillum multivorans	PCE	Activated sludge	Scholz-Muramatsu et al., 1995
Desulfomonile tiedjei strain DCB-1	3-chlorobenzoate	Sewage sludge	Deweerd et al., 1990
Anaeromyxobacter dehalogenans strain 2CP-1	2-chlorophenol	Stream sediment	Cole et al., 1994
Desulfomonas chloroethenica	PCE/TCE	Freshwater sediment	Krumholz, 1997
Desulfovibrio strain TBP-1	2,4,6-TBP	Estuarine sediment	Boyle et al., 1999
Desulfovibrio dechloracetivorans	2-CP	Sediment	Sun et al., 2000
Desulfoluna spongiiphila	Bromophenols	Marine sponge	Ahn et al., 2009
Trichlorobacter thiogenes K1	Trichloroacetic acid	Subsoil	De wever et al., 2000

Abbreviations: CP, chlorophenol; DCP, dichlorophenol; TCP, trichlorophenol; PCP, pentachlorophenol; OHPA, hydroxyl phenylacetate; PCE, tetrachloroethene; TCE, trichloroethene; TBP, tribromophenol; and PCB, polychlorinated biphenyl.

Table 1-5. Purified reductive dehalogenases.

Reductive dehalogenase	Substrate	Size (kDa)	Cofactor	Fe/S	Source	Reference
3-CBz RD	3-CBz	64,37	Haem		*Desulfomonile tiedjei*	Ni et al., 1995
PceA	PCE	57	Cobalamine	X	*Dehalospirillum multivorans*	Neumann et al., 1996
	o-CP	48	Cobalamine	X	*Desulfitobacterium dehalogenans*	Van de Pas et al., 1999
CprA	3,5-DCP	57	Corrinoid	X	*Desulfitobacterium frappieri* PCP-1	Thibodeau et al., 2004
	PCP	47	Corrinoid		*Desulfitobacterium hafniense* strain PCP-1	Bisaillon et al., 2010
CrdA	2,4,6-TriCP	33.8	Corrinoid		*Desulfitobacterium hafniense*	Boyer et al., 2003
CbrA	1,2,3-TriCB 1,2,3,4-TeCB	45	Corrinoid	X	*Dehalococcoides mccartyi* strain CBDB1	Adrian et al., 2007

Abbreviations: CBz, chlorobenzoate; RD, reductive dehalogenase; PCE, tetrachloroethene; CP, chlorophenol; DCP, dichlorophenol; PCP, pentachlorophenol; TriCP, trichlorophenol; TriCB, trichlorobenzene; and TeCB, tetrachlorobenzene.

"X" represents that the enzyme has iron-sulfur cluster.

CHAPTER 2

Reductive Dehalogenation Activity by Indigenous Microorganism in Sediments of the Hackensack River, New Jersey

Abstract

Organohalogen pollutants are of concern in many river and estuarine environments, such as the New York-New Jersey Harbor estuary and its tributaries. The Hackensack River is contaminated with various metals, hydrocarbons and halogenated organics, including polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins. The Hackensack River and its associated wetlands provide an important habitat for migratory birds and sediment and water contaminants are thus of concern. In order to examine the potential for microbial reductive dechlorination by indigenous microorganisms of the Hackensack River, sediment samples were collected from five different locations (designated H1 to H5) along the river. Hexachlorobenzene (HCB), hexabromobenzene (HBB), and pentachloroaniline (PCA) were selected as model organohalogen pollutants to assess anaerobic dehalogenating potential in the river sediments. Dechlorinating activity of HCB and PCA was observed in sediment microcosms for all sampling sites. HCB was dechlorinated via pentachlorobenzene (PeCB) and trichlorobenzene (TriCB) to dichlorobenzene (DCB). PCA was dechlorinated via tetrachloroaniline (TeCA), trichloroaniline (TriCA), and dichloroaniline (DCA) to monochloroaniline (MCA). However, no HBB debromination was observed over 12 months of incubation. *Dehalococcoides* species were detected by PCR-DGGE in sediments of the freshwater location (H1) but not in the estuarine site (H5). Other possible dehalogenators,

Dehalobacter and *Desulfitobacterium* spp., were not detected in the sediments. When HCB was as a co-substrate, slow HBB debromination was observed with production of TeBB and TriBB. Analysis targeting 12 putative *rdh* genes showed that more *rdh* genes were enriched concomitant with HCB or PCA dechlorination in H1 sediment microcosms than in H5 cultures. All 12 *rdh* genes were enriched in HCB+HBB cultures originated from H1 sediment. The CbrA gene was not detected in the Hackensack River sediment cultures.

Introduction

Halogenated aromatics have been widely used in industry and in agriculture and are of concern as pollutants in many river and estuarine environments. Due to their persistence and toxicity, the most problematic compounds have been listed as persistent organic pollutants (POP, Stockholm Convention, 2001). In river environments, organohalides typically accumulate in sediments due to their low solubility in water and sorption onto particulate material. However, these compounds can dissolve in lipids, thus they can be bioaccumulated and biomagnified in the food chain (ATSDR, 2000). Microbially mediated dehalogenation can reduce the toxicity of many halogenated aromatic compounds, and the dehalogenation products may also be more amenable to subsequent degradation by aerobic bacteria. Understanding the factors that affect the dehalogenation of organohalide pollutants by indigenous microorganisms is thus of interest. Here we studied the dehalogenation of potential of three problematic organohalide compounds: hexachlorobenzene (HCB), hexabromobenzene (HBB), and pentachloroaniline (PCA). HCB is listed as one of the POPs slated for elimination. HCB has been used as a pesticide (Bailey, 2001), while HBB is a flame retardant (Alaee, 2003), respectively. PCA is the first intermediate in the biodegradation of the fungicide, pentachloronitrobenzene (PCNB; Tamura et al., 1995; Susarla et al., 1996; and Okutman Tas and Pavlostathis, 2005).

The New York-New Jersey Harbor estuary and its tributaries have a long-term history of organohalogen pollution. Several factories have been discharged directly into the NY-NJ harbor area. These factories include American Cyanamid, Dupont, PSE&G, Colgate-

Palmolive Co (HydroQual, 1991). As one of the principal tributaries to Newark Bay, the Hackensack River is contaminated with various metals, hydrocarbons and halogenated organics, including PCBs and polychlorinated dibenzo-*p*-dioxins (Crawford et al., 1995 and Martello et al., 2007). Table 2-1 lists reported contaminant levels in sediments collected from the Hackensack River, fish and bird caught around the Hackensack River meadowlands. The Hackensack River is approximately 45 miles long, flowing from southeastern New York into Newark Bay, New Jersey. Early in its history, the Hackensack River supplied clean water to residents in the region. However, the water quality became worse after a construction of a dam in 1923. In addition, the Hackensack River has been polluted by both municipal and industrial wastewater since the river is located within the major New York metropolitan area, with a multitude of industrial sites in Elizabeth and Newark, NJ. Furthermore, the lower section of the Hackensack River, the Hackensack Meadowlands had been a hotspot of garbage dumping by factories and municipalities. As a result, the Hackensack River is contaminated by a variety of pollutants, such as total suspended solid, organic matter, nitrogen and ammonia, metals and chlorinated compounds including PCBs and dibenzo-*p*-dioxins. Contaminants can be found in water, sediment and even in fish and birds (Crawford et al., 1995; Weis et al., 2007; Tsipoura et al., 2008; Shin et al., 2013). The nutrient-rich estuary is used for fishing and the river and its associated wetlands provide an important habitat for migratory birds. Therefore, once an estuarine system gets contaminated, it can cause broad harmful effects on humans as well as wild life such as fish and birds. Once contaminated estuarine systems like the Hackensack River and Newark Bay need to be monitored.

In this chapter, the major objective was to understand the distribution and activity of dehalogenating bacteria in the Hackensack River sediments. The three main goals were to: determine whether indigenous microorganisms in Hackensack River sediments have dehalogenating activity; identify the key members of the microbial community active in degrading the halogenated model compounds hexachlorobenzene (HCB), hexabromobenzene (HBB), and pentachloroaniline (PCA) through 16S rRNA gene analysis; and obtained the reductive dehalogenase (*rdh*) genes profile for each halogenated model compound by PCR with specially designed 12 *rdh* gene targeted primer sets.

Table 2-1. Examples of contaminants detected in the Hackensack River (unit, ppb).

Samples			As	Cd	Cr	Pb	Hg	tPCBs	2,3,7,8-TCDD	2,3,7,8-TCDF	OCDD	OCDF
Sediments			ND	9900	499000	180700	3800	103000	0.14	0.145	11.5	3.5
	Species											
Fish (dw)	White perch		190	130	240	700	180	~2000				
	Brown Bullhead		500	110	240	690	670					
Bird (ww)	Canada geese	Feather	6.39	69.3	801	1650	200					
		Liver	6.24	122	201	208	36.8					
		Muscle	1.11	4.17	48.5	3.31	8.16					

ND, not detected.

Sediments were collected from the Hackensack River, Canada geese were from Harrier meadows in the Meadowland district, and fish were from the Hackensack meadowlands.

Data are compiled from Crawford et al., 1995, Martello et al., 2007, Weis and Ashley, 2007 and Tsipoura et al., 2011.

Materials and Methods

Chemicals and stock solutions

All chlorinated benzenes (hexachlorobenzene, pentachlorobenzene, tetrachlorobenzenes, trichlorobenzenes, dichlorobenzenes), 1,2,3,5-tetrabromobenzene and 1,3,5-tribromobenzene were purchased from Sigma-Aldrich (St. Louis, MO). All chlorinated anilines (pentachloroaniline, tetrachloroanilines, trichloroanilines, dichloroanilines, chloroanilines) were purchased from UltraScientific (North Kingstown, RI). Hexabromobenzene was from TCI America (Portland, OR). Stock solutions of chlorobenzenes and chloroanilines were prepared by dissolving the compounds in hexane to a final concentration of 10 mM. Stock solutions of bromobenzenes were prepared by dissolving the compounds in toluene to a final concentration of 5 mM.

Microcosm preparation

Sediment samples were collected from five different locations along the Hackensack River, New Jersey in July 2012 from North Hackensack to the mouth of the river near Newark Bay, designated H1 to H5, (Figure 2-1). GPS coordinates for the sampling sites are as follows: H1, 40.85, -74.03; H2, 40.82, -74.04; H3, 40.80, -74.07; H4, 40.78, -74.09; and H5, 40.74, -74.08. Sediments were stored in sealed glass jars at 4°C until setup of microcosms. Environmental monitoring data for the 5 sample locations is provided in Figure 2-2, which presents seasonal monitoring data for sulfate, salinity, dissolved oxygen, pH, and temperature in the Hackensack River over ten years (Meadowlands Environmental Research Institute, MERI, New Jersey Meadowlands

Commission). Due to tidal influence, sulfate concentrations and salinity increase in the river closer to Newark Bay.

Microcosms were prepared using sediment slurries in 60 mL serum bottles capped with Teflon coated rubber stoppers and crimped with aluminum seals. Sediment slurries (50 mL) contained 10 % (wet weight/volume) sediment and electron donor mixture in anaerobic media. Anaerobic media was made as described by Cole et al. (1994) with some modifications. The medium contained the following (per liter): NaCl, 1.0 g; $MgCl_2 \bullet 6H_2O$, 0.5 g; KH_2PO_4, 0.2 g; NH_4Cl, 0.3 g; KCl, 0.3 g, $CaCl_2 \bullet 2H_2O$, 0.015 g; resazurin, 1 mg; trace element solution A, 1 mL; trace element solution B, 0.1 mL; $Na_2S \bullet 9H_2O$, 0.048 g; cysteine hydrochloride, 0.035 g; $NaHCO_3$, 2.52 g; and vitamin solution, 1 mL. Trace element solution A contained the following (per liter): $FeCl_2 \bullet 4H_2O$, 1.5 g; $CoCl_2 \bullet 6H_2O$, 0.19 g; $MnCl_2 \bullet 4H_2O$, 0.1 g; $ZnCl_2$, 70 mg; H_3BO_3, 6 mg; $Na_2MoO_4 \bullet 2H_2O$, 36 mg; $NiCl_2 \bullet 6H_2O$, 24 mg; and $CuCl_2 \bullet 2H_2O$, 2 mg. Trace element solution B contained 0.03 g of $NaSeO_3$ and 0.08 g of Na_2WO_4 per liter. Vitamin solution contained the following (per 500 mL): d-biotin, 0.01 g; folic acid, 0.01 g; pyridoxine hydrochloride, 0.05 g; thiamin hydrochloride, 0.025 g; riboflavin, 0.025 g; nicotinic acid, 0.025 g, DL-calcium pantothenate, 0.025 g; vitamin B_{12}, 0.005 g; *p*-animobenzoic acid, 0.025 g; lipoic acid (thiotic acid), 0.025 g; 1,4-naphthaquinone, 0.02 g; nicotinamide, 0.05 g; and hemin, 0.005 g.

Four treatments were established in triplicate: halogenated electron acceptors hexachlorobenzene (HCB), hexabromobenzene (HBB), or pentachloroaniline (PCA), and

a control without organohalides. The structures for three model compounds are represented in Figure 2-3. All cultures were amended with a mixture of electron donors containing butyrate and propionate to a concentration of 500 μM each. Duplicate killed controls were also prepared by autoclaving of sediment slurries for 30 min at 121 °C on three consecutive days before addition of electron acceptors and donors.

To provide a carrier for the halogenated substrates, approximately 0.2 g of silica was added evenly into each serum bottle, and sterilized. The silica was then spiked with the respective halobenzene or chloroaniline stock solution to yield a final nominal concentration of 20 μM and the solvent was allowed to evaporate completely leaving a coating of the halogenated substrates. The serum bottles were then moved to an anaerobic chamber for the remaining steps. The chamber had a headspace of N_2:H_2 (97:3). 50 mL of sediment slurry of each site prepared in large stock bottles was transferred to the serum bottles. The microcosms were stirred, capped and then incubated upright without shaking in the dark at room temperature (~21 °C). Samples (0.5 mL) were collected every two weeks for chemical analysis. An additional 1 mL sample was collected from each microcosm every 4 weeks for molecular analysis. Samplings were done in the anaerobic chamber. Samples were stored at -20 °C or extracted immediately after sampling.

Additional microcosms were established later to determine whether HCB would stimulate HBB dehalogenation. Triplicate cultures originating from H1 sediment microcosms previously set up for monitoring HCB dechlorination were spiked with HBB and HCB. In these cultures, previously added HCB was already dechlorinated and the cultures had

accumulated dechlorination products. Culture bottles were supplemented with anaerobic media to approximately 50 mL and additional 10 μM of HCB and HBB were provided as electron acceptors. HCB and HBB were added as a coating on about 0.4 g of silica on the bottom of autoclaved serum bottles, as described before above, and the whole culture was transferred to the new culture bottle in the anaerobic chamber. Cultures were incubated as described above. Samples (0.5 mL) were collected every three or four weeks for chemical analysis. After 19 weeks and 39 weeks of incubation, 1 mL samples were collected for molecular analysis.

Chemical analytical methods

Samples were extracted by shaking at 180 rpm for two hours. To extract chlorobenzenes and chloroanilines, 500 μL of hexane was used. For extraction of bromobenzenes or the mixture of chloro- and bromobenzenes, 750 μL of a mixture of toluene and acetone (1:1(v:v)) was used. The supernatant was transferred to a GC vial. When solvent and aqueous/sediment phase were not clearly separated, brief sonication in a water bath was used. Samples were analyzed with an Agilent 6890 gas chromatograph equipped with a HP-5MS capillary column (0.250 mm × 60 m, 0.25 μm film thickness, J&W Scientific, Folsom, CA) and an Agilent 5973 Network Mass Selective Detector with helium as the carrier gas. For analyses of chlorinated benzenes and brominated benzenes, the GC column temperature was first held at 50 °C for 2 min, increased to 300 °C at a rate of 12 °C min^{-1}, and then held for 5 min. For analyses of chlorinated anilines, the GC column temperature was first held at 100 °C for 1 min and then increased to 300 °C at a rate of 20 °C min^{-1}. A calibration mixture containing chlorinated benzenes, chlorinated anilines, or

brominated benzenes was analyzed with samples. Each model compound and its dehalogenating products were combined in one vial. For example, HCB, PeCB, TeCB, TriCB and DCB were mixed in one vial to be used for calibration of HCB dechlorinating products. For quantification, standards of 10, 20, 30, 40, and 50 μM were used. Detection limits were ~0.1 μM for HCB and its intermediates, 0.1~0.5 μM for TeBB and TriBB, 0.5~1 μM for HBB, 0.1~0.5 μM for PCA and its intermediates.

Molecular analyses

Genomic DNA was extracted from sediment samples collected from HCB and PCA cultures from H1 and H5 sites at day 0 and day 112 using the PowerSoil^{TM} DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Extracted DNA was loaded on an agarose gel or its concentration was measured by a Nanodrop ND-1000 spectrophotometer (Nanodrop technologies, Wilmington, DE) to check its quality and quantity before PCR. Nested PCR was performed to amplify the DNA of the putative dechlorinating community (Krumins et al., 2009). Resulting PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE). First, DNA samples extracted from microcosms were amplified with primer 338F and Chl1101R targeting a 790 bp region of the *Chloroflexi* 16S rRNA gene. The resulting PCR products were re-amplified using general bacterial PCR primers 341F-GC and 534R. 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 was 94 °C for 5 min followed by 30 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.

Samples of PCR product (20 µL) were mixed with 2X DGGE dye and loaded onto 8% polyacrylamide gels in 1X Tris-acetate EDTA (TAE) buffer using 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 17 h at 60 °C and 40 V. After electrophoresis, the gels were stained with ethidium bromide and photographed on a UV transilluminator (BioRad Gel Doc™ EZ Imager, Bio-Rad Laboratories, Hercules, CA). Image analysis was conducted with Image Lab program version 5.1 (Bio-Rad Laboratories, Hercules, 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 loaded on DGGE to confirm and sequenced. Phylogenetic analyses were conducted using *MEGA* version 6 (Tamura et al., 2013). The sequences of the excised DGGE bands are listed in Appendix II.

Primers were selected to amplify the 16S rRNA gene of *Desulfitobacterium* and *Dehalobacter* spp. (Smits et al., 2004 and Nelson et al., 2011, respectively). *Dsb* 406F-GTACGACGAAGGCCTTCGGGT / *Dsb* 619R-CCCAGGGTTGAGCCCTAGGT and

Dhb 477F-GATTGACGGTACCTAACGAGG / *Dhb* 647R-TACAGTTTCCAATGCTTTACGG were used to amplify *Desulfitobacterium* and *Dehalobacter*, respectively. As templates, only one DNA sample among triplicates (D0, D112 ED control, D112 HCB treated and D112 PCA treated of H1 and H5) were tested. Total reaction volume was 20 μL. The PCR was performed in following conditions. The initial incubation was performed at 94 °C for 5 min, 30 cycles of 94 °C for 20 sec, 60 °C for *Dsb* or 53 °C for *Dhb* for 45 sec and 72 °C for 45 sec, final incubation was at 72 °C for 7 min, after which the DNA was stored at 4 °C. Samples of PCR products (5 μL) were examined on 1.5 % agarose gel containing ethidium bromide in 1X TAE. The electrophoresis was run for 35 min at 100 V. After electrophoresis, the gels were photographed on a UV transilluminator (BioRad Gel Doc™ EZ Imager, Bio-Rad Laboratories, Hercules, CA). Image analysis was conducted with Image Lab program version 5.1 (Bio-Rad Laboratories, Hercules, CA).

To determine the presence of the chlorobenzene reductive dehalogenase, *cbrA*, in enrichment cultures, primers were designed to amplify a whole *cbrA* gene (1467 bp) (*cbrA*-F: ATGAGTAACTTTCATTCAAC, *cbrA*-R: TTAATTACCGGGTACAGCCC). The total volume of reaction mixture was 20 μL. The PCR was performed at 94 °C for 5 min, 30 cycles of 94 °C for 20 sec, 45 °C for 45 sec and 72 °C for 90 sec, final incubation was at 72 °C for 7 min, after which the DNA was stored at 4 °C. PCR products were examined as described above but 0.7 % agarose gel in 1X TAE was used.

Rdh gene profile analysis

PCRs of extracted DNAs were done with 12 sets of primer designed to amplify reductive dehalogenase genes based on *rdh* gene sequence similarity pairs of *Dehalococcoides mccartyi* strain 195 and strain CBDB1 (Park et al., 2011). The DNA sequences of these 12 primer sets are listed in Table 2-2. The total volume of PCR mixture was 20 μL. The reaction mixture contained 2X premix (ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$, Sigma-Aldrich), 10 picomole of each primer and 1 μL template DNA. The premix contained the following: 20 mM Tris-HCl; pH 8.3; 100 mM KCl; 3 mM $MgCl_2$; 0.002 % gelatin; 0.4 mM dNTP mix (dATP, dCTP, dGTP, TTP); stabilizers; and 0.06 units Taq DNA polymerase/μL. The PCR was performed in following temperature profiles: initial incubation for 5 min at 94 °C, 35 cycles of 94 °C for 20 sec, 52 °C for 1 min, and 72 °C for 1 min, final extension for 7 min at 72 °C, after which the DNA was stored at 4 °C. Samples of PCR products (5 μL) were examined on 1.5 % agarose gels containing ethidium bromide in 1X TAE. The electrophoresis was run for 35 min at 100 V. After electrophoresis, the gels were photographed on a UV transilluminator (BioRad Gel Doc^{TM} EZ Imager, Bio-Rad Laboratories, Hercules, CA). Image analysis was conducted with Image Lab program version 5.1 (Bio-Rad Laboratories, Hercules, CA).

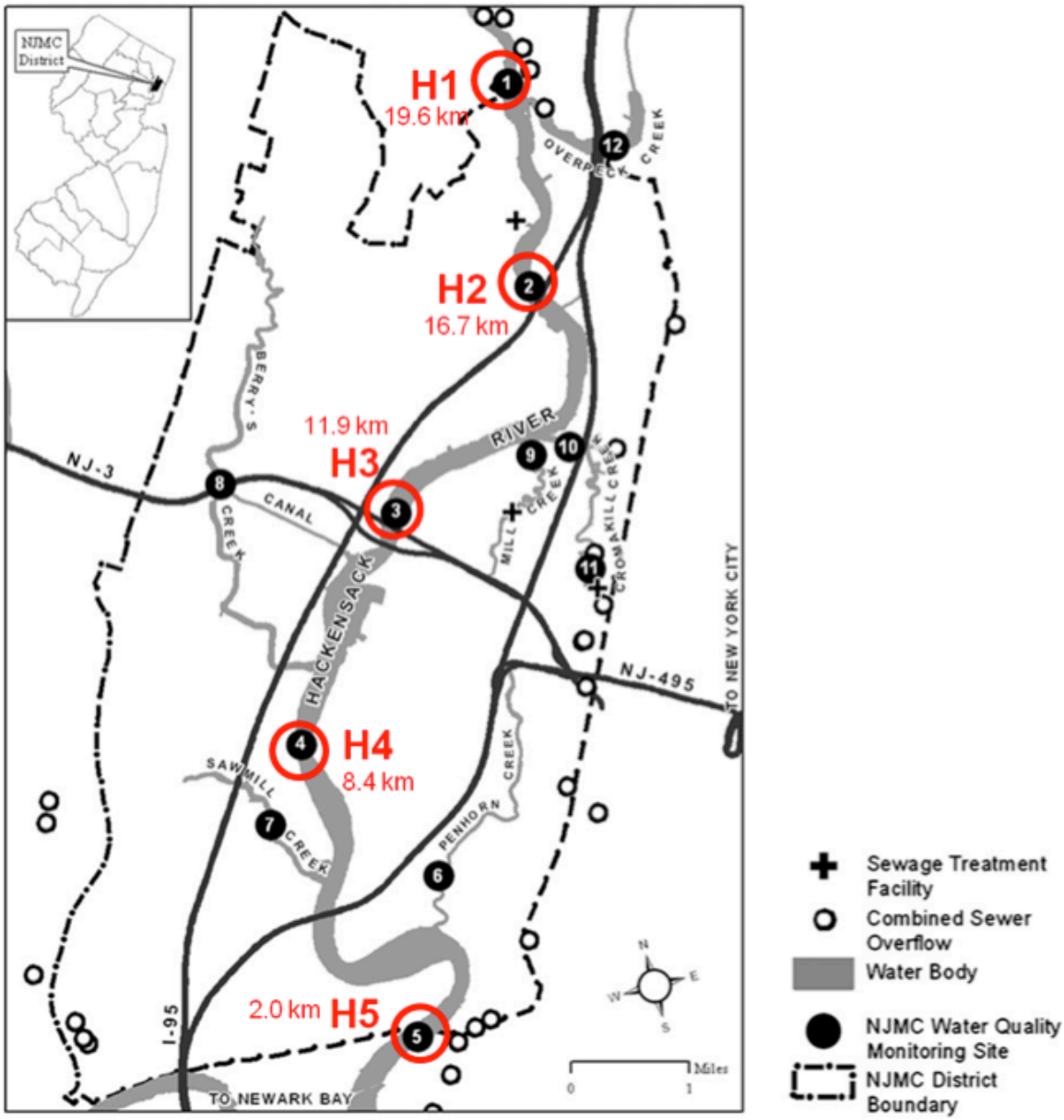


Figure 2-1. Five sampling locations along the Hackensack River, NJ (Map from Shin et al., 2013). Red numbers show distance from the mouth of the river.

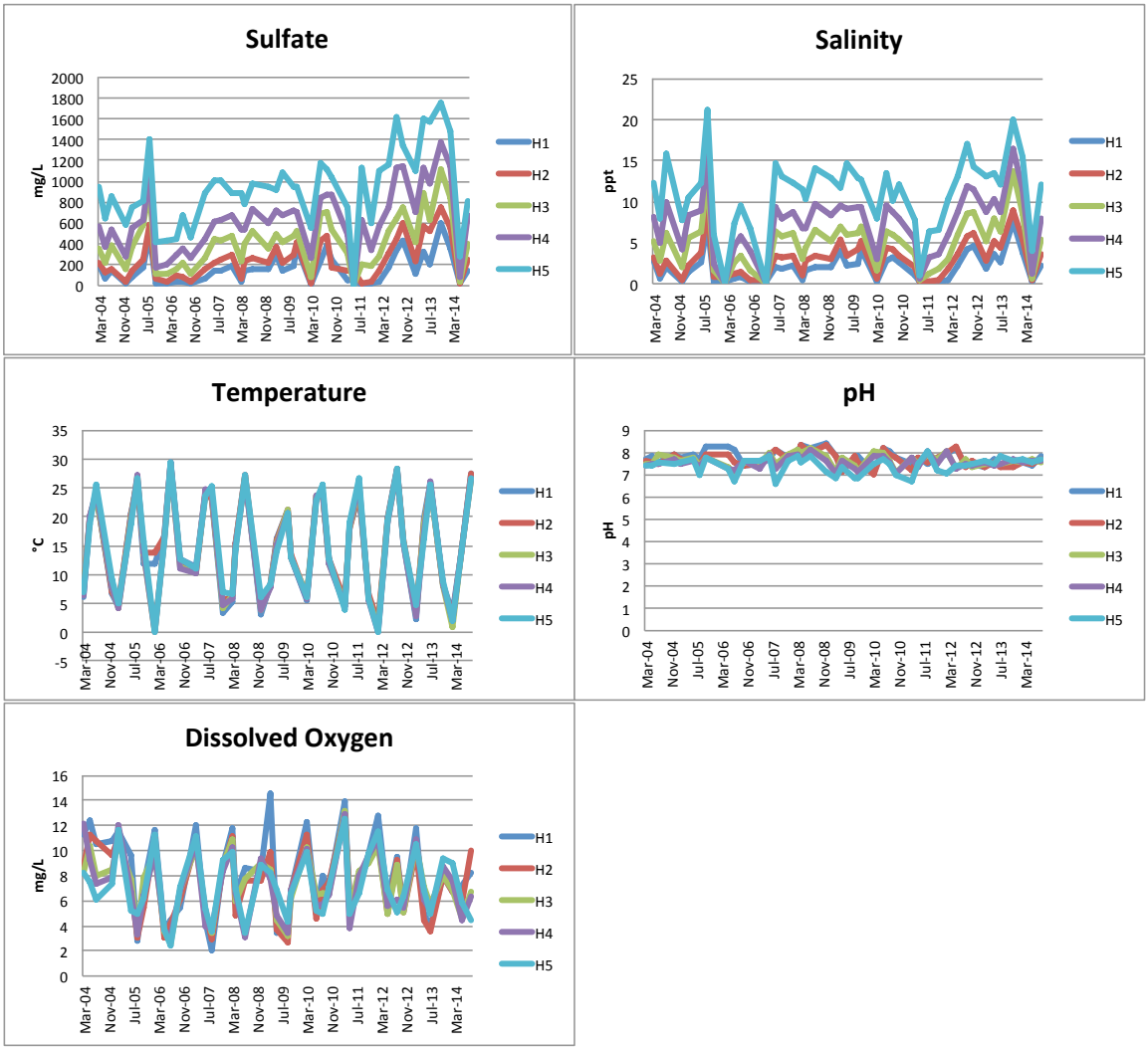


Figure 2-2. Environmental conditions of the Hackensack River at the five sampling locations over the last 10 years (from MERI, Meadowlands environmental research institute, New Jersey Meadowlands Commission).

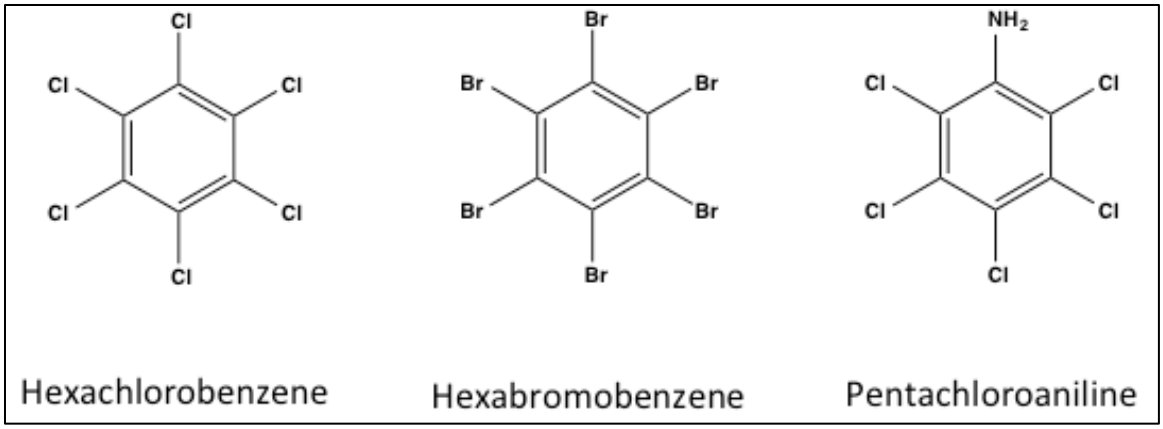


Figure 2-3. Structure of hexachlorobenzene (HCB), hexabromobenzene (HBB) and pentachloroaniline (PCA).

Table 2-2. Twelve primer sets used for *rdh* genes profile analysis (from Park et al., 2011).

Primers	Sequences (5' to 3')	Target	Expected size
RDH01F	TGGCTTATGGCTGTTCCAA	DET0180, cbdb_A187	148 bp
RDH01R	TATCTCCAGGGAGCCCATTC		
RDH02F	GCCGAATTCTGCCCTGT	DET0235, cbdb_A243	281 bp
RDH02R	CAGRRARCCATARCCAAAGG		
RDH03F	CAAGATGGATAGGCCTGCAT	DET0302, cbdb_A238	192 bp
RDH03R	ATGGTGCTATCCTGACCGAG		
RDH04F	GATGATACGATTTATGGCAATC	DET0306, cbdb_A1495	215 bp
RDH04R	CCRAACGGGAARTCTTCTTC		
RDH05F	AAGGATATCAAGTCCAGTATCC	DET0311, cbdb_A1495	215 bp
RDH05R	ATACCTTCRAGCGGCCARTAT		
RDH06F	CACCCCGGTTCGTTCATACA	DET0318, cbdb_A1588	102 bp
RDH06R	AGTCATCCACTTCRTCCCAC		
RDH07F	TGTCCGGCACTCTTAAACC	DET1171, cbdb_A1092	200 bp
RDH07R	GCYGCCGCYGGCAGTTACTG		
RDH08F	GGAAAGGCCATCATCAAAC	DET1519, cbdb_A1575	184 bp
RDH08R	GTCTTRCMGGRGTAACCYTG		
RDH09F	GGTGAGATTTAAAATTGTTGGC	DET1522, cbdb_A1570	136 bp
RDH09R	CTGGGTGCGGTWGCCGCAKC		
RDH10F	TCCTGAGCCGACAGGGT	DET1535, cbdb_A1595	223 bp
RDH10R	TTTCATTCMACACTYTCMCG		
RDH11F	ATTTACCCTGTCCCATCC	DET1538, cbdb_A1627	235 bp
RDH11R	TTTCACASTAGYCTKAGCCGMAG		
RDH12F	GCCCGTCATGGCGTTCCATC	DET1545, cbdb_A1638	187 bp
RDH12R	GAGCAAGTTTCATTCMATGG		

Results

Dehalogenating activities of microcosms

Dehalogenation of HCB and PCA was observed in Hackensack River sediment microcosms. Microcosm cultures set up with sediment sampled from locations H1to H4 (upper river) showed a start of HCB dechlorination earlier than cultures containing sediment from H5 (lower river; Fig 2-4). TriCB was detected as a dechlorination product of HCB on day 28 in the H1 to H4 microcosms. In the H5 microcosms, TriCB appeared later, at day 42. A further dechlorination product, DCB was detected in H2 cultures from day 28, and it was detected in H1, H3 and H4 cultures from day 42. In a consistent trend with TriCB, DCB was detected in H5 cultures much later, at day 77. In some of the cultures, PeCB was also detected as an early intermediate of dechlorination. There was no dechlorination activity in killed controls confirming that activity was microbial. Based on these results, dechlorination of HCB is mediated by indigenous microorganisms of Hackensack River sediments.

Dechlorination of PCA was observed, with TeCA, TriCA, DCA, and CA detected as dechlorination intermediates (Fig 2-5). H1 microcosms showed an earlier start of PCA dechlorination similar to what was observed for HCB. TeCA and TriCA were detected in H1 cultures at day 14. For the H2 to H4 cultures, dechlorinating products were detected at day 28. The H5 cultures showed a delayed start for PCA dechlorination with TriCA and DCA detected as dechlorinating products at day 44. These observations were consistent for all triplicate cultures. In every culture, CA was detected as a dechlorination

product. Killed controls showed no dechlorination of PCA. Therefore, we can conclude that indigenous bacteria in Hackensack River sediments mediated PCA dechlorination.

No dehalogenation of HBB or detection of intermediates was observed in any of the microcosms (data not shown) after up to 12 months of incubation. PeBB was, however, already detected as a contaminant of the HBB stock, but any increase in the PeBB concentration in microcosms was negligible.

Dechlorination pathways

HCB dechlorination pathways were established based on GC-MS analysis by comparison of mass spectral data and retention times of intermediates to those of authentic standards (Fig. 2-6). HCB was converted to PeCB, with subsequent detection of 1,3,5-TriCB and 1,3-DCB. One possible TeCB intermediate is 1,2,3,5-TeCB, since 1,3,5-TriCB would be a likely dechlorination product of 1,2,3,5-TeCB. In addition, a small amount of 1,4-DCB was also observed in some cultures. Hence, we could expect that 1,3,4-TriCB is also formed from 1,2,3,5-TeCB or from other intermediates not detected.

PCA dechlorination intermediates were also determined by comparison of retention times of intermediates to the retention time of authentic standards. Some intermediates were not observed, but predicted to propose a complete pathway. Overall, PCA was dechlorinated to 2,3,4,6- and 2,3,5,6-TeCA. 2,3,5,6-TeCA was dechlorinated via 2,3,5-TriCA to 3,5-DCA and 2,5-DCA. 2,3,4,6-TeCA was dechlorinated to 2,4,5-TriCA and 2,4,6-TriCA. 2,4,5-TriCA was dechlorinated to 2,4-DCA and 2,5-DCA. 2,4,6-TriCA was dechlorinated

to 2,4-DCA and 2,6-DCA. 3,5-DCA and 2,5-DCA were further dechlorinated to 3-CA (Figure 2-7). 2,4-DCA and 2,5-DCA cannot be distinguished by GC-MS since they have the same retention time. In the proposed dechlorination pathway presented in Figure 2-7, it is assumed that both 2,4- and 2,5-DCA are produced.

Enrichment of a Dehalococcoides community in dechlorinating cultures

In order to follow the dynamics of the active dechlorinating microorganisms, *Chloroflexi*-specific PCR-DGGE analysis was conducted for DNA extracted from H1 and H5 microcosms (D0; D112 (ED control, HCB, or PCA treated). DNA was extracted from all triplicate cultures. PCR-DGGE results indicated that several *Dehalococcoides* species were enriched in the microcosms containing sediment from H1 amended with HCB or PCA (Figure 2-8). Four PCR fragment bands were excised from the gel of H1 sediment. Two bands were cut from non-amended (ED) controls on day 112. And the other two bands were excised from HCB and PCA treated cultures on day 112, respectively. A second PCR was performed using primers 341F-GC and 534R to confirm the band and to increase the DNA amount before sequencing.

Sequence analysis demonstrated that *Dehalococcoides* species (*D. mccartyi*) were enriched in HCB and PCA dechlorinating sediment cultures. Based on the DGGE migration and phylogenetic analysis, the putative dechlorinators had 100% identity with the 16S rRNA gene of the Pinellas subgroup (Hendrickson et al., 2002) of *Dehalococcoides mccartyi*, which includes strain CBDB1 capable of TeCB dechlorination (Adrian et al., 1998; Figure 2-9). For cultures containing sediment

originating from site H5, there was no specific signal for *Dehalococcoides* after 112 days based on PCR-DGGE analysis (Figure 2-9).

Effect of HCB as a "haloprimer" for HBB dehalogenation

Since we observed no reductive dehalogenation of HBB over 12 months in any of the five sediment microcosms sets, a second set of microcosms were set up in an attempt to stimulate reductive debromination by adding HCB to the microcosms as a co-substrate (haloprimer). Triplicate cultures previously shown to dechlorinate HCB were re-spiked 10 μM HCB and HBB. Microcosms amended with both of HCB and HBB showed reductive dehalogenation of both of HCB and HBB (Figure 2-10). However, HCB dechlorination was stalled after 50 days compared to the initial cultures and remained at 50-60% mole fraction until 273 days. HBB debromination products such as 1,2,3,5-TeBB and 1,3,5-TriBB were detected although their concentrations were low (less than 0.1 μM).

PCR-DGGE was conducted to analyze the dehalogenating populations enriched in HCB+HBB cultures with DNA samples extracted at day 133 and day 273 (Fig 2-11). PCR-DGGE results showed a single band enriched at both time points. The bands migrated to the same distance in the acrylamide gel as the enriched band from HCB or PCA amended H1 cultures (comparison data not presented). Therefore, it is assumed that a *D. mccartyi* Pinellas subgroup –like dehalogenating bacterium was enriched in these cultures.

Presence of other putative dehalogenating bacteria in Hackensack River sediments

Dehalogenating activity was observed in all HCB- and PCA-amended cultures. However, PCR-DGGE analysis of H1 and H5 sediment microcosms only detected a *Dehalococcoides* spp. population in H1 microcosms. Since we didn't observe *Dehalococcoides* spp. in cultures from H5 sediment, additional PCR assays were conducted to determine the possible presence of other potential dehalogenating bacteria, such as *Dehalobacter* or *Desulfitobacterium*. However, neither *Dehalobacter* nor *Desulfitobacterium* were amplified with DNA templates from H1 and H5 samples.

Reductive dehalogenase gene profile

Even though indigenous bacteria originated from both H1 and H5 sediment had dehalogenating activity for HCB or PCA, they had different *rdh* gene profiles (Table 2-3). Twelve primer sets amplifying *rdh* genes were tested. In general, more *rdh* genes were detected in H1 sediment cultures than in H5 sediment cultures. All 12 *rdh* genes (designated here rdh 1 to 12) tested were detected in HCB amended H1 cultures but weak signal of rdh 4 and rdh 5 were only detected from one bottle among triplicates. In H1 sediments with PCA, 10 rdh genes (Except rdh 4 and rdh 5) were detected. Rdh 1, rdh 3, rdh 4, rdh 6, rdh 11, and 12 were detected even in D0 or/and control at D112 but they had weak signal and only detected in one or two bottles among triplicates. In H5 sediments, three *rdh* genes (rdh 1, rdh 3, and rdh 5) were enriched in PCA amended cultures. However, if we eliminate genes that were also amplified from non-amended controls, only one gene rdh 5, was specific to dehalogenating H5 cultures. In HCB-amended H5 cultures, weak signals of rdh 1 and rdh 3 were detected, but they were detected as well at

day 0 and in controls at day 112. All tested *rdh* genes were detected in cultures containing both HCB and HBB as electron acceptors. Primers targeting the whole *cbrA* gene was also designed and tested, but no signal was detected from either H1 or H5 sediment.

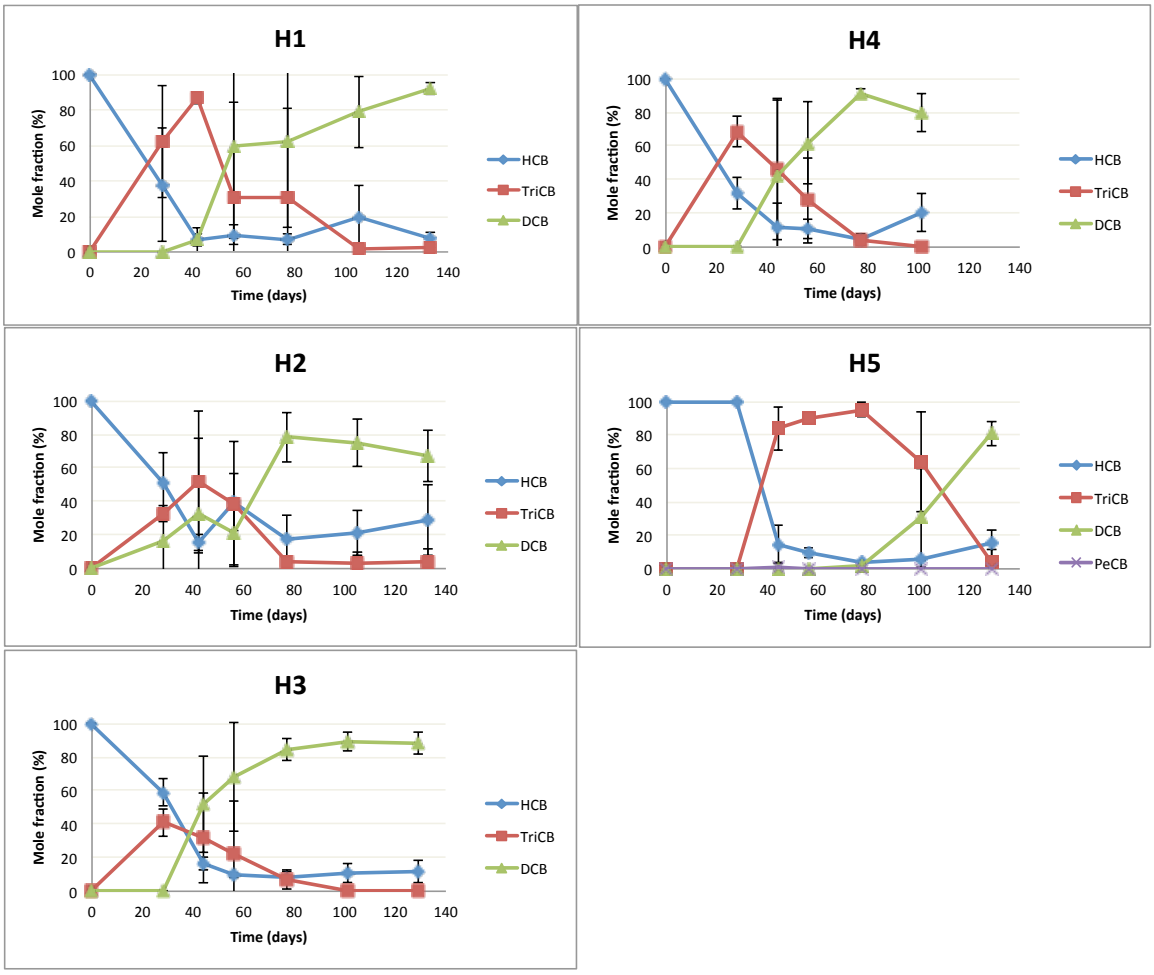


Figure 2-4. Dechlorination of HCB in the Hackensack River sediment microcosms. HCB and its dechlorination products are represented as the mole fraction of all chlorobenzenes detected. Data points are the mean ± standard deviation of three replicate microcosms.

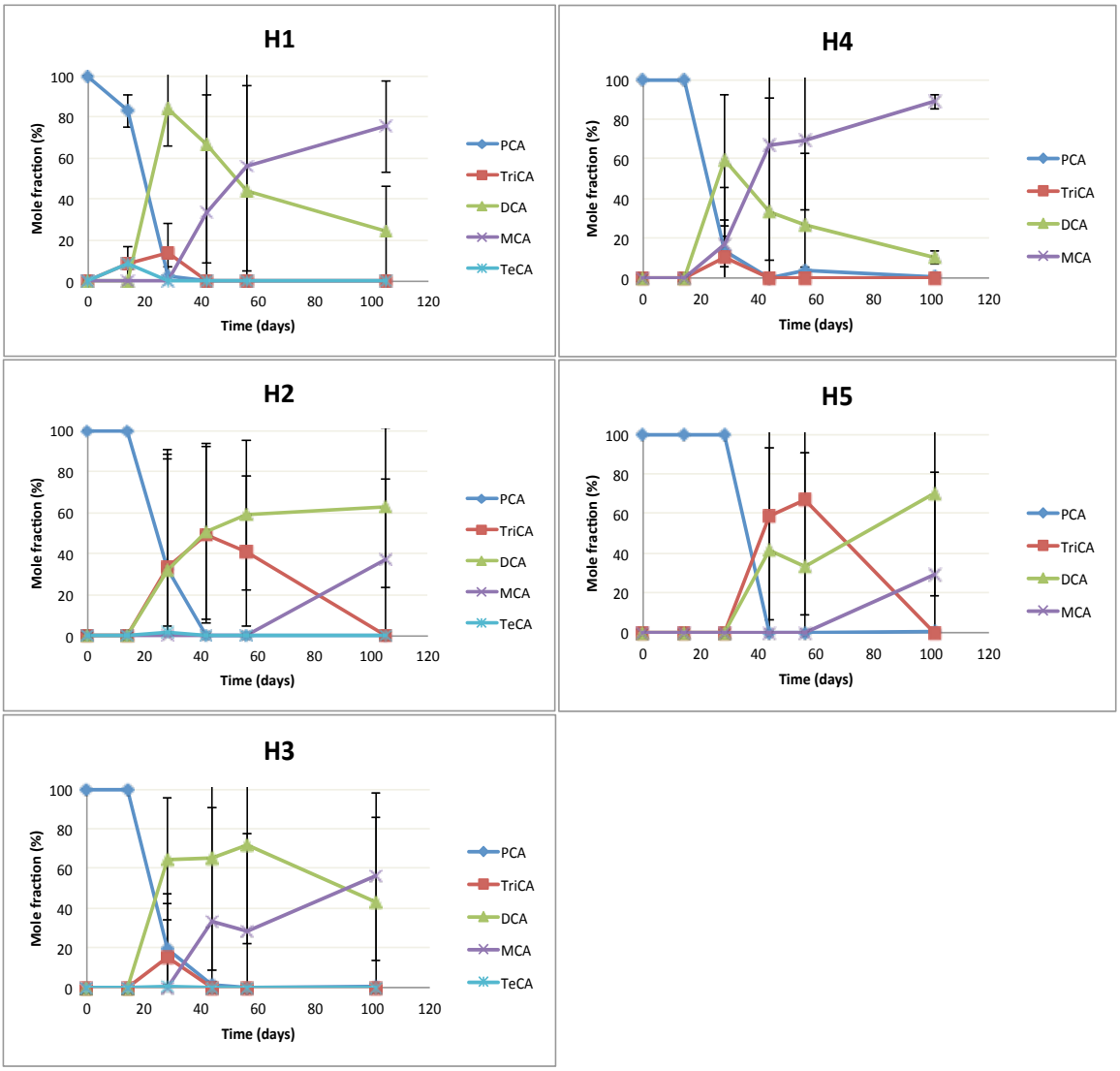


Figure 2-5. Dechlorination of PCA in Hackensack River sediments. PCA and its dechlorination products are represented as the mole fraction of all chloroanilines detected. Data points are the mean ± standard deviation of three replicate microcosms.

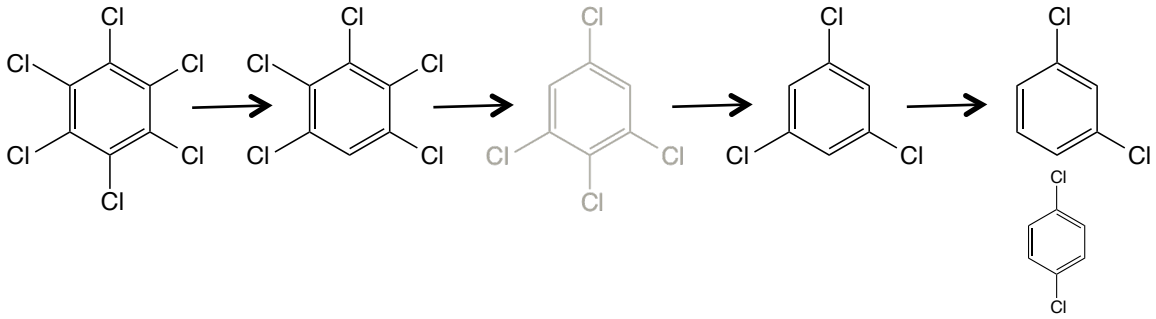


Figure 2-6. Suggested HCB dechlorination pathway based on observed intermediates: HCB→PeCB→1,2,3,5-TeCB→1,3,5→TriCB→1,3-DCB (a small amount of 1,4-DCB also found in some cultures). 1,2,3,5-TeCB (shown in grey) was postulated as an intermediate since it was the only isomer that could yield 1,3,5-TriCB, even though it was not detected in cultures over the course of dechlorination.

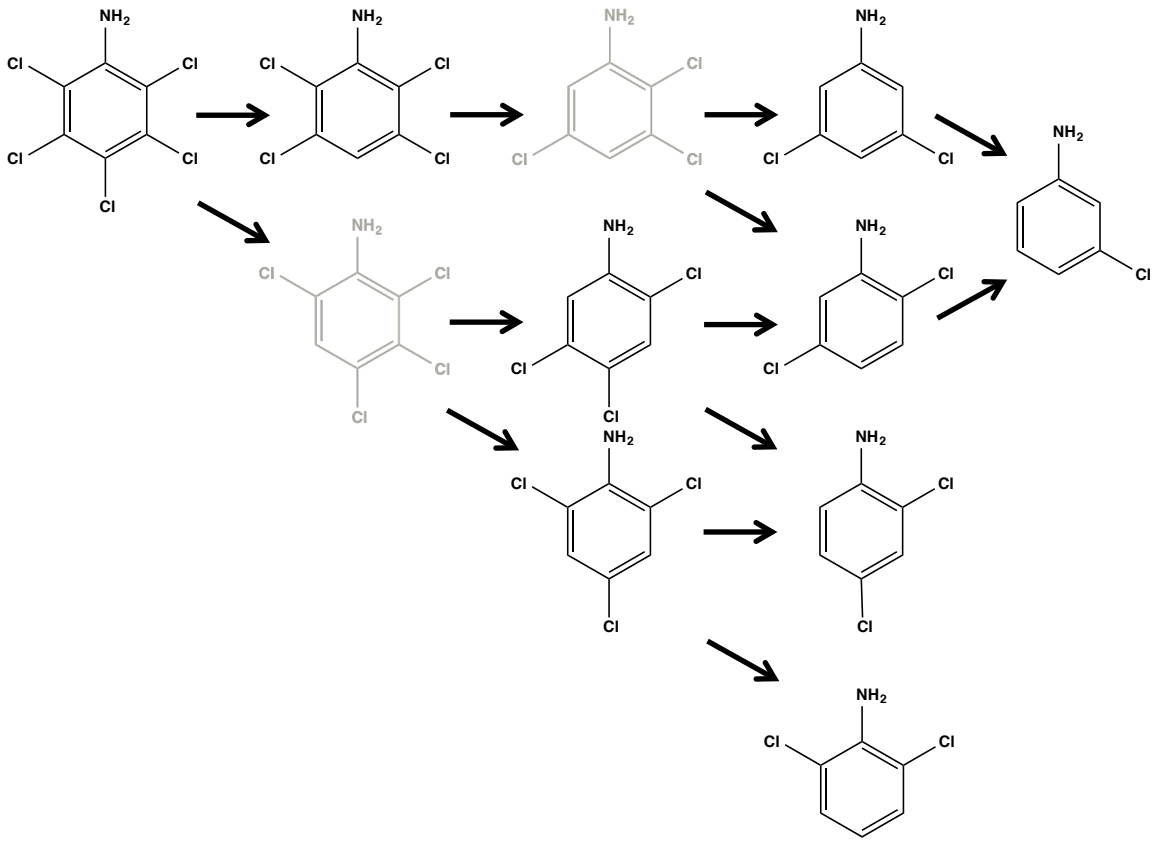


Figure 2-7. Suggested pathway for PCA dechlorination based on observed intermediates. Gray-colored isomers are postulated to make the pathway complete, but were not detected in cultures over the course of dechlorination.

Compound	Retention time (min.)
HCB	19.14
PeCB	17.06
1,2,3,5-TeCB	14.91
1,3,5-TriCB	12.45
1,3-DCB	10.62
1,4-DCB	10.71
HBB	25.4
PeBB	22.34
1,2,3,5-TeBB	19.32
1,3,5-TriBB	16.03
PCA	10.59
2,3,5,6-TeCA	9.28
2,4,6-TriCA	7.84
2,4,5-TriCA	8.68
3,5-DCA	7.76
2,4/2,5-DCA	7.29
2,6-DCA	6.65
3-MCA	6.33

Table 2-3. Retention times for HCB, HBB, PCA, and their dehalogenating intermediates. Abbreviations: HCB, hexachlorobenzene; PeCB, pentachlorobenzene; TeCB, tetrachlorobenzene; TriCB, trichlorobenzene; DCB, dichlorobenzene; HBB, hexabromobenzene; PeBB, pentabromobenzene; TeBB, tetrabromobenzene; TriBB, tribromobenzene; PCA, pentachloroaniline; TeCA, tetrachloroaniline; TriCA, trichloroaniline; DCA, dichloroaniline; and MCA, monochloroaniline.

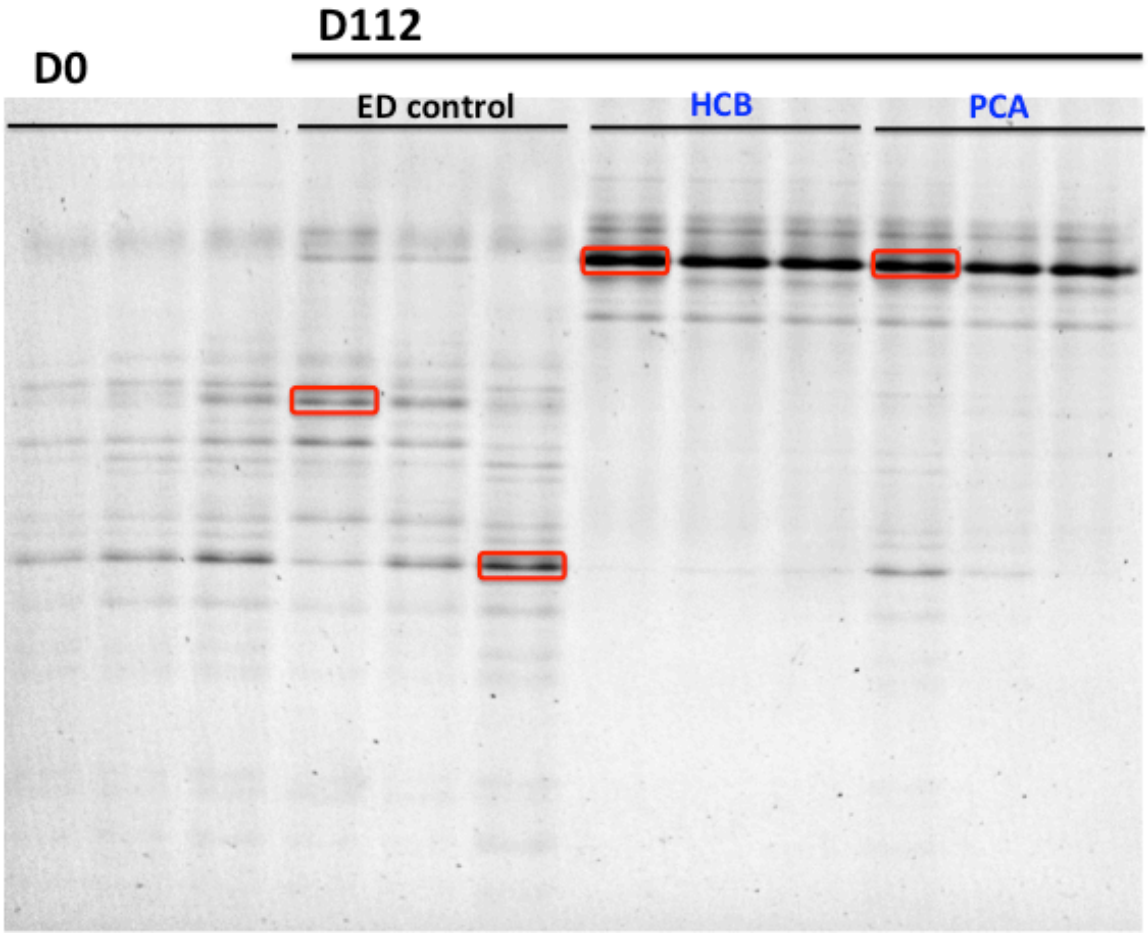


Figure 2-8. PCR-DGGE analysis of H1 sediment microcosms at day 0 and day 112. The red boxes represent four bands excised for sequence analysis.

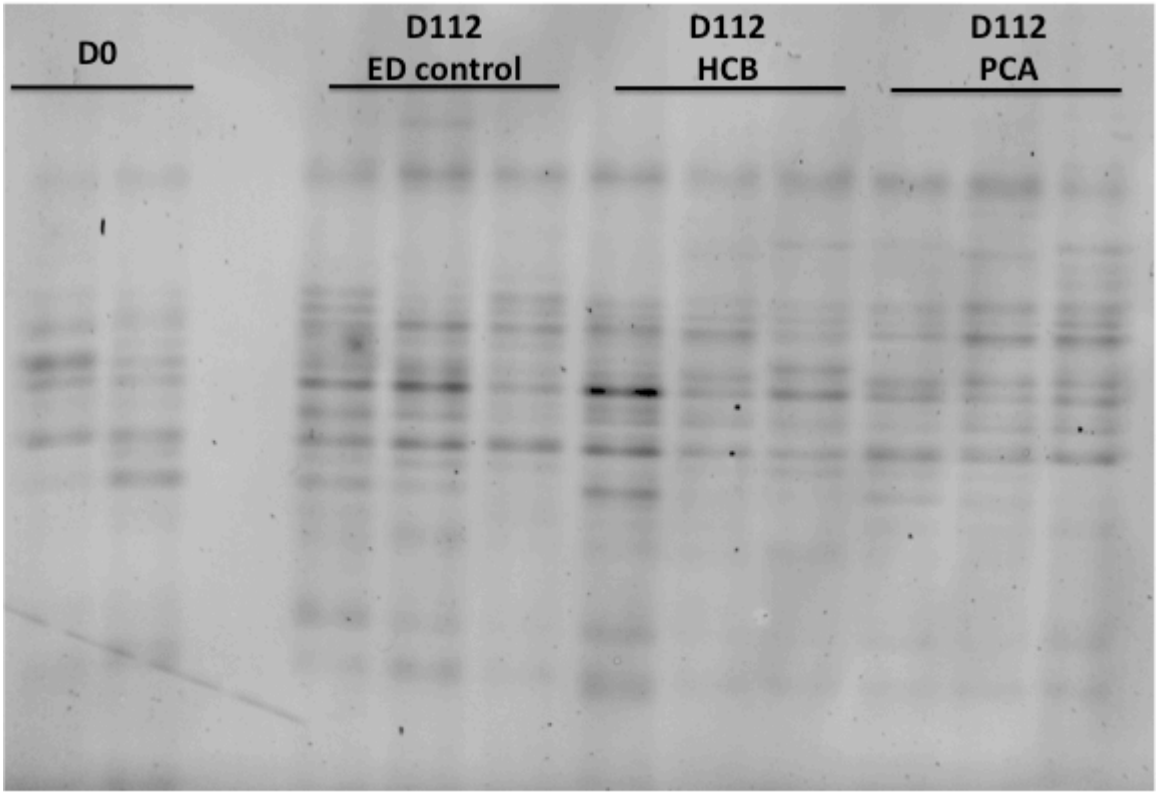


Figure 2-9. PCR-DGGE analysis of H5 sediment microcosms at day 0 and day 112.

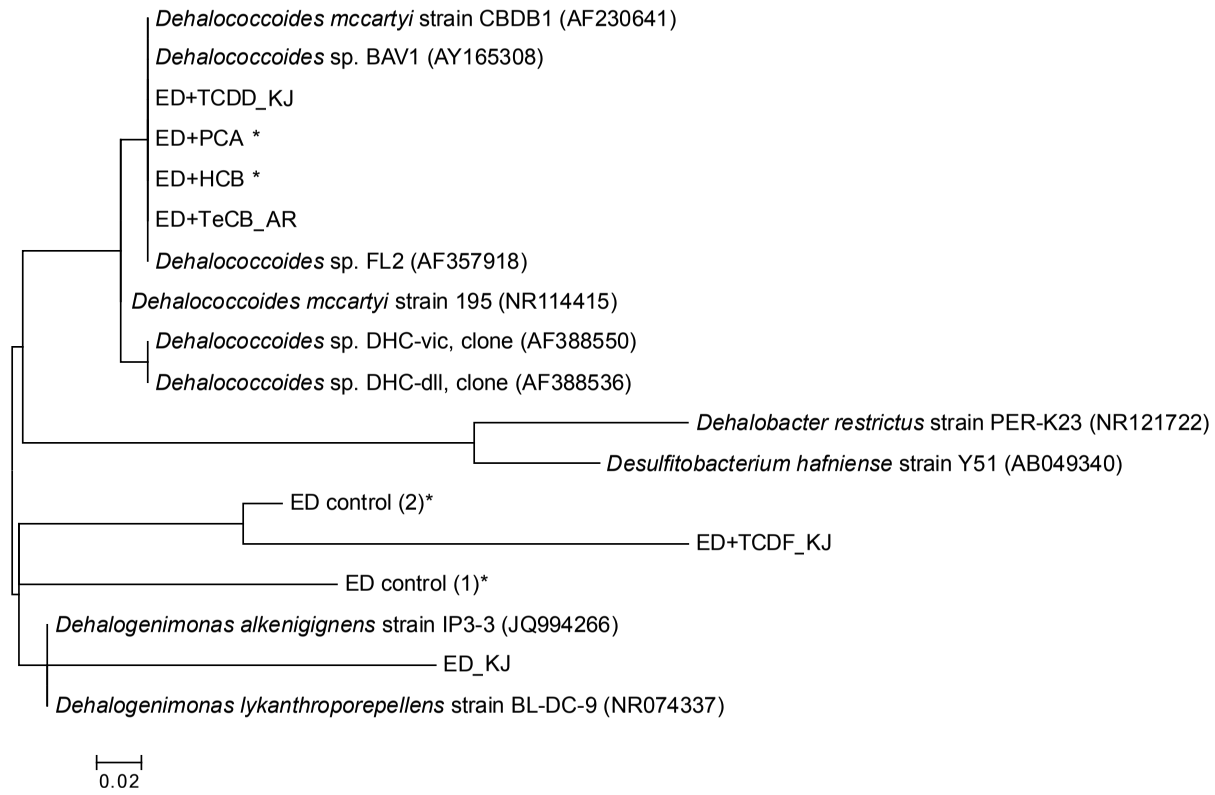
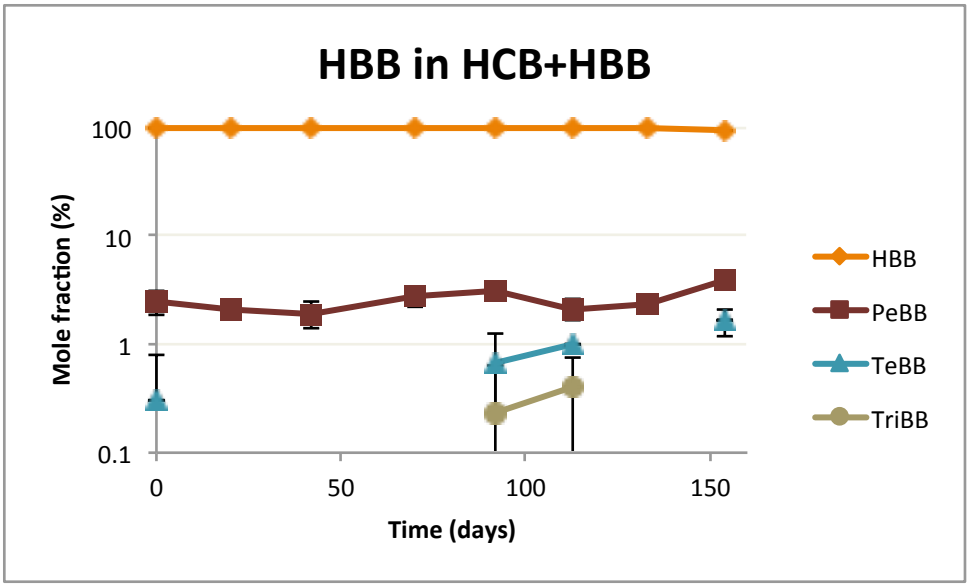


Figure 2-10. Phylogenetic analysis of 16S rRNA gene DGGE band sequences. The tree was constructed using the Maximum Likelihood method with *MEGA* 6 with 132 unambiguously aligned positions. Excised bands from previously analyzed dehalogenating cultures are indicated with an asterisk. KJ sequences were from Kymijoki River sediment, Finland (Liu et al., 2014). AR sequence was from Anacostia River sediment, DC (Park et al., 2011).



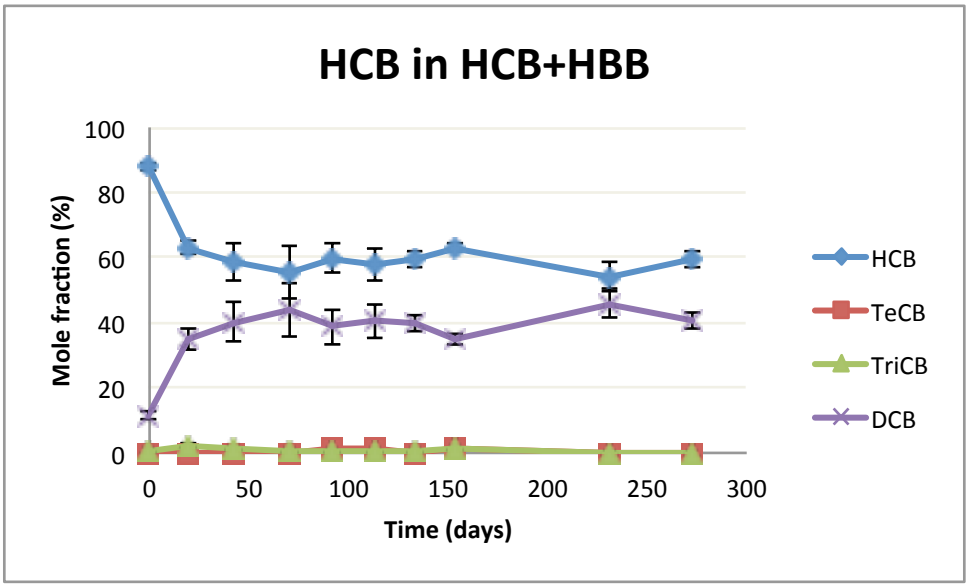


Figure 2-11. Change of HCB and HBB concentration and their intermediates in HCB+HBB cultures. HCB and HCB are represented with their intermediates in separate plots as the mole fraction. HBB dehalogenation intermediates were represented in a logarithmic scale. Data points are the mean ± standard deviation of three replicate microcosms.

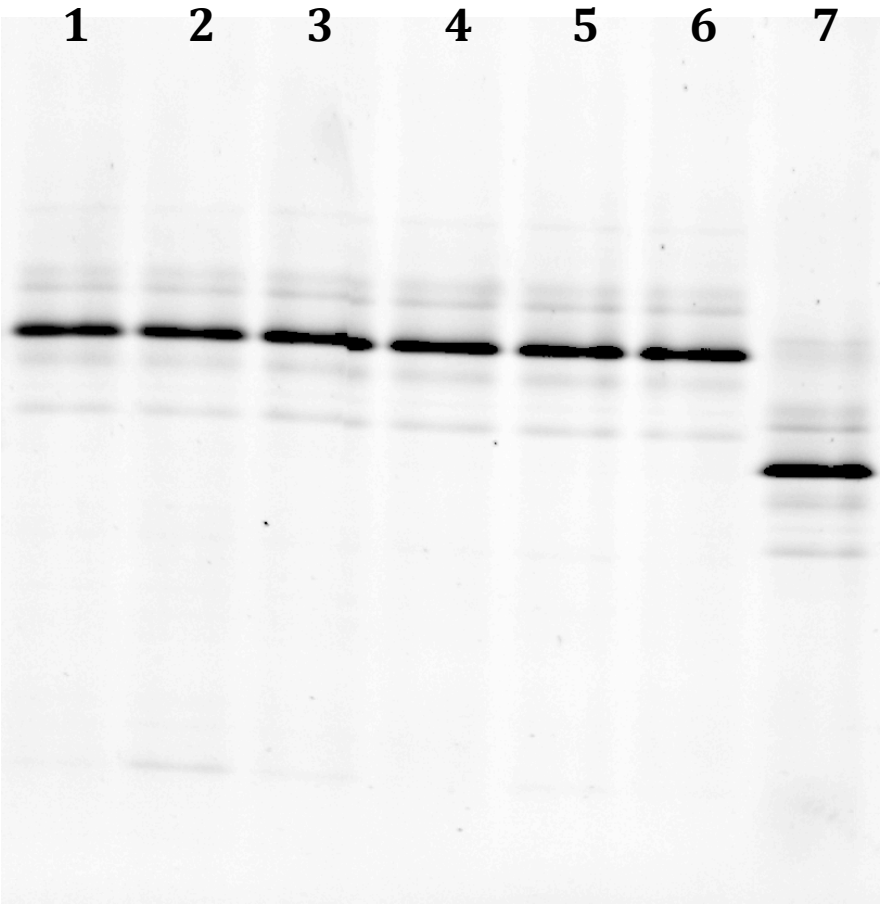


Figure 2-12. PCR-DGGE analyses of triplicate HCB+HBB cultures after 133 days and 273 days. Lane 1-3, D133; lane 4-6, D273; and lane 7, *Dehalococcoides mccartyi* strain 195.

Table 2-4. Reductive dehalogenase gene profile constructed with 12 *rdh* genes in H1 sediment (ED; HCB; PCA; and HCB+HBB) and H5 sediment (ED; HCB; and PCA).

	H1									H5								
	D0			D112						D0			D112					
				control			HCB			PCA				control			HCB			PCA	
Rdh 1	w	-	-	w	-	-	+	+	+	+	+	+	w	-	-	w	-	-	w	-	-	-	-	-
Rdh 2	-	-	-	-	-	-	+	w	w	+	w	w	-	-	-	-	-	-	-	-	-	-	-	-
Rdh 3	w	w	-	-	w	w	+	+	+	+	+	+	w	w	-	w	w	w	w	w	w	w	w	w
Rdh 4	w	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rdh 5	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-
Rdh 6	-	-	-	w	-	w	+	w	+	+	w	+	-	-	-	-	-	-	-	-	-	-	-	-
Rdh 7	-	-	-	-	-	-	+	w	w	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-
Rdh 8	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Rdh 9	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Rdh 10	-	-	-	-	-	-	+	+	+	w	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Rdh 11	-	-	-	w	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Rdh 12	-	-	-	-	w	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

X, signal; w, weak signal; and -, no signal.

Discussion

In this study, three persistent halogenated aromatic compounds, hexachlorobenzene (HCB), hexabromobenzene (HBB), and pentachloroaniline (PCA), were used to understand reductive dehalogenating activity of indigenous bacteria in Hackensack River sediments. These compounds were selected because they are problematic in the environment containing only one aromatic ring and that studying dehalogenation of these organohalide pollutants would also help to understand dehalogenation of more complex halogenated aromatics, such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins.

As an estuarine system, the Hackensack River contains both freshwater and saline locations. Sediment collected from freshwater site, H1 site has lower sulfate level and salinity (Figure 2-2) compared to other sampling sites, H2 to H5. These sulfate level and salinity gradually increases along the river. The most estuarine site, H5 site has the highest sulfate level and salinity among the five sampling sites.

Dechlorination activity was observed in most of the Hackensack River sediment microcosms within a few weeks after a short lag period (Figure 2-4 and 2-5). In contrast, Brahushi et al. (2004) reported HCB dechlorination after a lag period of 2 months in arable soil that had not been exposed to HCB before. Our sediment samples were collected from historically highly contaminated sites. Consistently, studies have shown that the Hackensack River sediment is contaminated by PCBs and dioxins (for review, see Crawford et al., 1995), which, we postulate, has resulted in the enrichment of

indigenous dehalogenating bacteria, and hence only a short lag period before the onset of dechlorination.

When comparing dechlorination rates observed in microcosms originating from H1 versus H5 sediment (see Figure 2-1 for different sampling sites), H5 showed a delay before the onset of dechlorination. This may be explained from the higher sulfate concentration at this site. Generally, studies have shown that sulfate inhibits reductive dechlorination (Gibson and Suflita, 1986; Alder et al., 1993), even though some other studies showed that sulfate did not affect reductive dechlorination (Kohring et al., 1989 and Häggblom and Young, 1990). Sulfate is thus likely to influence the activity of indigenous dehalogenating microorganisms. The Hackensack River sediments were collected from estuarine to freshwater sites. Fig 2-2 shows that sulfate level increases as the sampling site get closer to the mouth of the river in Newark Bay. Thus, we can conclude that the delayed onset of dechlorination in H5 sediment microcosms was likely caused by the high sulfate level in sediments at the site. The delayed onset of dechlorination might be explained by salinity as well. Prytula and Pavlostathis (1996) reported that the dechlorination rate of sediment bound HCB was two orders magnitude lower than that of freshly added, bioavailable HCB, with salinity causing increased contaminant hydrophobicity. Microbial reductive dechlorination of the sediment bound contaminants caused the long-term release of less chlorinated and more mobile products. By reducing the bioavailability of contaminants, salinity can inhibit indigenous microorganisms. Salinity level changes in the same manner with sulfate in distribution

along the Hackensack River system. Hence, we might explain the late dechlorination starting point observed in H5 sediment with the effect of salinity at sampling location.

HCB was sequentially dechlorinated to pentachlorobenzene (PeCB), 1,3,5-trichlorobenzene (TriCB) and finally 1,3-dichlorobenzene (DCB) and 1,4-DCB (Figure 2-6). A similar HCB dechlorination pathway has also been observed in previous studies (Fathepure et al., 1988; Beurskens et al., 1994; Tas et al., 2011). Based on their data, the most predominant dechlorination pathway follows HCB to 1,2,3,5-tetrachlorobenzene (TeCB) and finally to 1,3,5-TriCB. 1,2,3,5-TeCB was not detected as an intermediate in our HCB amended cultures, likely because it was rapidly dechlorinated further to 1,3,5-TriCB without significant accumulation. However, it was a likely dechlorination intermediate since 1,3,5-TriCB could be only formed from 1,2,3,5-TeCB.

In PCA amended cultures, sequential dechlorination was observed and it appeared more divergent than HCB dechlorination. Based on observations and reasonable estimations, PCA was sequentially dechlorinated to 2,3,5,6- and 2,3,4,6-tetrachloroaniline (TeCA), 2,3,5-, 2,4,5-, and 2,4,6-trichloroaniline (TriCA), 2,4-, 2,5-, 2,6-, and 3,5-dichloroaniline (DCA), and eventually to 3-chloroaniline (CA). Okutman Tas and Pavlostathis (2005) reported a PCNB degradation pathway under methanogenic conditions proceeding via reduction of PCNB to PCA, followed by sequential dechlorination to 2,3,4,5- and 2,3,5,6-TeCA, 2,4,5- and 2,3,5-TriCA, 2,4-, 2,5-, and 3,5-DCA, and eventually 3- and 4-CA. Liu et al. (2014) reported a similar conversion of PCNB to PCA, followed by dechlorination to 2,3,4,6- and 2,3,4,5-TeCAs, and then to 2,4,5- and 2,4,6-TriCA. The main product,

2,4,6-TriCA, accumulated and was slowly dechlorinated further to 2,4- or 2,5-DiCA (which could not be separated in analysis). Dehalogenating bacteria indigenous to different geographical locations thus mediate slightly different dechlorination pathways.

As shown in Figure 2-9, *Dehalococcoides mccartyi* phylotypes are consistently enriched from geographically different locations when chlorinated aromatics (polychlorinated dibenzo-*p*-dioxins, HCB, TeCB or PCA) are provided as electron acceptors. PCR-DGGE sequences obtained from sediments originated from Kymijoki River, Finland (Ahn et al. 2008; Kuokka et al., 2014; Liu et al., 2014) and Anacostia River, DC (Krumins et al., 2009; Park et al., 2011), and Hackensack River sediments, NJ (this study) were all highly similar. Chlorinated aromatic compounds used in these studies were TCDD for Kymijoki River sediment, TeCB for Anacostia River sediment, and HCB and PCA for Hackensack River sediment. All 16S rRNA gene PCR-DGGE sequences shared 100 % identity with the Pinellas subgroup of *Dehalococcoides mccartyi* (Hendrickson et al., 2002). Even though the 16S rRNA genes of the indigenous bacteria of these sediments are identical, their reductive dehalogenase gene profiles are likely distinct.

HBB debromination was not observed over incubation for up to 12 months (data not shown). There are some possible scenarios. By molecular weight, bromine is heavier than chlorine by 2 times and therefore, hexabromobenzene has a bulkier structure than hexachlorobenzene. It is possible that hexabromobenzene does not fit in the active site of the reductive dehalogenase of the indigenous microorganisms, or that HBB is poorly available and does not induce dehalogenating activity. When HCB and HBB were added

as co-substrates to HCB dechlorinating cultures, slow debromination of HBB (or PeBB; pentabromobenzene) was observed (Figure 2-10), with production of TeBB and TriBB after 92 days of incubation (although at low concentration, less than 0.1 μM). The slower rate of HCB compared to the original cultures could be due to depletion of electron donors. Several earlier reports have demonstrated that dehalogenation rates can be improved with amendment of halogenated co-substrates (Ahn et al., 2005, 2007, 2008; Bedard et al., 1998; Kuokka et al., 2014; Liu et al., 2014; Wu et al., 1997).

Over a hundred different reductive dehalogenase gene homologs have been described (for review, see Hug et al., 2013). One of these, CbrA, is a reductive dehalogenase isolated for its capability of dehalogenating 1,2,3,4-TeCB and 1,2,3-TriCB (Adrian et al., 2007). Since the Hackensack River sediment cultures generate TeCB and TriCB as intermediates of HCB dechlorination, we determined whether a *cbrA* gene was present in these cultures. Therefore, *cbrA* primers were designed to amplify the whole gene of *cbrA* and tested with one culture set. However, no PCR product was detected. We can explain it with observed intermediates in HCB dechlorination pathway. The TriCB intermediate in our HCB cultures was 1,3,5-TriCB, not 1,2,3-TriCB. This may explain why *cbrA* was not amplified, since that gene was originally isolated for 1,2,3-TriCB dechlorination. Some of the HCB cultures showed 1,4-DCB as one of intermediates of HCB dechlorination. This 1,4-DCB may not come from 1,2,3,4-TeCB in our cultures since the cultures did not have *cbrA (cbrA* was isolated for 1,2,3,4-TeCB dechlorination as well as 1,2,3-TriCB dechlorination). In my experiment, DNAs were extracted from sediment sampled at day 112. According to Figure 2-4, dechlorination was almost complete at day

112. Tas et al. (2010b) detected *cbrA* the early phase of dechlorination. Hence, another explanation for the absence of *cbrA* in our culture was that DNA was extracted from the late phase of dechlorination. There is another TriCB dehalogenase gene, cbdbA1624, which has been reported for dechlorination of 1,2,4-TriCB (Wagner et al., 2009), but it was not tested in this study.

The *rdh* gene profile analysis indicated that sediments from sites H1 and H5 likely do not contain *Dehalococcoides mccartyi* strains 195 or CBDB1. Since the twelve primer sets were designed based on the *rdh* gene sequences common to *Dehalococcoides mccartyi* strain 195 and strain CBDB1 (Park et al., 2011), all of the twelve primer sets should be amplified if *Dehalococcoides mccartyi* strain 195 or strain CBDB1 are present. In our H1 samples, ten *rdh* genes were amplified clearly but rdh 4 and rdh 5 were detected in one of triplicate cultures with weak signal. This result was consistent with the PCR-DGGE analyses. Enriched bands had different GC contents from strain 195 loaded as a control (data not shown). In general, there were more *rdh* genes detected in H1 cultures than in H5 cultures, suggesting that there is a more active dehalogenating *Dehalococcoides* community in H1 sediment. Since dechlorination of HCB and PCA was also observed in H5 cultures, we can conclude that different kinds of *rdh* genes were enriched in H5 cultures, but not detected by our 12 primer sets. Previously, these 12 primer sets were also used to analyze the *rdh* gene profile in different dehalogenating cultures (Park et al., 2011; Liu et al., 2014). In Anacostia River sediment, 9 *rdh* genes were detected in cultures amended with TeCB, pentachloronitrobenzene (PCNB), PCNB with bioaugmentation (*Dehalococcoides* mccartyi strain 195), or bioaugmentation. Rdh 4 and

rdh 5 were only detected in PCNB with or without bioaugmentation and bioaugmented cultures. In Kymijoki River sediment, 11 *rdh* genes excluding rdh 9 were enriched in 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (TeCDD) or 1,2,3,4-tetrachlorodibenzofuran (TeCDF) and/or PCNB. Rdh 9 was clearly enriched in 1,2,3,4-TeCDD plus PCNB. From the above studies and this work, it can be concluded that rdh 4 and rdh 5 can be enriched for dehalogenation of fully substituted halogenated aromatic compounds containing heavy substituents such as bromine or a nitro group.

In this chapter, we aimed to understand the distribution and activity of dehalogenating bacteria in Hackensack River sediments. In conclusion, indigenous bacteria were responsible for dehalogenation processes in sediment originating from the Hackensack River, NJ. Molecular analyses with *Chloroflexi* specific PCR-DGGE and *rdh* gene profile represented that different microbial populations were enriched corresponding to the sampling locations.

Acknowledgments

We thank Prof. John Reinfelder (Dept. of Environmental Sciences) for providing the Hackensack River sediment samples, Prof. Lily Young (Dept. of Environmental Sciences) for supplying hexabromobenzene, and Dr. Joong-Wook Park for guidance on DGGE analyses.

CHAPTER 3

Evaluation of Carbon Isotope Fractionation during Dehalogenation of Chlorinated and Brominated Benzenes in Hackensack River Sediment Cultures

Abstract

Compound specific isotope analysis (CSIA) has been established as a useful tool to evaluate *in situ* biodegradation. Here, CSIA was used to determine microbial dehalogenation in microcosms derived from Hackensack River sediments. Two sets of experiments were established: the first experiment was conducted with hexachlorobenzene (HCB) as substrate and a follow-up experiment with HCB, pentachlorobenzene, 1,2,3,5-tetrachlorobenzene (TeCB), 1,2,3,5-tetrabromobenzene, and 1,3,5-tribromobenzene (TriBB) as substrates. Gas chromatography-isotope ratio mass spectrometry (GC-IRMS) was used to determine carbon isotope fractionation during reductive dehalogenation. Strong evidence of isotope fractionation coupled to dehalogenation was not observed, possibly due to the low solubilities of the halobenzene substrates and a dilution of the isotope signal. Nonetheless, the following results were observed: a depletion of the $\delta^{13}C$ value in the dichlorobenzene product during dechlorination of HCB; the sequential depletion and enrichment of $\delta^{13}C$ value for trichlorobenzene in TeCB dechlorinating cultures; and the enrichment of $\delta^{13}C$ during debromination of TriBB. This indicates that a measurable isotope fractionation occurred during reductive dehalogenation of highly halogenated chloro- and bromobenzenes. Thus, the data suggests that CSIA can have application for monitoring *in situ* microbial reductive dehalogenation of highly halogenated benzenes.

Introduction

Tools for evaluating and demonstrating *in situ* biodegradation are important for the assessment of bioremediation, in particular natural attenuation as a remediation option. The general strategy to confirm *in situ* biodegradation should include three types of evidence: (1) documented loss of contaminants from the site, (2) laboratory assays showing potential of the indigenous microbial community to transform contaminants under expected site conditions, and (3) one or more sets of evidence showing that biodegradation potential is actually realized in the field (NRC, *In situ* bioremediation, 1993). Compound specific stable isotope analysis (CSIA) is a promising option for monitoring and quantification of *in situ* biodegradation of contaminants at polluted sites. There are six major elements of organic compounds: carbon; hydrogen; nitrogen; oxygen; sulfur; and chlorine. These elements have at least two stable isotopes which can be distinguished by mass spectrometry. Table 3-1 lists these elements and their relative isotope abundances.

Stable isotope signatures may be either conservative or fractionating. Conservative isotopic signatures can be used to provide insight into sources of contamination, while isotopic fractionation may be used to assess the progress of biodegradation in the environment (Wang et al., 2004). For the use of conservative isotopic signatures, individual signatures should retain their isotopic integrity under environmental conditions and the contributions from different sources should be understood. In principal, the rate of biodegradation is reduced by the presence of the heavier isotope since in biological systems lighter isotopes are preferentially reacted. Hence, this results in the accumulation

of the heavier isotope in the residual substrate (Hunkeler et al., 1999) and the lighter isotope is enriched in the product of degradation. This is referred to as compound specific stable isotope fractionation, which can be analyzed by isotope ratio mass spectrometry (IRMS).

Isotope analysis offers an opportunity to obtain information on physical and (bio-) chemical processes making use of kinetic and equilibrium isotope effects. The magnitude of the isotope fractionation during degradation depends on the nature of the bond cleavage. A number of factors impact isotope fractionation. These include the redox condition, temperature and growth kinetics, sorption, evaporation and chemical reactions (for review, see Meckenstock et al., 2004). However, physical removal processes do not cause significant isotope fractionation, and CSIA is thus a strong tool for proving *in situ* biodegradation. The extent of isotope fractionation can be quantified using the Rayleigh approach and expressed as the isotope enrichment factor ε (Mariotti et al., 1981; Rayleigh, 1896). Multi-dimensional CSIA, by combining the analysis of two or more different stable isotopes within a molecule, can be used to identify specific pathways of *in situ* biodegradation (Vogt and Richnow, 2014).

Over the last decade CSIA has been applied to study degradation of halogenated compounds. Most studies have focused on carbon stable isotope fractionation during the reductive dehalogenation process. The focus has mainly been on the use of CSIA for assessing tetrachloroethene (PCE) dechlorination (e.g., Hunkeler et al., 1999; Slater et al., 2001; Nijenhuis et al., 2005; Renpenning et al., 2015), but there are some recent reports

on chlorinated benzenes, such as trichlorobenzene (TriCB), dichlorobenzene (DCB), and monochlorobenzene (MCB). Griebler et al. (2004) showed significant carbon isotope fractionation during reductive dehalogenation of 1,2,3-trichlorobenzene (TriCB) by *Dehalococcoides* sp. strain CBDB1. Braeckevelt et al. (2007) combined carbon stable isotope analysis with a microcosm study to assess *in situ* MCB biodegradation in a wetland system. The combined results from CSIA and microcosms supported that MCB biodegradation is occurred *in situ*. Liang et al. (2014) reported carbon isotope fractionation during anaerobic degradation of DCB isomers. They converted ε_{bulk} values to apparent kinetic isotope effects for carbon ($AKIE_C$) in order to characterize the carbon isotope effect at the reactive positions for the DCB isomers.

To date, there have been no CSIA studies reported for highly halogenated benzenes in spite of their harmful health effects. For example, hexachlorobenzene is highly toxic and causes several health problems, such as immune system disruption and cancer (ATSDR, 2013). This problematic compound needs to be monitored in environment. Therefore, there is a need for CSIA developed for HCB dechlorination. In this study, carbon isotope fractionation during reductive dehalogenation of highly halogenated benzenes was determined with anaerobic cultures originating from Hackensack River sediment. CSIA was conducted for dehalogenation of HCB and its intermediates, pentachlorobenzene and 1,2,3,5-tetrachlorobenzene, and brominated benzenes including 1,2,3,5-tetrabromobenzene and 1,3,5-tribromobenzene.

Table 3-1. Six major elements in nature and the relative natural abundance and mass differences of the international standards.

Element	Relative isotope abundance	Relative mass difference	Standard
H/D	99.9844/0.0156	2.00	V-SMOW
$^{12}C/^{13}C$	98.89/1.11	1.08	V-PDB
$^{14}N/^{15}N$	99.64/0.36	1.07	Air
$^{16}O/^{18}O$	99.76/0.02	1.13	V-SMOW
$^{32}S/^{34}S$	94.02/4.21	1.06	V-CTD
$^{35}Cl/^{37}Cl$	75.53/24.47	1.06	24.47

Materials and Methods

Establishment of dehalogenating cultures

The dehalogenating cultures investigated in this study were established from anaerobic microcosms using sediment slurries from Hackensack River sediments. Sediments from sites H1, H2 and H3 (Chapter 2) were used. The microcosms were set up to examine the potential of indigenous microorganisms to dehalogenate chlorinated and brominated benzenes. Two sets of carbon isotope fractionation experiments were established separately with carbon compound specific isotope analyses (CSIA) conducted in March 2013 and January 2014. The first set was prepared to examine compound specific isotope analysis of carbon for hexachlorobenzene (HCB) and hexabromobenzene (HBB) in Hackensack River sediment. The second experiment investigated HCB, and three intermediates of HCB dechlorination (pentachlorobenzene (PeCB); 1,2,3,5-tetrachlorobenzene (TeCB); and 1,3,5-trichlorobenzene (TriCB)) and two bromobenzenes (1,2,3,5-tetrabromobenzene (TeBB) and 1,3,5-tribromobenzene (TriBB)).

For the first set, sediment slurry microcosms for CSIA were established in anaerobic medium as previously described in Chapter 2 with a culture volume of 40 mL and final substrate concentration of 10 μM. Sediment slurries were made with sediment in anaerobic media, amended with a mixture of electron donors and then transferred to each bottle. Each serum bottle contained 10 % sediment from H1 in media with 500 μM of electron donors (a 1:1 mixture of propionic acid and butyric acid) and 10 μM of electron acceptors. Two halogenated benzenes, HCB and HBB, were used as electron acceptors. A set of triplicate monitoring cultures amended with HCB or HBB were also prepared when

cultures were set up for CSIA. These three cultures were monitored over the time course to determine the time points for sampling for CSIA. For eight time points and a set for monitoring, 27 cultures were prepared for each electron acceptor. Triplicate autoclaved killed controls were also prepared for two time points (6 bottles) for each electron acceptor. At each time point, three replicate cultures were sacrificed. A 0.5 mL sample was taken for GC-MS analysis to determine the extent of dechlorination and the remainder of the culture frozen at -20 °C for later CSIA analysis.

The second set of carbon compound specific isotope analysis was prepared to examine HCB and three intermediates of HCB dechlorination (PeCB; 1,2,3,5-TeCB; or 1,3,5-TriCB), and two bromobenzenes (1,2,3,5-TeBB or 1,3,5-TriBB). Since no HBB debromination was observed in the first set of analysis, additional cultures amended with less brominated benzenes were set up to monitor debromination. In HCB dechlorination, 1,3,5-TriCB was detected as one intermediate and 1,2,3,5-TeCB was predicted as the TeCB intermediate. Based on this information about the HCB dechlorination pathway, the corresponding bromobenzenes 1,2,3,5-TeBB and 1,3,5-TriBB were chosen for CSIA experiments. 20 cultures were prepared with H2 sediment for each of TeBB and TriBB for ten time points (duplicated). Killed controls containing both TeBB and TriBB were also prepared for two time points by autoclaving for 30 min at 121 °C on three successive days. At each time point, two culture bottles were sacrificed. A 0.5 mL sample was taken for GC-MS analysis and the remainder of the culture frozen at -20 °C for later CSIA analysis.

For CSIA of HCB and each intermediate of HCB dechlorination, a set of 80 cultures was prepared with sediment mixture of H2 and H3 (7:9; w:w). Four halogenated benzenes (HCB; PeCB; 1,2,3,5-TeCB; 1,3,5-TriCB) detected as intermediates in HCB dechlorination were used. For duplicated 10 time point samples, 20 cultures were prepared for each substrate. At each time point, two cultures were sacrificed, a 0.5 mL sample was taken for GC-MS analysis and the remainder of the culture frozen at -20 °C for later CSIA analysis. Duplicate killed controls containing HCB, PeCB, TeCB, and TriCB together were prepared for two time points as described above.

Analytical methods

For the first experiment conducted in 2013, a 0.5 mL sample was taken every few days from the three monitoring cultures and analyzed by GC-MS as described below in order to determine time points for destructive sampling of cultures for CSIA. The second experiment set up in 2014 did not have monitoring cultures, and sample time points were estimated from previous data. At each time point, 0.5 mL sample was taken to determine the extent of dehalogenation by GC-MS, and the whole remaining culture was sacrificed for CSIA analysis. For analysis of chlorinated benzenes, the culture was extracted three times with 5 mL hexane. For analysis of brominated benzenes, 7.5 mL of a mixture of toluene and acetone (1:1; v:v) was used.

The extraction procedures were started with addition of solvent to the culture. Then, the bottle was sealed with Teflon coated rubber stopper and crimped with an aluminum seal. Sealed bottles were laid on shaker at 180 rpm overnight. After shaking, the bottles were

returned to upright position and supernatant was removed to a clean glass test tube. This procedure was repeated twice. For the second and third extraction, the time was 2 hours. All three extracts (~15 mL total) were combined into a test tube, and briefly sonicated in a water bath for clearer separation of solvent and any residual aqueous phase. Supernatants were transferred from the test tubes to a clean glass extraction bottle. The ~15 mL extracts were concentrated by evaporation of the solvent to less than 0.5 mL, and then sealed, packed on ice in a Styrofoam box, and shipped to Germany for CSIA.

The concentration of brominated benzenes and chlorinated benzenes was determined with 0.5 mL samples taken from cultures. Chloro- and bromobenzenes were extracted into hexane and toluene, respectively, following by the procedure described in Chapter 2. Briefly, 0.5 mL sample was mixed with solvent, shaken for 2 hours at 180 rpm, and the supernatant was transferred to a new GC-vial. Samples were analyzed with an Agilent 6890 gas chromatograph equipped with a HP-5MS capillary column (0.250 mm × 60 m, 0.25 μm film thickness, J&W Scientific, Folsom, CA) and an Agilent 5973 Network Mass selective detector. The GC column temperature was first held at 50 °C for 2 min, increased to 300 °C at a rate of 12 °C min^{-1}, and then held for 5 min. For chlorinated benzenes, 1,2,3,5-TeBB, and 1,3,5-TriBB, intermediates were identified by comparison of their retention times to authentic standards. Dibromobenzene (DBB) and bromobenzene (BB) were determined by their mass spectra. Standard curves for each compound except DBB and BB were generated with standards of 10, 20, 30, 40, 50 μM. For dibromobenzene and bromobenzene, response curves were generated with the calculated average value of standard sets of TeBB and TriBB.

To determine whether the extraction and concentration process affected isotope composition, two sets of controls were prepared for CSIA. To test for potential extraction effects, triplicate serum bottles were prepared with mixture of 10 μM HCB, PeCB, 1,2,3,4-TeCB, 1,2,4-TriCB, and 1,3-dichlorobenzene (DCB) and 500 μM electron donor mix in 40 mL anaerobic media. Extraction was conducted as described above. Extracts were concentrated by evaporation in fume hood to a final volume of less than 0.5 mL.

To test for any effects resulting from solvent evaporation, the mixture of HCB, PeCB, TeCB, TriCB, and DCB was added in 15 mL hexane. The volume of mixture added was same with previously described extraction test. Hexane containing the mixture was evaporated in a fume hood till its final volume reached less than 0.5 mL. Evaporation and extraction tests were performed in triplicate and analyzed by CSIA as described below.

Stable Carbon Isotope Analysis

Prepared samples concentrated to less than 0.5 mL were shipped to Germany and stable isotope analyses conducted at the Stable Isotope Laboratory of the Center for Environmental Research (UFZ), Leipzig-Halle, Germany. Before analyses, sample cleanup through a Florisil column (~1 g of Florisil in a Pasteur pipette eluted with 5 mL of pentane-diethyl ether; 90:10 (v:v)) was conducted to remove organic matter from samples that interfered with CSIA. Samples were concentrated to a volume of 50 to 100 μL prior to analysis. The GC-IRMS system consisted of a gas chromatograph (7890 series, Agilent Technology) coupled via a Conflow IV Interface combustion oven (ThermoFinnigan GC-combustion IV, Bremen, Germany) to a Finnigan MAT 252

isotope ratio mass spectrometer (ThermoFinnigan, Bremen). The organic substances in the GC effluent were oxidized to CO_2 on a CuO/Ni/Pt catalyst held at 960 °C. A Zebron ZB5 column (60 m × 0.32 m; 0.25 μm film thickness) was used for separation with helium at a flow rate of 1.5 mL min^{-1} used as the carrier gas. The GC temperature program was held at 60 °C for 5 min, then increased to 320 °C at a rate of 10 min^{-1}, and then held for 5 min isothermally. Samples were injected (2 μL) in split mode with a split ratio 1:1 into a hot injector held at 320 °C. Each sample was analyzed at least in triplicate.

The carbon isotopic compositions (R) are reported as δ notation in parts per thousand (denoted as ‰) enrichments or depletions relative to a standard (V-PDB; Vienna Pee Dee Belemnite standard) of known composition. δ values of carbon were calculated as follows:

$$\delta(^{13}C)\ (‰) = (R_{sample}/R_{standard} - 1) \times 1000$$

where R_{sample} and $R_{standard}$ represent the ratios of the heavy isotope to light isotope in sample and the international standard, respectively.

The extent of isotope fractionation during degradation can be expressed as an isotopic enrichment factor (ε) calculated based on the Rayleigh equation for a closed system (Rayleigh, 1896; Mariotti et al., 1981):

$$R_t/R_0 = (C_t/C_0)^{(1/\alpha - 1)}$$

where R is the isotope ratio, C is the concentration, and the index (0 and t) describes the incubation time of experiment (t). The isotope ratios (R_t/R_0) are determined from the equation $R_t/R_0 = (\delta_t/1000 + 1)/(\delta_0/1000 + 1)$. When $\ln R_t/R_0$ versus $\ln C_t/C_0$ is plotted, the isotopic enrichment factor (ε) can be determined from the slope of the curve (b), with $b = 1/\alpha - 1$ and $\varepsilon = 1000 \times b$. Linear regression is used to estimate the slope.

Results

Reductive dehalogenation of chlorobenzenes

In the first experiment conducted in 2013, dehalogenation of hexachlorobenzene (HCB) or hexabromobenzene (HBB) was monitored first in monitoring cultures (data not shown). Monitoring cultures showed dehalogenation of HCB, while no activity was found for HBB cultures. Thus, only HCB dechlorinating cultures were analyzed by carbon compound specific isotope analysis. Based on the rate of HCB dechlorination in monitoring cultures, the time points for CSIA culture sampling were determined. No changes in C isotope composition were observed due to the extraction, sample concentration or Florisil clean-up procedures (See Appendix III).

HCB was sequentially dechlorinated to dichlorobenzene (DCB) over a period of 33 days (Figure 3-1). (However, since the dechlorination rate was slower in monitoring cultures (data not shown) and the first CSIA culture was not sacrificed until day 17, data for isotope fractionation at HCB residual substrate concentrations between 100 and 50 mole % were not be obtained). From the CSIA analyses at 50 to 10 % residual HCB concentration, no clear C isotopic enrichment in HCB (at ~-26 ‰) could be observed. However, pentachlorobenzene (PeCB) detected at approximately 50 % dechlorination had a $\delta^{13}C$ value of -29 to -26 ‰. Similarly, DCB detected at 70 to 90 % dechlorination of HCB also had a depleted $\delta^{13}C$ value, at around -28 to -30 ‰ when approximately 12 % of HCB remained in the culture (Figure 3-2). For tetrachlorobenzene (TeCB) and trichlorobenzene (TriCB) dechlorination intermediates, there was no measurable change in isotope composition over the course of reductive dechlorination.

In the second experiment from 2014, dechlorination of PeCB (Figure 3-4) and TeCB (Figure 3-5) occurred over 30 days, while HCB dechlorination took longer (Figure 3-3). After 30 days, the mole fraction of HCB was still at 50 % of all CBs (Figure 3-3). No significant change in the $\delta^{13}C$ value of HCB was observed over the course of dechlorination. The PeCB intermediate was slightly enriched, as observed in the 2013 experiment, however, isotope composition data for the other dechlorination products was inconclusive.

During PeCB dechlorination (Figure 3-4), the $\delta^{13}C$ value for the TeCB intermediate slightly decreased from -26 to -27 ‰ at around 70 % of dechlorination of PeCB, and the value increased to -24 ‰ when PeCB dechlorination was almost complete. However, no significant changes in the isotope composition of the substrate, other intermediates and products, PeCB, TriCB, and DCB, were observed over the course of PeCB dechlorination.

Over the course of dechlorination of TeCB (Figure 3-5), changes in the $\delta^{13}C$ value for TeCB substrate and TriCB intermediate were detected. The $\delta^{13}C$ value for TeCB was enriched from -29 to -27 ‰ at approximately 10 % dechlorination of TeCB. The $\delta^{13}C$ value for TriCB was depleted at first from -33 to -34 ‰ when ~90 % of TeCB was decreased to ~70 % in the culture. Then, the $\delta^{13}C$ value was enriched to -28 ‰ at 4 % residual TeCB in the culture.

Reductive dehalogenation of bromobenzenes

Previous experiments with HBB, including the first CSIA set up, had shown only limited debromination activity (Chapter 2), possibility due to the very low solubility of HBB or due to steric hindrance that blocked reductive dehalogenation. We therefore tested tetrabromobenzene (TeBB) and tribromobenzene (TriBB) as electron acceptors in microcosms originating from the Hackensack River sediment (H2). 1,2,3,5-TeBB and 1,3,5-TriBB were selected based on the dechlorination pattern of HCB shown in previous experiments. Debromination was observed for both compounds. TriBBs, dibromobenzene (DBBs), and monobromobenzene (MBB) were detected as dehalogenation intermediates and end-products (see below).

TeBB and TriBB were debrominated via DBB to MBB. At 31 days of incubation, approximately 50 % of the substrates remained (the last sampling point for CSIA; Figure 3-6 and 3-7). From the CSIA for TeBB, no clear C isotopic enrichment in TeBB (at average ~-26 ‰) could be observed over the course of reductive debromination. However, a sequential depletion and enrichment of the $\delta^{13}C$ value was detected for the TriBB intermediate. With approximately 80 to 70 % TeBB remaining in culture, the $\delta^{13}C$ value for TriBB was -25 to -27 ‰ suggesting a slight depletion from the value for the intermediate TriBB. Then this value increased to -25 ‰ at 60 % debromination of TeBB.

Over the course of TriBB debromination (Figure 3-7), the value of $\delta^{13}C$ for TriBB in cultures became more enriched from an initial value of -27 ‰ and increasing to -23 ‰ when approximately 68 % of TriBB remained in the culture. For the DBB intermediate

detected, there was no significant change of isotope fractionation over the course of debromination. The isotope enrichment factor (ε) was calculated to describe the debromination of TriBB (Figure 3-8), yielding an estimated ε value for TriBB debromination of -6.5 ‰ ($r^2 = 0.43$).

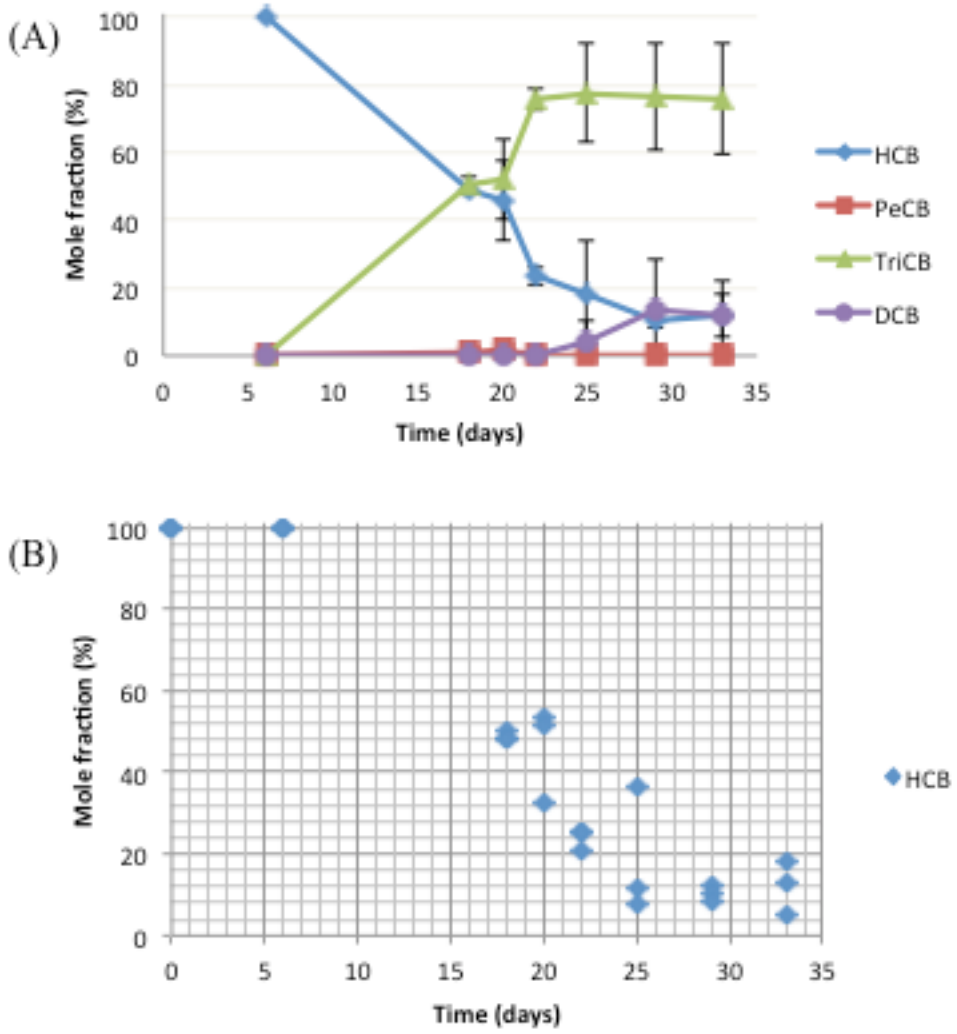


Figure 3-1. Dechlorination of HCB in cultures set up for CSIA in 2013. (A) Mole fractions of remaining HCB and generated dechlorination intermediates. (B) Mole fraction of remaining HCB.

Abbreviations: HCB, hexachlorobenzene; PeCB, pentachlorobenzene; TeCB, tetrachlorobenzene; TriCB, trichlorobenzene; and DCB, dichlorobenzene

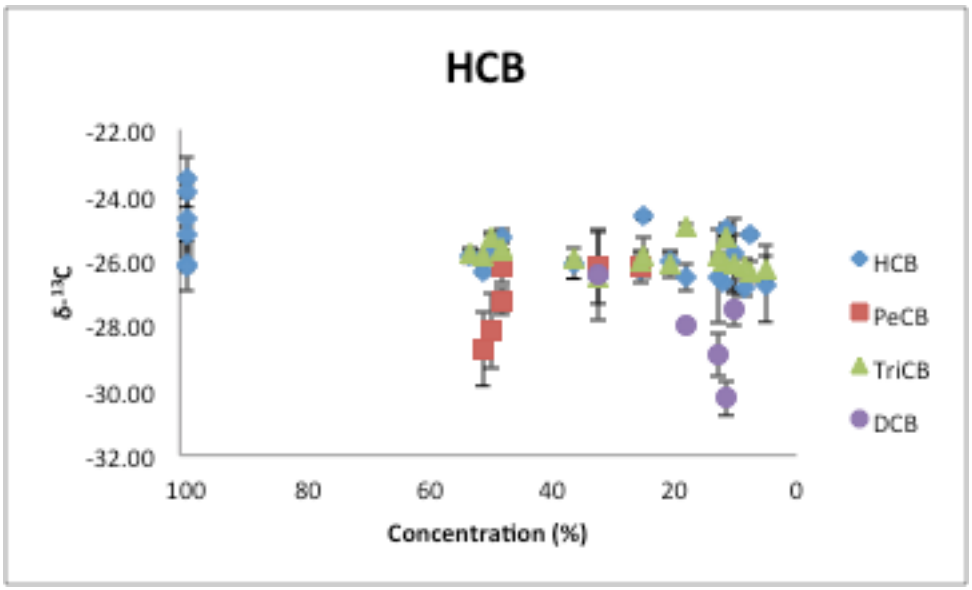


Figure 3-2. CSIA for HCB conducted in 2013. X-axis represents residual HCB substrate concentration in culture and Y-axis represents the measured $\delta^{13}C$ value (of triplicate analyses for each sample).

Abbreviations: HCB, hexachlorobenzene; PeCB, pentachlorobenzene; TriCB, trichlorobenzene; and DCB, dichlorobenzene

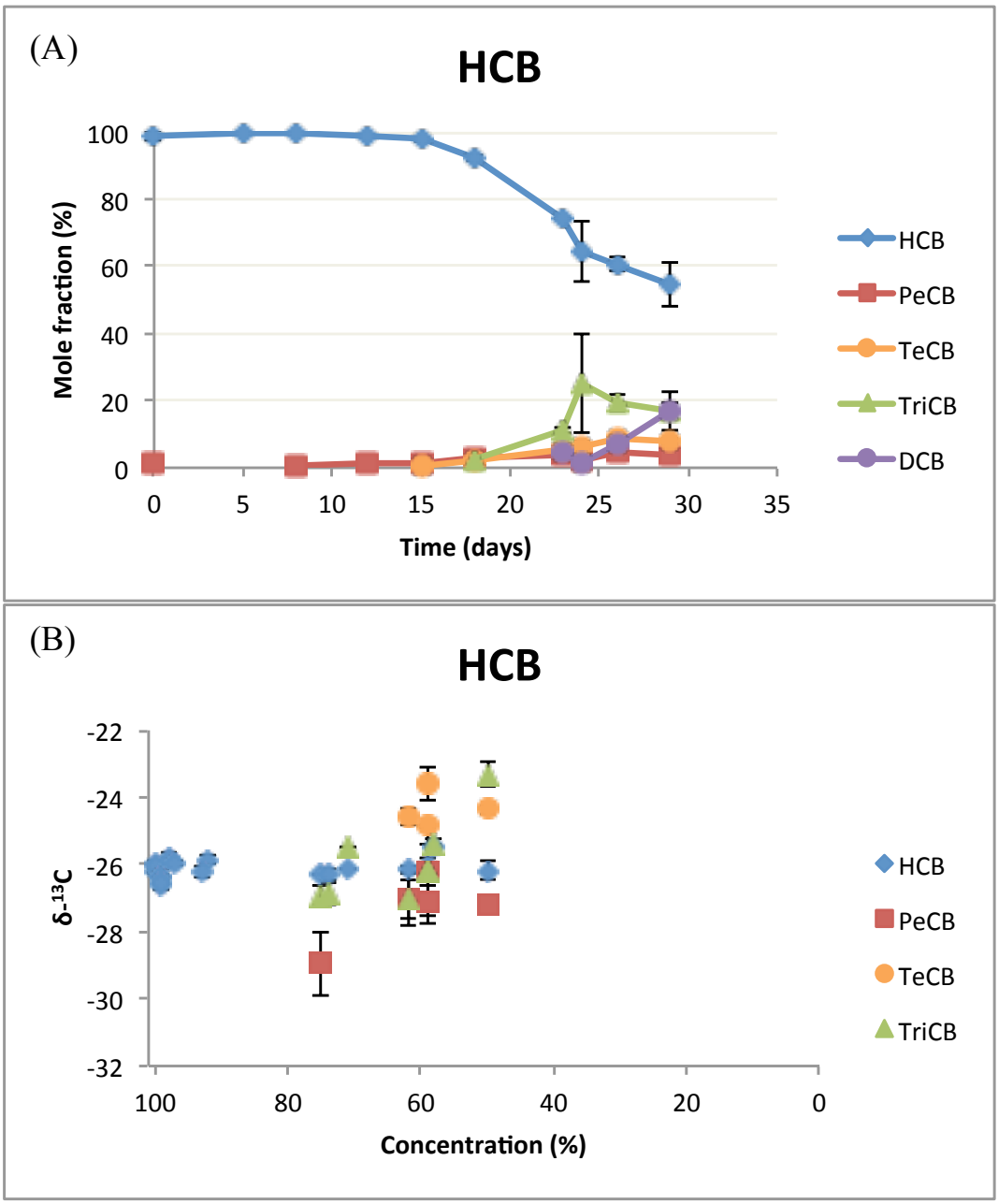


Figure 3-3. HCB dechlorination and CSIA in 2014. (A) Dechlorination of HCB in cultures. Each data point represents single culture bottle. (B) CSIA data. X-axis represents residual substrate concentration in culture and Y-axis represents the measured $\delta^{13}C$ value (of triplicate analyses for each sample).

Abbreviations: HCB, hexachlorobenzene; PeCB, pentachlorobenzene; TeCB, tetrachlorobenzene; and TriCB, trichlorobenzene.

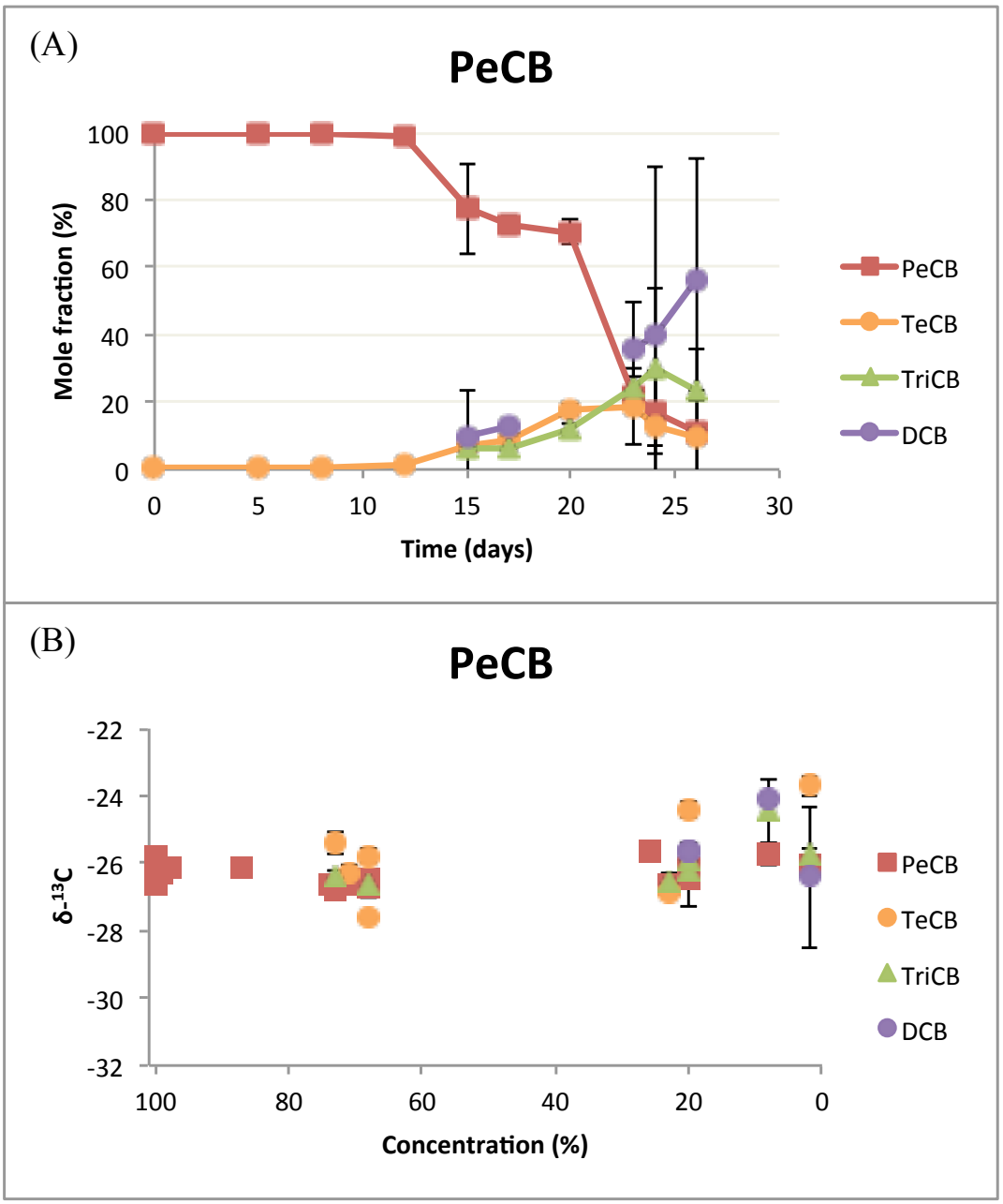


Figure 3-4. PeCB dechlorination and CSIA in 2014. (A) Dechlorination of PeCB in cultures. Each data point represents single culture bottle. (B) CSIA data. X-axis represents residual substrate concentration in culture and Y-axis represents the measured $\delta^{13}C$ value (of triplicate analyses for each sample).

Abbreviations: PeCB, pentachlorobenzene; TeCB, tetrachlorobenzene; TriCB, trichlorobenzene; and DCB, dichlorobenzene.

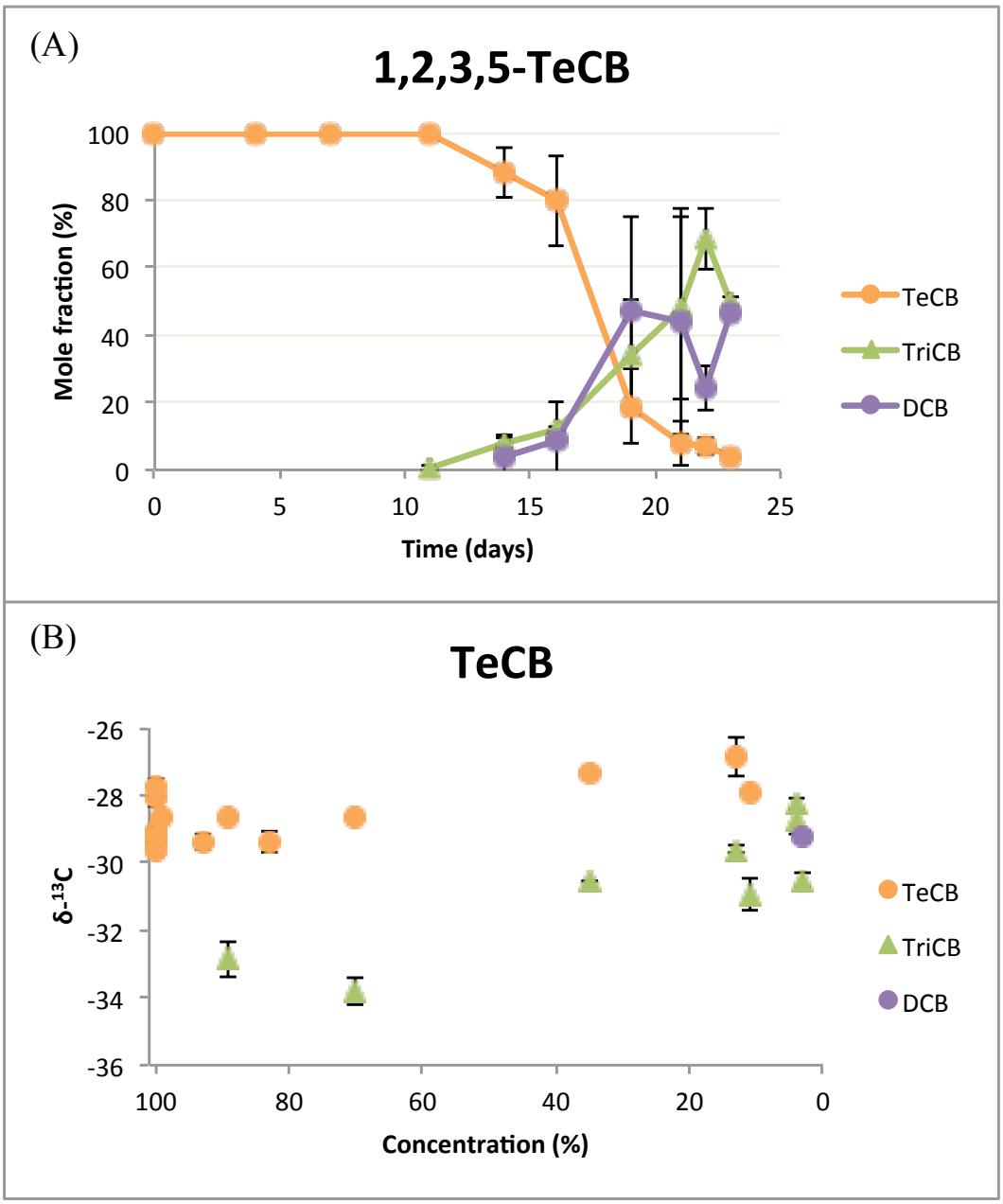


Figure 3-5. TeCB dechlorination and CSIA in 2014. (A) Dechlorination of TeCB in cultures. Each data point represents single culture bottle. (B) CSIA data. X-axis represents residual substrate concentration in culture and Y-axis represents the measured $\delta^{13}C$ value (of triplicate analyses for each sample).

Abbreviations: HCB, hexachlorobenzene; PeCB, pentachlorobenzene; TeCB, tetrachlorobenzene; TriCB, trichlorobenzene; and DCB, dichlorobenzene.

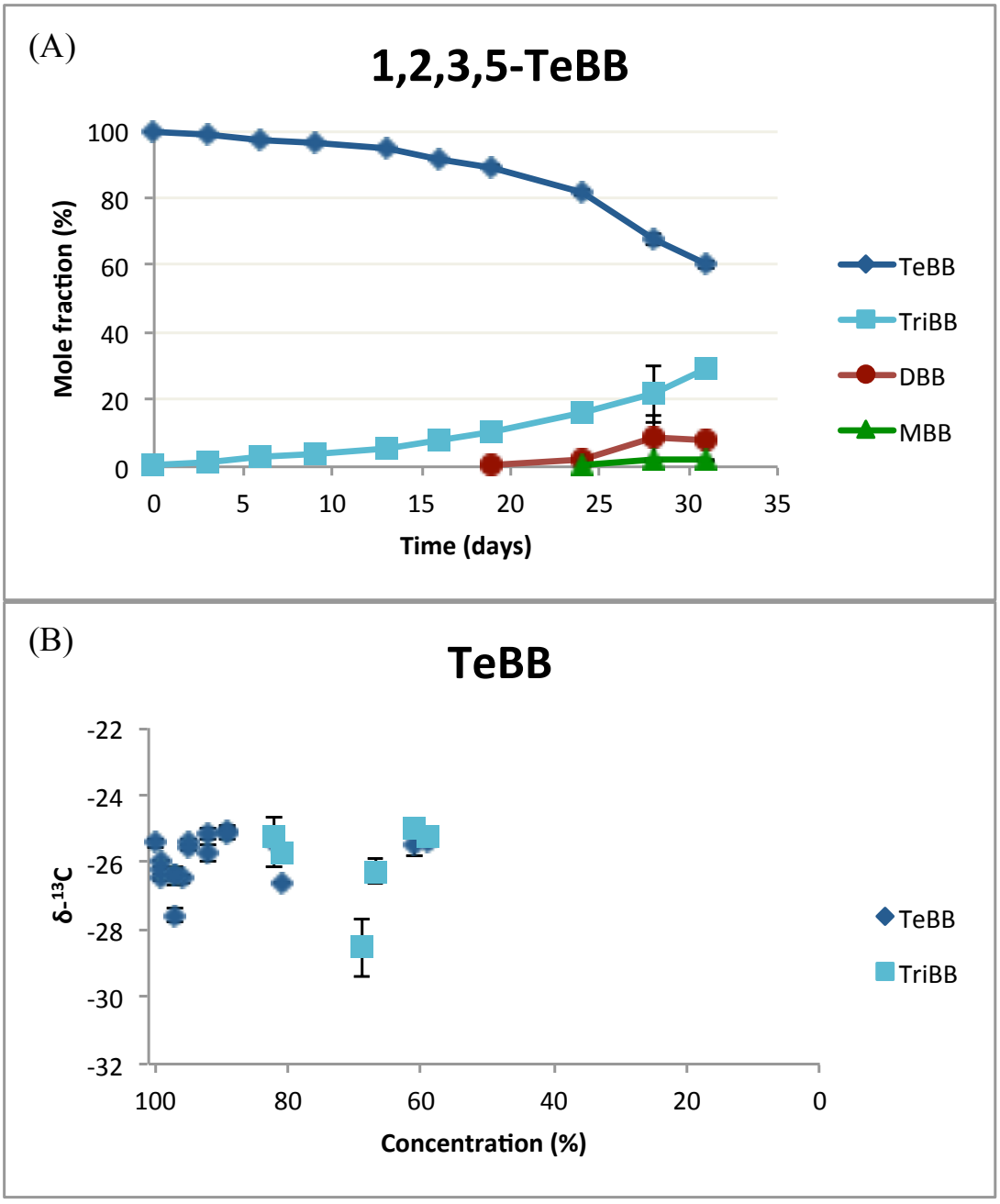


Figure 3-6. TeBB debromination and CSIA in 2014. (A) Debromination of TeBB in cultures. Each data point represents single culture bottle. (B) CSIA data. X-axis represents residual substrate concentration in culture and Y-axis represents the measured $\delta^{13}C$ value (of triplicate analyses for each sample).

Abbreviations: TeBB, tetrabromobenzene; and TriBB, tribromobenzene.

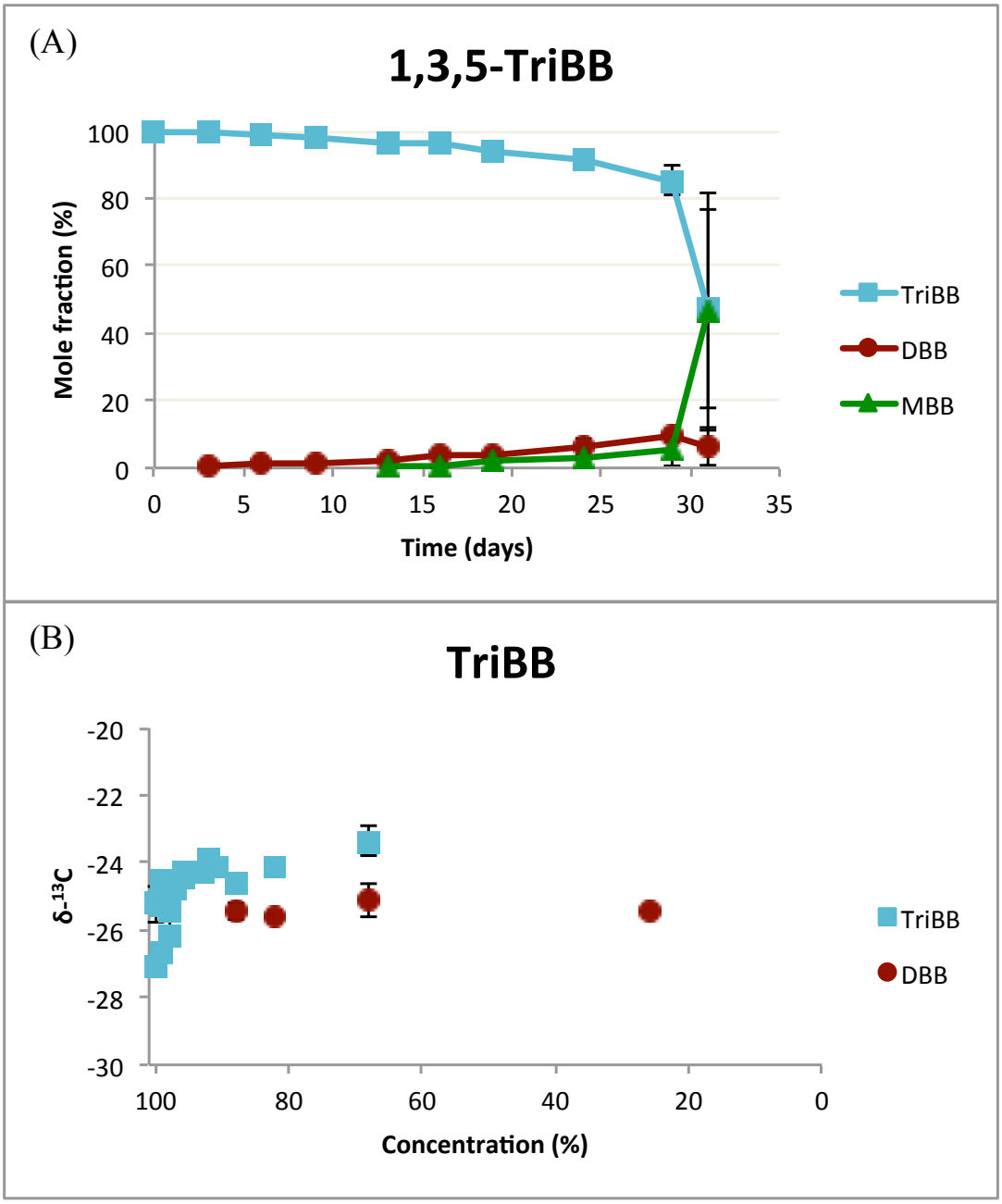


Figure 3-7. TriBB debromination and CSIA in 2014. (A) Debromination of TriBB in cultures. Each data point represents single culture bottle. (B) CSIA data. X-axis represents residual substrate concentration in culture and Y-axis represents the measured $\delta^{13}C$ value (of triplicate analyses for each sample).

Abbreviations: TriBB, tribromobenzene; and DBB, dibromobenzene.

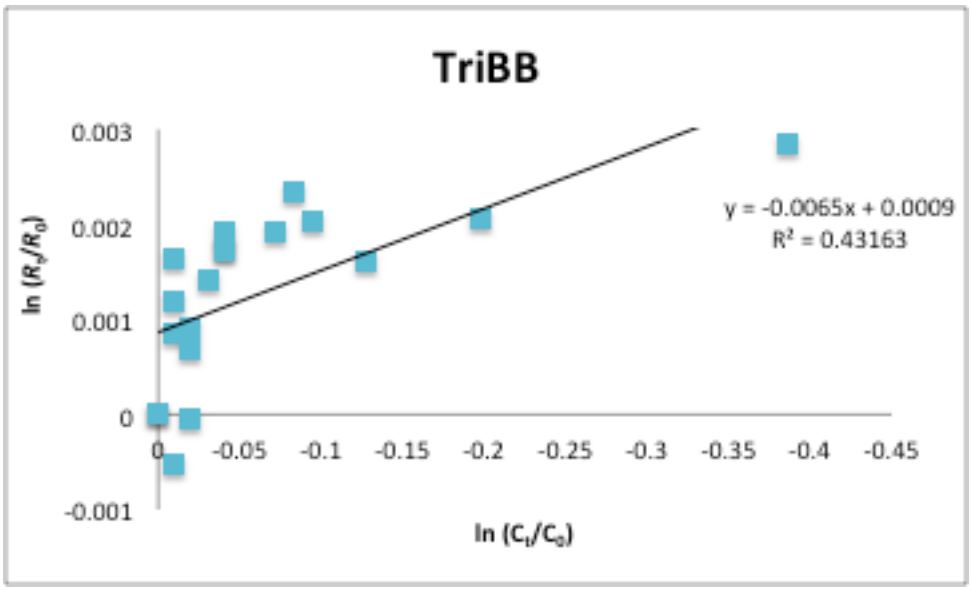


Figure 3-8. A double logarithmic plot for TriBB in TriBB debrominating cultures according to Rayleigh equation of the isotopic composition versus the residual concentration of substrate.

Discussion

In this study, carbon stable isotope signatures were obtained for the reductive dehalogenation of chlorinated benzenes (hexachlorobenzene (HCB); pentachlorobenzene (PeCB); or tetrachlorobenzene (TeCB)) and brominated benzenes (tetrabromobenzene (TeBB); or tribromobenzene (TriBB)). Carbon compound specific stable isotope analysis (CSIA) has been proved as an effective tool to evaluate *in situ* biodegradation in many studies (for review, see Meckenstock et al., 2004). CSIA for chlorobenzenes have been reported as well, but the studies have been limited to monochlorobenzene, dichlorobenzene, and trichlorobenzene (TriCB; Griebler et al., 2004, Braeckevelt et al., 2007, and Liang et al., 2014). This is thus the first attempt of CSIA for highly chlorinated and brominated benzenes.

CSIA of HCB showed depletion in the $\delta^{13}C$ value of the dichlorobenzene (DCB) dechlorination product (Figure 3-2). There are some studies representing $\delta^{13}C$ depletion of product. Hunkeler et al. (1999) conducted CSIA for microcosm and field study of groundwater, and they observed the $\delta^{13}C$ depletion of dechlorination products in tetrachloroethene (PCE) dechlorination. Ewald et al. (2007) analyzed carbon isotope composition of trichlorinated dibenzo-*p*-dioxins during reductive dehalogenation by an anaerobic mixed culture. The dehalogenation product, monochlorinated dibenzo-*p*-dioxins (MCDD), showed a significant depletion in ^{13}C.

In CSIA data from 2014, two clear changes of isotope fractionation were observed. One is from trichlorobenzene (TriCB) in TeCB cultures, and the other is from TriBB in TriBB

cultures. First, the $\delta^{13}C$ value for TriCB showed sequential decrease and increase as the TeCB dechlorination proceeded (Figure 3-5). It can be explained by the role of TriCB in TeCB dechlorinating cultures. As an intermediate of TeCB dechlorination, TriCB plays two roles: as a product of TeCB dechlorination; and as a substrate of TriCB dechlorination to release less chlorinated benzene. In the initial stage of TeCB dechlorination, $\delta^{13}C$ depletion for TriCB occurred, reflecting the accumulation of ^{12}C in the product TriCB as a result of TeCB dechlorination. In the latter stage, TriCB is a substrate for dechlorination and the $\delta^{13}C$ value was increased. Liang et al. (2013) reported the same tendency in the $\delta^{13}C$ for benzene during degradation of monochlorobenzene (MCB). $\delta^{13}C$ values for benzene decreased until 45 % of the MCB was degraded. As both MCB and benzene were further degraded, the $\delta^{13}C$ values of benzene became enriched. Second, the $\delta^{13}C$ value for TriBB in TriBB cultures became more enriched as debromination advanced (Figure 3-7). This suggests that TriBB debromination is a result of biological activities. Kaschl et al. (2005) reported the increase of $\delta^{13}C$ for MCB during anaerobic MCB degradation. Liang et al. (2014) showed $\delta^{13}C$ value for DCB became enriched during anaerobic DCB degradation.

The isotope enrichment factor (ε) was calculated to describe the debromination of TriBB (Figure 3-8), yielding an ε value for TriBB debromination of -6.5 ‰ ($r^2 = 0.43$). The low r^2 value suggested that Rayleigh model is not suitable for carbon isotope fractionation during reductive dehalogenation of TriBB. Since there was no reference of ε values for brominated benzenes, ε values reported for chlorinated benzenes are compared instead. Griebler et al. (2004) reported carbon isotope enrichment factor (ε) for TriCB

dechlorination. The values are between -3.1 and -3.7 ‰ for 1,2,3-TriCB and 1,2,4-TriCB during anaerobic dechlorination by *Dehalococcoides* sp. strain CBDB1. Liang et al. (2011) reported ε values for MCB and 1,2,4-TriCB during anaerobic degradation: -5.0 ± 0.2 ‰; and -3.0 ± 0.4, respectively. Their follow-up report contained ε values for 1,2-DCB, 1,3-DCB, and 1,4-DCB during anaerobic degradation: -0.8 ± 0.1 ‰; -5.4 ± 0.4 ‰; and -6.3 ± 0.2 ‰, respectively (Liang et al., 2014). Our ε value for TriBB was more agreeable with the ε value for DCB or MCB than the ε value for TriCB. However, the low r^2 value is still an obstacle to apply our ε values for comparison.

For dehalogenation intermediates, it is difficult to observe isotope enrichment or depletion since the intermediates have both a role as a substrate and a product. Thus, Stelzer et al. (2009) proposed a concept of isotope balance to interpret CSIA during chlorobenzene biodegradation. Sequential dehalogenation of chlorinated benzenes complicates the evaluation with the Rayleigh model because the isotope signature of single chlorobenzene species is affected by both simultaneous depletion and enrichment of ^{13}C. The isotope balance can be calculated by following equation: $\delta^{13}C_{CB}[‰] = \Sigma(C_i \times \delta^{13}C_i) / C_{CB}$, where $\delta^{13}C_{CB}$ is the isotope signature of the total CBs, C_i is the molar concentration of each compound, $\delta^{13}C_i$ is the respective carbon isotope signature of each compound, and C_{CB} is the total molar concentration of all chlorinated benzenes. With isotope balance, *in situ* biodegradation was determined by the enrichment of the cumulative isotope composition of all chlorobenzenes. They used this concept to interpret CSIA of MCB and confirm biodegradation of MCB in both laboratory and field.

Theoretically, the substrate of the reaction shows $\delta^{13}C$ value enrichment; the product of the reaction shows $\delta^{13}C$ value depletion; and the intermediates show a sequential depletion and enrichment of the $\delta^{13}C$ value. Not all of our CSIA data were agreeable with the theory. There are some possible reasons to explain this. A low rate of reaction, a high hydrophobicity and low solubility of substrate can cause low isotope fractionation (Renpenning et al., 2015).

From this chapter, CSIA data were obtained for halogenated benzenes including HCB, PeCB, TeCB, TeBB, and TriBB with anaerobic cultures originated from the Hackensack River sediment. We could observe the evidence of carbon isotope fractionation suggesting *in situ* microbial reductive dehalogenation even though the little changes of $\delta^{13}C$ value were detected.

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CHAPTER 4

Discussion

In this dissertation, anaerobic dehalogenation by indigenous microbes was observed in microcosms set up with sediments from the Hackensack River, New Jersey. For laboratory experiments, sediments were collected from five different sites along the Hackensack River, designated H1 ~ H5. Microcosms were set up to study dehalogenation of halogenated aromatics, including hexachlorobenzene (HCB), hexabromobenzene (HBB), and pentachloroaniline (PCA). Dehalogenation of HCB and PCA by indigenous microorganisms was detected in sediments at all of the locations. The freshwater site H1 sediment showed a more rapid initial start of dechlorination, while the estuarine site H5 sediment showed a delayed onset of dechlorination. The increase in sulfate concentration and salinity may explain the different rates of dehalogenation between the sites.

The intermediates and the products during reductive dehalogenation of HCB and PCA were detected by GC-MS and dechlorination pathways were deduced. HCB was dechlorinated to final dechlorinating products 1,3- and 1,4-dichlorobenzene (DCB) via 1,2,3,5-tetrachlorobenzene (TeCB) and 1,3,5-trichlorobenzene (TriCB). 1,2,3,5- TeCB was assumed as a TeCB intermediate after reasonable inference based on the detected production of 1,3,5-TriCB. A PCA dechlorination pathway was constructed based on observed intermediates. Additional intermediates were postulated to fill gaps in detected intermediates to complete whole dechlorination process. Overall, PCA was sequentially dechlorinated to 2,3,4,6-tetrachloroaniline (TeCA), 2,4,5-trichloroaniline (TriCA), 2,4-

and 2,5-dichloroaniline (DCA) and 3-chloroaniline (CA) or to 2,3,5,6-TeCA, 2,3,5-TriCA, 3,5- and 2,5-DCA, and 3-CA. 2,3,4,6-TeCA from PCA was also dechlorinated to 2,4,6-TriCA, and finally to 2,4- and 2,6-DCA. In contrast to the rapid dechlorination of HCB and PCA, there was no observed HBB debromination after up to approximately 12 months of incubation. However, when both HBB and HCB were amended to previously established HCB dechlorinating cultures, HBB debromination was stimulated and 1,2,3,5-tetrabromobenzene (TeBB) and 1,3,5-tribromobenzene (TriBB) were detected at low levels as intermediates.

Molecular community analyses were conducted for H1 and H5 cultures amended with HCB or PCA and for electron donor controls. Analysis of the dehalogenating bacterial communities using 16S rRNA gene PCR-DGGE and sequence analysis demonstrated that *Chloroflexi* from the so-called Pinellas subgroup of *Dehalococcoides mccartyi* were enriched in the H1 cultures with both electron acceptors. This result of enrichment of *Dehalococcoides* spp. is consistent with previous work suggesting that members of the Pinellas subgroup of *Dehalococcoides* are ubiquitous and enriched by different halogenated aromatic compounds (Park et al., 2011; Liu et al., 2014). Additional PCR assays showed that there neither *Dehalobacter* nor *Desulfitobacterium* could be detected in the cultures. When twelve reductive dehalogenase (*rdh*) gene-amplifying primers were tested, H1 sediment had a more diverse *rdh* gene profile than H5 sediment. Ten *rdh* genes were clearly enriched in H1 cultures amended with HCB or PCA, while only 1 ~ 3 *rdh* genes were detected in H5 sediment. It suggested that H5 sediment had different enriched *rdh* gene profile from H1 since H5 sediment showed dehalogenation activity as well. All

12 *rdh* genes were detected in cultures amended with both HBB and HCB suggesting and additional 2 *rdh* genes (rdh 4 and rdh 5) might be enriched by the combination of HCB and HBB. Previously, these 12 primer sets were also used to analyze *rdh* gene profile in different dehalogenating conditions (Park et al., 2011; Liu et al., 2014). In Anacostia River sediment, rdh 4 and rdh 5 were only detected in PCNB with or without bioaugmentation and bioaugmented cultures. In Kymijoki River sediment, rdh 9 was clearly enriched only in 1,2,3,4-TeCDD plus PCNB while the remaining 11 *rdh* genes were enriched in other treatments including 1,2,3,4-TeCDD plus PCNB without specificity.

Carbon compound specific isotope analysis (CSIA) of dehalogenating cultures was conducted to evaluate whether it could be used to assess *in situ* reductive dehalogenation of highly halogenated benzenes. Cultures were set up for HCB, pentachlorobenzene (PeCB), 1,2,3,5-TeCB, 1,2,3,5-TeBB, and 1,3,5-TriBB. Although the results are not conclusive, likely due to poor solubility of the substrates resulting in mass transfer limitations and due to isotopic dilution (only 1 carbon of 6 involved in the reaction), it clearly showed isotope fractionation during HCB dechlorination as observed in the depleted of $\delta^{13}C$ values of the DCB dehalogenation product. CSIA of TeCB dechlorinating cultures showed sequential depletion and enrichment of the $\delta^{13}C$ value for the TriCB intermediate. The enrichment of $\delta^{13}C$ in the residual TriBB of debrominating cultures was detected, as well. The isotopic enrichment factor (ε) was calculated for TriBB, but limited fractionation and large variation inhibit its application.

Dehalogenating bacterial communities are ubiquitous in the environment. Therefore, bioaugmentation is typically not necessary for bioremediation, but instead one of the key goals is to stimulate indigenous dehalogenating communities. Table 1-3 and 1-4 demonstrate that there are numerous indigenous dehalogenating bacteria in the environment. Oliver and Nicol (1982) represented that the ratio of less chlorinated benzenes to highly chlorinated benzenes increased with sediment depth. There are several reports of the dechlorination of weathered PCBs by indigenous dehalogenating microorganisms in the environment (e.g., Brown Jr. et al., 1987; Tiedje et al., 1993; Fava et al., 2003; Krumins et al., 2009). Continuing studies represented that the anaerobic bacteria of the phylum *Chloroflexi* including *Dehalococcoides* and more distant taxons are responsible for PCBs dechlorination (Wu et al., 2000; Fagervold et al., 2007; Park et al., 2011; Zanaroli et al., 2012). Indigenous bacteria are responsible for HCB dechlorination at contaminated sites (Holliger et al., 1992; Prytula and Pavlostathis, 1996). These studies clearly show that microbial communities are responsible for dehalogenation of different organohalide contaminants. Therefore, we can expect to observe diverse dehalogenating activities by diverse microbial communities in different environmental samples.

Molecular community analyses by PCR-DGGE and reductive dehalogenase gene profile analysis of H1 and H5 cultures showed different microbial communities enriched in each site (Chapter 2). Even though dehalogenating activities were detected for HCB or PCA in both H1 and H5 cultures, their *rdh* gene profile did not look similar. H1 cultures had more *rdh* genes enriched than H5 cultures. It suggested that there were different

dehalogenating populations bearing different *rdh* genes enriched in H5 cultures. The suite of *rdh* genes might not be detected by our 12 tested primer sets. Furthermore, the consistent result of the enrichment of 16S rRNA gene of *Dehalococcoides* and the enrichment of *rdh* genes detected by primer sets designed from *Dehalococcoides mccartyi* strain 195 and strain CBDB1 in H1 cultures suggested that *Dehalococcoides* species was one of the dehalogenators responsible for the fast onset of dechlorination in H1 cultures. The enrichment of members of the Pinellas subgroup of *Dehalococcoides,* which includes *Dehalococcoides mccartyi* strain CBDB1 as a member, has been observed in organohalide-amended sediments from many different geographical locations (Figure 2-9; Park et al., 2011; Liu et al., 2014).

HCB dechlorination was observed in cultures containing sediment from the Hackensack River with several intermediates and the final dechlorination product was DCB in this study (Chapter 2). For complete remediation, DCB needs to be mineralized to carbon dioxide and methane. Generally, a combination of anaerobic-aerobic techniques has been used to obtain complete detoxification of halogenated organics (Beeman and Bleckmann, 2002; Master et al., 2002; Van der Zee and Villaverde, 2005). This approach has been proved feasible, but application of the treatment is limited because of relatively high energy consumption and cost of aerobic treatment. To overcome limitation, combining two different enrichment cultures to obtain a complete mineralization of chlorinated compounds under anaerobic conditions had been introduced. Yang et al. (2009) showed the potential for complete anaerobic detoxification. They combined an anaerobic pentachlorophenol (PCP) dechlorinating enrichment culture and an anaerobic phenol

degrading enrichment culture and obtained complete mineralization of PCP under both iron-reducing and sulfate-reducing conditions. PCP dechlorination and phenol degradation occurred both simultaneously and sequentially. Liang et al. (2013) combined chlorobenzene dechlorinating enriched culture and benzene degrading culture to complete detoxification of chlorobenzene. Active chlorobenzene dechlorinating microcosms were inoculated with active methanogenic benzene degrading culture. It resulted in the transformation of chlorobenzene to carbon dioxide and methane via benzene. Sustainable degradation of chlorobenzene was confirmed by carbon isotope fractionation. They observed the values of $\delta^{13}C$ for monochlorobenzene (MCB) enriched as biodegradation proceeded. $\delta^{13}C$ values for benzene decreased first and then enriched during the time reflecting a dual role of benzene as the product of MCB dechlorination and as the substrate for further degradation.

In Chapter 2, we observed that HCB dechlorinating cultures stimulated debromination of HBB, which was persistent in cultures amended with HBB only (Figure 2-10). It is not clear whether this stimulation was due to the growth of a debrminating community and/or the induction of the perquisite dehalogenases. Morris et al. (1992) suggested that different microbial strains were selected for dehalogenation of polybrominated biphenyls (PBB) and PCBs. When PBB debromination by anaerobic microorganisms was tested in microcosms derived from PBB contaminated and PCB contaminated sediments, PBB debromination was observed in all cultures but with different extents of debromination. PBB contaminated sediments had a higher debrominating activity than PCB contaminated ones. Different extent of debromination suggested that previous exposure

to PBBs enhances the debromination capability of the sediment microbial community through selection for different strains of microorganisms. This result suggested one possible clue why our HBB cultures did not show debromination, while HCB cultures showed dechlorination though they were originated from same sites. The chlorinated contaminants in the Hackensack River sediments (Table 2-1) may enrich for dehalogenators specifically selected for chlorinated compounds, while no dehalogenators for brominated compounds were enriched at the sites.

Fathepure et al. (1988) suggested a similarity between HCB dechlorination and HBB debromination, since HBB had intermediates consistent with HCB dechlorination, including 1,3,5-TriBB, 1,2,4,5-TeBB, and pentabromobenzene (PeBB). Therefore, it was reasonable to postulate intermediates of HBB debromination based on the data from HCB dechlorination. In Chapter 3, 1,2,3,5-TeBB and 1,3,5-TriBB were tested as possible dehalogenating substrates since we observed corresponding congeners of chlorinated benzenes from HCB dechlorinating cultures. The results demonstrated the debrominating activity for 1,2,3,5-TeBB and 1,3,5-TriBB in Hackensack River sediments. These brominated benzene intermediates were confirmed by cultures amended with both HCB and HBB.

In Chapter 3, using CSIA we could detect depletion of the $\delta^{13}C$ value in the DCB product from dechlorination of HCB; the sequential depletion and enrichment of $\delta^{13}C$ value for TriCB in TeCB dechlorinating cultures; the enrichment of $\delta^{13}C$ during debromination of TriBB. CSIA analyses did not provide strong evidence of isotope fractionation coupled to

dehalogenation, possibly due to low solubility and dilution of the isotope signal. The hydrophobicity of halogenated benzenes inhibits dissolution of substrates in water. According to the CSIA study for tetrachloroethene (PCE) by Nijenhuis et al. (2005) with reduced cyanocobalamin and bacterial strains, the largest isotopic fractionation was observed during the reaction with cyanocobalamin. For bacteria, the isotope fractionation of PCE was increased as the cell integrity was decreased. It suggested the inhibition effect of cell components on isotope fractionation. In follow-up study, Renpenning et al. (2015) concluded that masking of the isotope fractionation was due to hydrophobicity of the substrate, properties of the cell envelope, and the location of the reacting enzyme.

Overall, we conclude that indigenous microorganisms in the Hackensack River, NJ have dehalogenating activities for HCB and PCA. There are different dehalogenating populations enriched from H1 and H5 sediments. *Dehalococcoides* species are one of the responsible bacteria for the faster onset of dehalogenation in H1 sediment. The priming effect of HCB on HBB dehalogenation was observed. CSIA data for highly halogenated benzenes suggested *in situ* microbial dehalogenation.

These results suggest that dehalogenation of halogenated aromatic contaminants may be mediated by indigenous microbial populations as a means for *in situ* bioremediation of sediments contaminated with halogenated compounds. The microcosm experiments allow us to conclude that dehalogenation of halogenated aromatics in Hackensack River sediments by indigenous microorganism was occurring and can be stimulated by halogenated co-substrates. The results from microcosm experiments and CSIA analyses

suggest the potential for *in situ* microbial reductive dehalogenation of highly halogenated benzenes in Hackensack River sediments. Further characterization of the microbial communities and *rdh* gene profiles involved in dehalogenation process and conclusive isotopic fractionation data will aid in the assessment of contamination by persistent halogenated compounds and *in situ* bioremediation.

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Appendix I

Microbial Transformation of Pentachloronitrobenzene (PCNB) and 1,2,3,4-Tetrachlorobenzene (TeCB) via Reductive Dehalogenation

Sediment samples from Anacostia River, Washington, D.C. were used to establish anaerobic microcosms (Krumins et al., 2009 and Park et al., 2011). Microcosms were established with 20 % sediment slurries with 20/50 μM PCNB/1,2,3,4-TeCB in methanogenic media as previously described. The original microcosms were maintained and substrates were respiked when initial substrates were completely transformed to dehalogenation products. Every 4~5 rounds of dehalogenation, a small portion of the cultures were transferred and diluted to 1/5 in fresh medium and amended with electron acceptor and 100 μM mix of butyrate and propionate as electron donors. The original and a subset of transfer cultures were maintained under dechlorinating conditions. Culture protocols and analytical methods were as described in Chapter 2.

Microbial transformation of PCNB via reductive dehalogenation

Five enriched cultures had activity to transform PCNB to PCA and further dehalogenating products among 8 cultures (Table AI-1). Chemical analyses by GC-MS represented that PCNB dechlorination in diluted cultures were maintained stable after 8 rounds of dechlorination. PCNB was completely transformed to PCA or to dehalogenating products in 4 weeks. In detail, PCNB was transformed to pentachloroaniline (PCA) and PCA was dechlorinated to 2,3,5,6-tetrachloroaniline

(TeCA), 2,4,6-trichloroaniline (TriCA), and to 2,4-/2,5- and 3,5-dichloroaniline (DCA) (Figure AI-1). 2,4- and 2,5-DCA had the same retention times and could not be distinguished. These dechlorination products and intermediates were determined based on their standards' retention times on GC-MS. There were one TeCA and one TriCA intermediate peak detected, but these did not match available standards. 2,3,4,6-TeCA and 2,3,5-TriCA were assumed to make a dechlorination pathway complete.

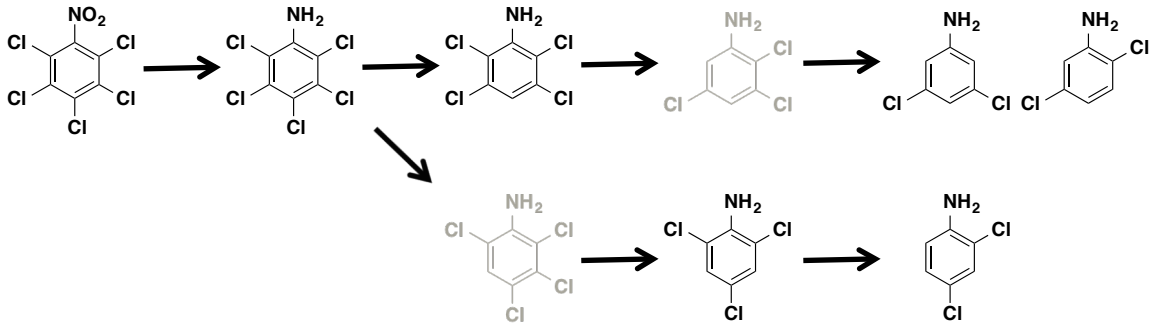
Reductive dehalogenation of TeCB

A total of 8 enriched cultures were maintained for TeCB dechlorination and most of these cultures were successfully maintained for dehalogenating activity over several rounds of transfer (Table AI-1). TeCB dechlorination in diluted cultures were stabilized after 4 rounds of dechlorination. TeCB was completely dechlorinated to less chlorinated products in 4 weeks. In detail, 1,2,3,4-TeCB was dechlorinated to 1,2,4-Trichlorobenzene (TriCB) and to 1,3-Dichlorobenzene (DCB) and 1,4-DCB (Figure AI-1).

Table AI-1. Diluted cultures enriched with PCNB and TeCB

				PCNB				
# of spikes before transfer	4th	4th	4th	8th	8th	12th	13th	13th
% of sediment	4	4	2	4	2	2	4	4
Concentration of substrate (µM)	20	50	50	20	20	20	20	50
				TeCB				
# of spikes before transfer	4th	4th	5th	5th	8th	9th	8th*	9th*
% of sediment	4	4	4	4	4	4	0.8	0.8
Concentration of substrate (µM)	20	50	20	50	20	20	20	20

(A) PCNB



(B) TeCB

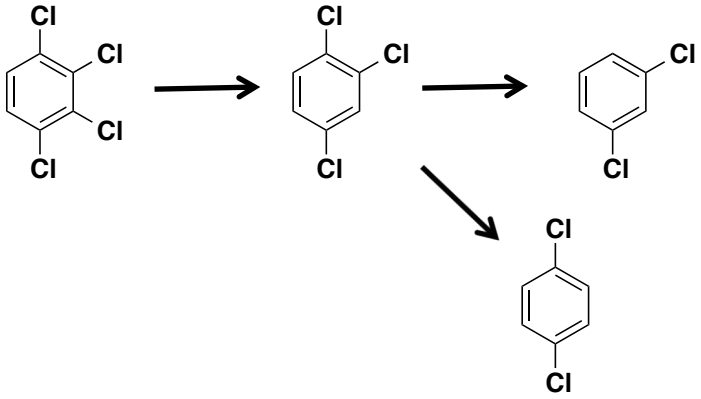


Figure AI-1. Suggested microbial transformation pathways for PCNB and TeCB. Gray-colored intermediates were proposed to make the pathway complete.

Appendix II

Sequence data of excised bands from PCR-DGGE

ED control (1)*

GAAAGCCTGACGCAGCGACTCCGCGTGGGGGATGAAGGCCttCGGGTTGTAtA
CCCCTTTTCCCGAGGAAGAATTATGAcGGTACCCGcGGAATAAGTCTtGGCTAA
CTACGTGCCAGCAGCCGCGGTAAta

ED control (3)*

GCAAGCCTGATGCAGCAACGCCGCGTGAGGGACGAAGGCCTTCGGGTCGTA
AACCTCTTTTCTGAGGGACGAGAAAGGACGGTACCTCAGGAATAAGTCTCGG
CTAACTACGTGCCAGCAGCCGCGGTAATa

HCB (1)*

CGAAAGCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCTTTCGGGTTGTA
AACCTCTTTTCATAGGGAAGAATAATGACGGTACCTGTGGAATAAGCTTCGG
CTAACTACGTGCCAGCAGCCGCGGTAATa

PCA (1)*

GCAATGGGCGAAAGCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCTTT
CGGGTTGTAAACCTCTTTTCATAGGGAAGAATAATGACGGTACCTGTGGAAT
AAGCTTCGGCTAACTACGTGCCAGCAGCCGCGGTAATaa

Appendix III

C isotope composition for extraction, evaporation, and Florisil clean-up tests

1. C isotope composition for extraction and evaporation tests.

	Cal		Extr 1		Extr 2		Extr 3		Evap 1		Evap 2		Evap 3	
	Ave	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
DiCB	-28.08	0.15							-28.57	0.55	-29.39	0.08	-29.38	0.04
TrCB	-28.64	0.12	-28.27	0.50	-28.78	0.22			-29.28	0.58	-30.09	0.16	-30.43	0.13
TeCB	-31.93	0.18	-31.75	0.11	-31.68	0.26	-31.51	0.08	-32.10	0.51	-32.85	0.10	-33.20	0.10
PeCB	-26.31	0.15	-26.13	0.22	-25.80	0.10	-25.78	0.25	-26.17	0.05	-26.34	0.11	-26.66	0.16
HxCB	-26.32	0.22	-26.09	0.13	-25.67	0.13	-25.39	0.37	-25.57	0.12	-25.85	0.07	-26.14	0.15

Cal, calibration mix; Extr, extraction control; Evap, evaporation control

2. C isotope composition for Florisil clean-up tests.

	Evap 1	Evap 1_Fl	Evap 2_Fl	Extr 1	Extr 1	Extr 1_Fl	Extr 1_Fl
DiCB	-27.76	-27.49	-27.69				
TrCB	-28.79	-27.87	-27.95				
TeCB	-32.83	-31.37	-31.54	-31.64	-31.78	-31.38	-31.37
PeCB	-26.28	-25.98	-25.85	-26.13	-25.98	-25.70	-25.58
HxCB	-26.01	-25.88	-25.99	-25.61	-25.69	-25.42	-25.49

Fl, with Florisil clean-up