REDUCTIVE DECHLORINATION OF POLYCHLORINATED DIBENZO-*P*-DIOXINS

BY *DEHALOCOCCOIDES*-ENRICHED CULTURES

FROM CONTAMINATED SOILS AND SEDIMENTS

By

HANG THUY DAM

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ABTRACT OF THE DISSERTATION

Reductive Dechlorination of Polychlorinated Dibenzo-*p*-dioxins

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By HANG THUY DAM

Dissertation Director

Max M. Häggblom

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are pervasive environmental pollutants and resistant in soil and sediment environments. Under anaerobic conditions, PCDDs can undergo reductive dechlorination which produces less chlorinated dibenzo-*p*-dioxin congeners, these compounds are more susceptible to further degradation. The process is mediated by dehalogenating bacteria, some of which gain energy from the process by using PCDDs as terminal electron acceptors. The objective of our study was to use a conventional enrichment approach to enrich for dehalogenating bacteria, to investigate the PCDD dechlorination activity and the microbial community of enrichment cultures, and to investigate genomes of the abundant dehalogenating bacteria in enrichment cultures from metagenomics data of the corresponding enrichment cultures.

PCDD dechlorination was observed in five sets of enrichment cultures established from Hackensack River sediments collected along the tidally influenced river, in which dechlorination activity was negatively affected by high salinity and high sulfate concentrations. Dechlorination activity was also recorded in enrichment cultures established from freshwater sediments of the Kymijoki River (Findland), a water reservoir (Vietnam), as well as from rice paddy soil (Vietnam). Reductive dechlorination of PCDDs was mediated by organohalide-respiring bacteria. PCDD dechlorinating anaerobic bacteria appeared to be ubiquitous, and their activity was observed in

almost all enrichment cultures established using soils and sediments collected from sites of different contamination backgrounds. *Dehalococcoides* spp. were identified as the main bacterial species that couple dechlorination of PCDDs to growth.

Genomes of two *Dehalococcoides* spp. were reconstructed from metagenomes of the Hackensack and the Kymijoki River enrichment cultures in which they were present at relatively high abundance. Comparative genome analysis suggested that a reductive dehalogenase *cbrA* ortholog is responsible for reductive dechlorination of 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin in a respiratory process. Genome analysis provided preliminary evidence for the function of reductive dehalogenases in PCDD dechlorination which have not been studied before due to the complex nutritional requirements and slow growth of *Dehalococcoides* spp.

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Chapter 1

Introduction

Abstract

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are ubiquitous pollutants and resistant to biodegradation in soils and sediment environments. They bioaccumulate and biomagnify through the food chain, causing health problems in humans and animals. The binding of dioxin compounds to the Aryl Hydrocarbon Receptor upregulates expression of genes that are involved in aromatic metabolisms, and triggers toxic responses in mammalian cells. Even though the production of PCDDs increased in the 1960s and has since reduced over the past five decades, PCDDs are still present in various contaminated sites. Microbial processes that can transform PCDDs include aerobic degradation and mineralization of less chlorinated compounds, enzymatic oxidation and ring cleavage by lignin peroxidase or manganese peroxidase, or reductive dechlorination of highly chlorinated dibenzo-*p*-dioxins. PCDDs are extremely hydrophobic and have the tendency to absorb onto organic matter in highly reduced environments such as sediments. These conditions favor the growth of a specialized group of microorganisms which can remove chlorines from PCDDs by respiratory reductive dechlorination. Although microorganisms that are able to perform reductive dechlorination are phylogenetically diverse, only members of *Dehalococcoides* and *Dehalobacter* have been shown to grow on PCDDs as terminal electron acceptors. Reductive dehalogenases (RdhA) are terminal reductases which catalyze the last step of electron transfer to chlorinated compounds to yield energy for growth of dehalogenating bacteria. Reductive dehalogenase genes (*rdhA*) are usually found in cluster with *rdhBs* which encode for small and highly hydrophobic proteins and presumably serve as anchoring protein to RdhA on the cell membrane. The majority of *rdhAB* gene clusters are located on High Plasticity Regions (HPRs) or

sandwiched by mobile gene elements, indicating that horizontal gene transfer is the major means of *rdhAB* acquisition and evolution. Reductive dehalogenase genes are showed to be tightly regulated at the transcriptional level.

Chemistry and toxicology of PCDDs

PCDDs refer to a group of chlorinated aromatic compounds that are made of a dibenzo-1,4-dioxin skeleton (Figure 1.1). There can be up to eight chlorines in a PCDD molecule which makes the compound complex in structure, highly oxidized, and resistant to biological attack. There are 75 congeners of PCDDs, their physical and chemical properties, therefore, vary in a wide range (Table 1.1). Overall, PCDDs are poorly soluble or volatile. Their high octanol-water partition coefficients indicate their tendency to absorb onto organic matter and their potential of bioaccumulation in biological systems (Shiu *et al*., 1988). PCDDs have been shown to accumulate in fatty tissues as well as in other organs in edible marine organisms as well as terrestrial animals (Stephens *et al*., 1995; Bonn, 1998). The bioaccumulation factor is dependent on the number and position of chlorine in the PCDD molecule. Tetra- and penta-CDDs were observed to accumulate more strongly than higher chlorinated congeners in fatty tissues of Atlantic salmon. This poses more problematic issues to human's health when salmon are consumed as food, since the large fraction of PCDDs produced in industry have 4 chlorines and are more toxic than higher chlorinated dioxins produced in natural processes such as forest fires (Isosaari *et al*., 2004). In the environment, PCDDs are usually co-produced and co-exist with polychlorinated dibenzofurans (PCDFs) which have only one ether bond in their molecules. The chemical properties as well as toxicity of these two chemical groups are similar and they are often referred to as PCDD/Fs in this review as well as in the literature.

PCDDs cause toxicity via the Aryl Hydrocarbon Receptor (Ahr) mediated signaling pathway (for review see Mandal, 2005). Figure 1.2 illustrates the toxicity mechanism in mammalian cells. PCDDs entering the cell cause dissolution of a complex of Ahr, 2 heat shock proteins and a chaperone protein called XAP2. PCDDs then bind to Ahr, translocate into the nucleus, and bind to the Aryl Hydrocarbon Receptor Nuclear Translocator protein (Arnt). The heteromer binds to the Dioxin Responsive Element (DRE) located upstream of the promoter that regulates expression of genes involved in xenobiotic metabolisms. An example is Ahr-dependent P450 cytochrome 1A1. PCDDs are not metabolized in mammalian cells, but they regulate gene expression of processes that are involved in production of metabolites. These metabolites interact and alter cellular macromolecules, which leads to the alteration of regular cell functions. PCDDs cause various adverse effects on human and animal such as endocrine disruption, immune damage, nervous system degeneration, reproductive toxicity, sexual development, and controversially cancer (for review, see Mandal, 2005). Toxicity of PCDDs differs among different test animals. LD50 via oral route is 0.6 μg/kg body weight of guinea pigs and is more than 5000 μg/kg in hamsters (Schwetz *et al*., 1973; Poland & Knutson, 1982).

PCDDs are present in the environment and living organisms as mixtures. Each congener contributes to the toxicity of the mixture differently. Therefore, concentrations of PCDDs are presented taking into consideration the toxicity they contribute. Toxic equivalents (TEQs) were developed by World Health Organization (WHO). Each PCDD congener is assigned a toxic equivalency factor (TEF) in which the most toxic congener, i.e., 2,3,7,8-TeCDD, is designated a TEF of 1 and others have TEFs of fractions of 1. PCDD levels in the environment and biota is presented in TEQ and is the sum of each congener's mass concentration multiplied by its toxic equivalency factor (van den Berg *et al*., 1998, 2006).

PCDDs in the environments

Anthropogenic sources of PCDDs

PCDDs have never been intentionally produced, except for research purposes. They were often produced as by-products of many industrial activities, and disposal of PCDDs into the environment has caused heavy contamination in some former industrial zones all over the world. Table 1.2 summarizes some "hot spots" of PCDD pollution and their sources of contamination.

PCDDs are impurities produced during chemical synthesis of chlorinated phenols such as tri-, tetra-, and pentachlorophenols. Ring condensation between the benzene rings of two trichlorophenols at high temperature leads to the formation of 2,3,7,8-TeCDD (Figure 1.3). The production and utilization of chlorinated compounds as herbicides and wood preservatives in large quantities during the 1950s to 1970s caused many historical PCDD contaminations in Europe and North America. In many cases, factories that manufactured chlorinated compounds were located near rivers within highly populated areas. The release of PCDDs into the rivers often resulted in contamination of the water column and deposition of contaminants in sediments. For example, Kymijoki River (Finland), Bitterfeld industrial region (Germany), and Passaic River (USA) became heavily contaminated with PCDDs from production of organochlorine based herbicides and wood preservatives (Bopp *et al*., 1991; Götz *et al*., 1994, 2007; Verta *et al*., 2009).

Application of chlorophenol-based herbicides, pesticides, or wood preservatives resulted in contamination of PCDDs in larger areas. Soils in Southern Vietnam became contaminated with PCDDs as impurities of Agent Orange (2,4,5-Trichlorophenoxyacetic acid containing herbicide) during the Vietnam War. A total quantity of 366 kg 2,3,7,8-TeCDD was estimated to be sprayed from 1961 to 1971 (Stellman *et al*., 2003). Rice paddy soils in Japan received PCDDs due to utilization of pentachlorophenol and chlornitrofen in agriculture, which resulted in accumulation of PCDDs at 1.3 – 435 ng TEQ/kg dry weight (Seike *et al*., 2007).

Industrial thermal processes, such as combustion of municipal, hazardous, and medical waste, as well as metallurgy and other anthropogenic activities, contribute greatly to the emission of PCDDs into the environment. Municipal waste incineration is the primary source of PCDD/Fs released into the environment in Japan since the start of applying this method in municipal waste treatment by which 80% of waste in this country is incinerated (Takeda & Takaoka, 2013). Soils outside of a municipal waste incineration facility in Nose, Osaka, Japan were contaminated with PCDDs at an average concentration of 8,500 ng TEQ/kg dw, and up to 52,000,000 ng TEQ/kg dw (Kishida *et al*., 2010). Followed the improvement in waste incineration technology, PCDDs emitted from this waste treatment method has reduced significantly since the 1990s (Takeda and Takaoka, 2013). A magnesium production plant was the point source of PCDD contamination in Frierfjord, Norway with up to 50 kg 2,3,7,8-TeCDD equivalents being accumulated over the course of 30 years (Knutzen & Oehme, 1989). Metallurgy introduced a significant amount of PCDD/Fs into the environments in Korea. Approximately 35 g I-TEQ PCDD/Fs were released annually from the four major metal production in this country, i.e., copper, lead, aluminum, and zinc (Yu *et al*., 2006). In China, metallurgy contributes the largest portion (46%) of the total PCDDs produced (Liu G *et al*., 2013). Other industrial processes in which chlorine or oxidized species of chlorines are used also contribute to the emission of PCDDs in the environment. Bleaching of pulp and paper as well as disinfection of sewage waste water results in the formation of PCDD/Fs when chlorine reacts with phenolic compounds that are often present in wood pulp and sewage water (LaFleur *et al*., 1990). Non-industrial combustions from sources such as diesel vehicles, open burning of agriculture waste and residential yard burning also emit PCDDs into the atmosphere (Booth *et al*., 2013).

Due to improvements of incineration technologies and other dioxin releasing industrial processes, the release of PCDDs into the environment has decreased since the 1970s. However, triclosan-derived PCDDs (2,8-DiCDD, 1,2,8-TriCDD, and 2,3,7-TriCDD) were found to increase

(Buth *et al*., 2010). PCDD profiles in sediment cores from waste water impacted part of the Mississippi River revealed that profiles of the three PCDD congeners matched with triclosan usage in the area since the 1970s and did not correlate with PCDD profiles from industry and incineration (Buth *et al*., 2010). Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a commonly used and controversial antimicrobial agent which was first synthesized in the 1960s and employed shortly after that in hygiene products (Latch *et al*., 2003). Triclosan containing products are discharged into waste water stream. Photolysis of triclosan in surface water leads to total loss of triclosan, mostly by formation of triclosan-derived polymers. However, this process also causes the formation of radical O^- followed by ring closure and eventually forms 2,8-DiCDD (Figure 1.4; Latch *et al*., 2003). In the bottom layer of a water column of more than 10 meters with elevated concentration of dissolved organic matters, indirect photolysis of triclosan appears to play the major role in PCDD formation. Indirect photolysis of triclosan in subsurface water accounts for up to 90% triclosan-derived PCDDs (Bianco *et al*., 2015). Chlorination of triclosan can occur when triclosan is exposed to residual chlorine in tap water or when chlorine is used to disinfect wastewater. Chlorinated triclosans are also photolyzed into higher chlorinated dibenzo-*p*-dioxins, including 1,2,8-TriCDD, 2,3,7-TriCDD, and 1,2,3,8-TriCDD (Buth *et al*., 2009). The total yield of PCDDs derived from triclosan and its derivatives is estimated to be 46 – 92 gTEQ per year, which contributes about 1.8 – 3.7% of air emission in the U.S. (Buth *et al*., 2009).

Natural sources of PCDDs

Chlorine is the 17th most abundant element on earth. Because it is a strong oxidant, the majority of chlorine is in the form of chloride. Elemental chlorine is highly reactive and can easily interact with a wide array of organic chemicals to form organochlorines, including PCDDs, especially at high temperature. In contrast to conventional opinion, PCDDs are ubiquitous, even in pristine environments where industrialization has little to no effects (Dopico & Gómez, 2015).

They are produced from various natural events such as forest fires, volcano eruptions, photo – induced transformation of dioxin precursors, oxidation of chlorinated aromatic compounds by peroxidases, or biosynthesis of PCDDs by marine organisms. Approximately 60 kg of PCDDs were estimated to be produced annually in Canada in forest fires according to a study in 1985 (Sheffield, 1985) and from 29 to 837 g TEQ per year in the US recently (Dwyer & Themelis, 2015).

In vitro incubation of chlorophenols with hydrogen peroxide in the presence of horseradish peroxidase, manganese peroxidase, or lignin peroxidase results in the increase of PCDDs than control reactions without chlorophenols (Svenson *et al*., 1989; Öberg & Rappe, 1992; Muñoz *et al*., 2014). These studies suggested the possible source of PCDDs from composting of sewage sludge or municipal wastes, especially when pentachlorophenol treated wood is used as a bulking agent.

Sponges have been known to produce a variety of brominated compounds. Brominated and several mixed brominated/chlorinated dibenzo-*p*-dioxins (PBDDs) were also found in sponge systems at a higher level than the background environment. Concentrations of PBDDs are higher in samples collected from the marine environment but are undetectable in samples from the freshwater environment. Marine sponges contain precursors for PBDDs such as bromophenols, MeO-polybrominated diethyl ethers, and OH-polybrominated diethyl ethers. Condensation of those precursors can form PBDDs even though it is not clear if sponges are the sources of PBDD (Unger, 2009).

Fate of PCDDs in the environment

Figure 1.5 describes fate of PCDDs in the environments. PCDDs are highly hydrophobic and have low volatility. They tend to accumulate in sediments which later become major sinks of PCDDs. Direct discharge of PCDDs into water columns results in deposition of the contaminants into sediments at elevated concentration. Historical contaminated sites, therefore, can be found

all over the world from production and overuse of chlorinated aromatic compounds which contain PCDDs as impurities (Table 1.2). PCDD deposition into sediments increased and peaked in 1960 – 1970, and has decreased since then due to the improvement of chemical manufacturing and waste combustion technologies (Götz *et al*., 2007).

PCDDs still can be dispersed from sediments and re-distributed into the water column of rivers, they can then translocate downstream and expand contaminated areas. Aproximately 4 – 8 kg PCDDs were reported to release into Newark Bay from the Passaic River since the 1940s (Bopp *et al*., 1991). The Bitterfeld industrial region (Germany) became heavily contaminated with PCDDs from chlorophenol production along with other industrial activities such as chloralkali and metallurgy. PCDD contamination from this region expanded to a large area of the Mulde River and its tributary Spittelwasser, released to the Elbe River and the Harbor of Hamburg (Götz *et al*., 1994, 2007). Even though the input of PCDDs by re-suspending from sediments to water column is considerably lower than that from water into sediments, they still become bioavailable to aquatic organisms and thus raise a threat to aquatic life and humans. PCDDs from sediments and water columns can also volatilized into the atmosphere and migrate to locations that are not close to the source of contamination (Friedman *et al*., 2012).

In addition to the minor contribution from sediments, the majority of PCDDs emitted into the atmosphere is contributed by direct emissions from anthropogenic and natural combustion events. The total amount of PCDDs released into the atmosphere is estimated to be 17,226 kg, which equals 287 kg-TEQ annually, 93% of which is deposited onto land (57%) and ocean water (40%) (Booth *et al*., 2013). Dispersion of PCDDs into the atmosphere is not confined to the area with the source, PCDDs can be carried away and impact locations with low PCDD input level such as Africa (Booth *et al*., 2013). However, unlike many persistent organic pollutants such as DDT, PCDDs do not have a “grasshopper” or global distillation effect. They are found at a very low

concentrations and are not enriched in cold latitudes (Wagrowskii & Hites, 2000). Only 1 x 10^{-8} mg TEQ/km^2 was deposited in the Antarctic, compared to the highest deposition of 146 mg TEQ/km^2 in Europe and South Korea (Booth *et al*., 2013).

Microbially mediated transformation of PCDDs

Aerobic degradation of PCDDs

PCDDs are persistent organic pollutants and tend to bind tightly to organic materials in sediments which makes them difficult to be attacked by microorganisms. Non-chlorinated or less chlorinated dibenzo-*p*-dioxins are more vulnerable to degradation by aerobic microorganisms such as *Sphingomonas wittichii* RW1 (Wittich *et al*., 1992), *Terrabacter* sp. DBF63, *Pseudomonas* sp. CA10 (Habe *et al*., 2001), *Rhodococcus opacus* SAO 101 (Kimura & Urushigawa, 2001). Aerobic degradation of dibenzo-*p*-dioxins is usually initiated with the formation of dihydrodiols by angular dioxygenases which weakens the aromatic ring and enables ring cleavage (Habe *et al*., 2001).

White rot fungi are also responsible for aerobic degradation of PCDDs. Extracellular oxidative enzymes (lignin peroxidases, manganese peroxidases) produced by white rot fungi are non-substrate specific. They are able to attack the aromatic rings of PCDDs which leads to ring cleavage and mineralization of PCDDs (Valli *et al*., 1992). Aerobic degradation of PCDDs by white rot fungi is not only limited to non to less chlorinated dibenzo-*p*-dioxins, highly chlorinated dibenzo-*p*-dioxins can also be degraded via this mechanism (Takada *et al*., 1996).

Anaerobic degradation of PCDDs

PCDDs are resistant to oxidation under anaerobic conditions, so far, there's no report on ring cleavage of PCDDs under strictly anaerobic conditions (Bunge & Lechner, 2009). PCDDs are highly oxidized, hence, they can serve as terminal electron acceptors for microbial respiration under strictly anaerobic conditions. Table 1.3 summarizes research on microbially mediated reductive

dechlorination of PCDDs. Twelve years after microbial reductive dechlorination was first demonstrated by Suflita et al. (1982), reductive dechlorination of PCDDs was demonstrated in anaerobic microcosms using contaminated sediments from the Hudson River (New York) and a dioxin contaminated aquifer (Pensacola, Florida). Even though no dechlorinating bacteria were revealed, the authors demonstrated that biotic reductive dechlorination exceeded abiotic dechlorination (Adriaens & Grbić-Galić, 1994). Barkovskii and Adriaens (1996) provided the first evidence that non-methanogenic, non-spore forming bacteria were likely responsible for reductive dechlorination of trichlorodibenzo-*p*-dioxins in their enrichment cultures.

Reductive dechlorination can be a respiratory process in which chlorinated compounds serve as terminal electron acceptors; a process that is carried out by only a few anaerobic microorganisms. Reductive dechlorination of PCDDs has been reported in well-characterized enrichment cultures or in pure cultures of obligate organohalide-respiring bacteria, i.e., *Dehalococcoides* and *Dehalobacter*. Among phylogenetically diverse reductive dehalogenating bacteria, only these two species are shown to be able to respire PCDDs so far (Figure 1.6) (Bunge *et al*., 2003, 2007; Ballerstedt *et al*. 2004; Fennell *et al*., 2004; Yoshida *et al*., 2009b; Pöritz *et al*., 2015).

Reductive dechlorination of PCDDs may play an important role in bioremediation of PCDD contaminated environments. The products of PCDD reductive dechlorination are less hydrophobic and therefore are more likely to be released into the water column where they are prone to complete aerobic degradation.

Reductive dechlorination as an electron accepting process

Reductive dehalogenases are the terminal reductases in respiratory reductive dechlorination. They transfer 2 electrons and a proton to halogenated compounds which serve as terminal

electron acceptors to create a proton motive force across the cytoplasmic membrane. Protons are then translocated through ATP synthase into the cytoplasm and generate ATP for organohalide-respiring bacterial cells. Reductive dehalogenases are membrane bound, iron sulfur cluster containing proteins with a corrinoid co-factor. Biochemical analysis of a few purified and partially purified reductive dehalogenases revealed that each reductive dehalogenase contains two iron sulfur clusters and one corrinoid in its molecule (Magnuson *et al*., 1998; Krasotkina *et al*., 2001; Müller *et al*., 2004). The corrinoid co-factor plays the vital role in the catalytic reaction of reductive dehalogenases. The crystalographic structure of a soluble reductive dehalogenase from *Nitratireductor pacificus* (NprdhA) revealed the direct interaction between halogen and the cobalamin cobalt (Payne *et al*., 2015). Bommer *et al*. (2014) revealed the conformation of PceA of *Sulfurospirillum multivorans* in the presence of trichloroethene as the substrate and the involvement of distal amino groups in binding to corrinoid.

Although the corrinoid is essential for respiratory reductive dechlorination and for growth of organohalide-respiring bacteria (He *et al*., 2007), many obligate organohalide-respiring bacteria such as *Dehalococcoides* and some strains of *Dehalobacter* lack the ability to synthesize the corrinoid *de novo* and have to depend on exogenous corrinoids amended in culture media or provided by supporting organisms of the consortium such as *Acetobacterium woodii* (He *et al*., 2007; Hug *et al*., 2012; Rupakula *et al*., 2015). They can then uptake exogenous corrinoid from the environment and remodel them into functional forms of corrinoid for reductive dehalogenases (Yi *et al*., 2012). A complete corrinoid *de novo* synthesis pathway, however, was found in facultative organohalide-respiring bacteria such as *Sulfurospirillum multivorans* and *Desulfitobacterium* (Nonaka *et al*., 2006; Goris *et al*., 2014; Keller *et al*., 2014).

Electrons are transferred from electron donors (hydrogen, short chain organic acids) through cytoplasmic membrane to reductive dehalogenases. Hydrogen is commonly used as electron

donor by most organohalide-respiring bacteria. Hup-type hydroxygenases, which are oriented outward of the cytoplasmic membrane, are responsible for hydrogen oxidation and electron transfer from hydrogen to the electron transport chain (Jayachandran *et al*., 2004; Nijenhuis & Zinder, 2005; Seshadri *et al*., 2005). Due to the slow growth rate of organohalide-respiring bacteria, little is known about electron transport during their respiratory dechlorination. Menaquinone seems not to be involved in the electron transport chain in *Dehalococcoides* (Adrian *et al*., 2007b). In contrast, Kruse *et al*. (2015) and Goris *et al*. (2015) found the involvement of menaquinone in the electron transport chain of *Desulfitobacterium dehalogenans* and *Sulfurospirillum multivorans*, respectively. They also postulated that electrons are transferred from this quinol pool to reductive dehalogenases via extracellular flavoproteins serving as electron shuttles (Kruse *et al*., 2015) or via a putative quinol dehydrogenase (Goris e*t al*., 2015). Figure 1.7 illustrates respiratory reductive dehalogenation of PCDDs by *Dehalococcoides* spp.

Reductive dehalogenase homologous genes (*rdhA*s)

Reductive dehalogenase homologous gene (*rdhA*) sequences contain 2 conserved motifs for iron-sulfur cluster binding ($CX_2CX_2CX_3CP$ and CX_2CX_3CP), which suggests that there are two iron sulfur clusters present in the enzyme that contribute to catalytic activity of reductive dehalogenases (as illustrated in Figures 1.8 and 1.9). Unlike many other corrinoid dependent enzymes, reductive dehalogenase sequences do not contain the corrinoid binding motif $(DXHX_2G)(X_{41\text{-}42})SXL(X_{26\text{-}28})GG$. However, a truncated corrinoid binding motif was found in a few putative reductive dehalogenase gene sequences of *Dehalococcoides mccartyi* CBDB1 (Hölscher *et al*., 2004; Adrian *et al*., 2007b).

The number of different *rdhA* genes varies among organohalide-respiring bacteria. Facultative organohalide-respiring bacteria, such as *Desulfitobacterium* and *Sulfurospirillum*, contain up to 7

rdhA genes in their genomes (Kim *et al*., 2012). In contrast, as many as 38 *rdhA* genes have been found in obligate organohalide-respiring bacteria, such as *Dehalococcoides*, *Dehalogenimonas* and *Dehalobacter* (McMurdie *et al*., 2009, Siddaramappa *et al*., 2012; Kruse *et al*., 2013). In addition to truncated *rdhA* genes, the function of the majority of full length *rdhA* still remains unknown, mostly because organohalide-respiring bacteria grow very slowly and reductive dehalogenases are extremely sensitive to oxygen (Maillard *et al*., 2003) and thus difficult to characterize. A few reductive dehalogenases were partially purified and their catalytic activities were tested using conventional biochemical approaches. Most of them are from faster growing organisms on more water soluble chlorinated substrates, such as CprA from various *Desulfitobacterium* spp., PceA from *Sulfurospirillum multivorans*, PceA from *Dehalobacter restrictus* PER-K23, TceA and PceA from *Dehalococcoides mccartyi* 195 (Neumann *et al*., 1998; Magnuson *et al*., 1998, 2000; Suyama *et al*., 2002; Maillard *et al*., 2003). Blue Native PAGE followed by enzymatic assay were first applied to determine substrate specificity of CbrA produced by *Dehalococcoides mccartyi* CBDB1 (Adrian *et al*., 2007b). The method is highly sensitive and was later applied systematically to determine the substrate-enzyme specificity of several other reductive dehalogenases including TdrA from *Dehalogenimon*as WBC-2 (Molenda *et al*., 2016), CfrA and DcrA from *Dehalobacter* containing mixed culture (Tang & Edwards, 2013), BvcA from *Dehalococcoides mccartyi* BAV1 (Tang *et al*., 2013), DcpA from *Dehalogenimonas lykanthroporepellens* BL-DC-9, *Dehalococcoides mccartyi* strains KS and RC (Pallida-Crespo *et al*., 2014).

The substrate specificity of some reductive dehalogenases were inferred at the transcriptional level, for example, *Dehalococcoides mccartyi* strains VS, 11a, JNA, and MB (Müller *et al*., 2004; Chow *et al*., 2010; Wang *et al*., 2015; Low *et al*., 2015), *Desulfitobacterium* DCA-1 (Marzorati *et al*., 2007), *Desulfitobacterium* sp. KBC1 (Tsukagoshi *et al*., 2006). However, it has been known that

there are a lot of reductive dehalogenase homologous genes that are expressed constitutively. This makes it difficult to infer their substrate specificity (Wagner *et al*., 2009; Mukherjee *et al*., 2014). It is postulated that organohalide-respiring bacteria evolved long before chlorinated compounds of industrial origin were released into the environment, and their substrates for respiration during the pre-industry era were naturally produced chlorinated compounds (Öberg, 2002). Constitutive expression of *rdhA* genes help them readily adapt to grow on limited sources of organohalides in the environment (Krzmarick *et al*., 2012, 2013).

The *rdhB* gene, which encodes for a small hydrophobic protein that functions as an anchoring protein for RdhA, is usually found within a few nucleotides from *rdhA* and co-transcribed with *rdhA* (Neumann *et al*., 1998; Van de Pas *et al*., 1999; Maillard *et al*., 2003; Kube *et al*., 2005). In addition to *rdhB*, several other genes are also found in the vicinity of *rdhA*. Most of their functions have not been confirmed experimentally. The gene cluster *pceABCT* was found in *Dehalobacter restrictus* PER-K23 and *Dehalobacter* strain E1 that grew on PCE (Maphosa *et al*., 2012; Rupakula *et al*., 2013). The gene cluster *cprTKZEBACD* in which *cprA* encodes for ortho-chlorophenol reductive dehalogenase was found in several *Desulfitobacterium* strains. PceC and CprC of the above-mentioned *Dehalobacter* and *Desulfitobacterium* strains are similar in amino acid sequence to the membrane bound FMN binding transcription regulator of the NosR/NirI family. PceT and CprT are postulated to be chaperone protein, and take part in maturation of PceA and CprA, and then transporting them to the cell membrane. *rdhK* of *Dehalobacter restrictus* PER-K23 which is also found in the close proximity to *pceA* and *cprK* of *Desulfitobacterium* spp. are postulated to encode for transcriptional regulatory proteins (Smidt *et al*., 2000; Pop *et al*., 2004; Rupakula *et al*., 2013). They have amino acid sequences similar to CRP/FNR regulatory protein. In *Desulfutobacterium*, binding of CprK to ortho-chlorophenol compounds leads to the change in conformation which causes the binding of CprK to the promotor region of the *cprA* gene cluster

and triggers gene transcription. In obligate dehalogenating bacteria belonging to the *Chloroflexi* phylum, however, transcriptional regulatory proteins belonging to the CRP/FNR family are not found near *rdhA* genes. Instead, transcriptional regulators belonging to the multiple antibiotic resistant regulator (MarR) family and two-component regulatory system (TCS) family are commonly found in close vicinity of *rdhA* genes (Seshadri *et al*., 2005; Kube *et al*., 2005; Siddaramappa *et al*., 2012; Wagner *et al*., 2013). This suggests that transcription of *rdhA* genes is tightly regulated.

Genome analysis of dehalogenating bacteria belonging to the *Chloroflexi* phylum reveals that the majority of *rdhAs* are located on the two high plasticity regions (HPR) near and on two sides of the origin of replication. The two HPRs are also flanked by the core genomes of *Dehalococcoides* spp. and *Dehalogenimonas* spp. HPRs contain a high number of *rdhA* genes and are densely occupied by genes that are related to genetic transfer such are insertion sequence (IS) and repeated elements, and genomic islands with their own integration sites (Seshadri *et al*., 2005; Kube *et al*., 2005; McMurdie *et al*., 2009; Siddaramappa *et al*., 2012; Molenda *et al*., 2016). This suggests that HPRs are strain-specific and allow for the evolution of *rdhA* genes distinctively from other core genes of *Dehalococcoides* and *Dehalogenimonas*, which enables rapid adaptation of these dehalogenating bacteria to chlorinated substrate found in the environment. Genome analysis of a facultative dehalogenating bacterium, i.e., *Desulfitobacterium hafniense* Y51 revealed that *pceA* is located in a gene cluster with *pceB*, *pceC*, and *pceT,* which is sandwiched by two transposases, suggesting that horizontal gene transfer is also the method to acquire *pceA* by this strain (Nonaka *et al*., 2006).

Hypotheses

PCDDs are not only products of anthropogenic activities, they are also produced naturally during chemical, biological or thermal transformation from chlorine and aromatic precursors. They are found at different concentrations in all types of environments. Microorganisms have evolved to inhabit a wide variety of environments on earth, to utilize not only naturally produced chemicals but also chemicals of industrial origin, including chlorinated compounds such as PCDDs. Highly chlorinated dibenzo-*p*-dioxins have high redox potentials, when coupled with oxidation of a reduced chemical species such as hydrogen, they can provide sufficient energy for microbial growth. I hypothesized that microbially mediated anaerobic reductive dechlorination of PCDDs is prevalent in the environment, and that PCDD dehalogenating bacteria can be found and enriched from various contaminated anaerobic environments.

Overall Goals and Objectives

The overall goal of this study was to enrich for dehalogenating bacteria using a model chlorinated dibenzo-*p*-dioxin compound by a conventional enrichment approach and to study dechlorination activity, microbial communities, and the genomes of dehalogenating bacteria from metagenomics data of dehalogenating enrichment cultures. 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) was used as a model dibenzo-*p*-dioxin compound throughout this dissertation research because it has similar physicochemical properties as of the most toxic congener of this chemical group (2,3,7,8-TeCDD).

The three objectives of this study are as follows:

1. To enrich for dehalogenating bacteria and monitor 1,2,3,4-TeCDD dehalogenation activity in soils and sediments collected from various locations with different contamination

background. Sampling sites in this study include southern and central Vietnam, the Hackensack River (New Jersey, USA), and the Kymijoki River (Finland).

2. To Investigate the microbial communities of 1,2,3,4-TeCDD dehalogenating cultures and elucidate microorganisms that are directly involved in reductive dechlorination of 1,2,3,4-TeCDD in the Hackensack River enrichment cultures.
3. To compare genomic information of *Dehalococcoides* strains that are enriched in 1,2,3,4-TeCDD dechlorinating cultures established from the Hackensack and the Kymijoki River sediments.

Table 1.1. Selected physical and chemical properties of PCDD congeners (Shiu *et al*., 1988).

Congener group	**Molecular weight (g/mole)**	**Solubility at 25°C (µg/l)**	**Henry's law constant ($Pa.m^3/mol$)**	**LogK_{ow} at 25°C**
Dioxin (DD)	184	842 – 900	12.29	4.3
Monochlorodibenzo-*p*-dioxins (MoCDDs)	218.5	278 – 318	8.38 – 14.82	4.75 – 5
Dichlorodibenzo-*p*-dioxins (DiCDDs)	253	3.75 – 16.7	2.13 – 8.11	5.6 – 5.75
Trichlorodibenzo-*p*-dioxins (TriCDDs)	287.5	8.41	3.84	6.35
Tetrachlorodibenzo-*p*-dioxins (TeCDDs)	322	0.00791 – 0.63	0.71 – 10.34	6.6 – 7.1
Pentachlorodibenzo-p-dioxins (PeCDDs)	356.4	0.118 (20°C)	0.264	7.4
Hexachlorodibenzo-*p*-dioxins (HxCDDs)	391	0.00442 (20°C)	4.52	7.8
Heptachlorodibenzo-*p*-dioxins (HpCDDs)	425.2	0.0024 (20°C)	0.133	8
Octachlorodibenzo-*p*-dioxins (OcCDDs)	460	0.000074	0.683	8.2

Table 1.2. Examples of PCDD contaminated sites.

Contaminated site	Contamination source	Concentration/quantity	References
Seveso, Italy	Chemical manufacture	30 kg TCDD deposited	Domenico *et al*., 1980
Frierjorden, Norway	Magnesium production	50 – 100 kg TEQ (Nordic model) was emitted in 30 – 35 years	Knutzen & Oehme, 1989
Bitterfeld industrial region, Germany	Chemical manufacture	5,080 – 14,500 pg TEQ/g dw (Spittelwasser Creek)	Götz *et al*., 1998, 2007
	Metallurgy	7,680 pg TEQ/g dw (flood plain of the Elbe River)	
	Chloroalkali	600 pg I-TEQ/kg dw (Mulde River sediment)	
Byker incinerator, UK	Municipal waste incineration	6 – 1,911 ng I-TEQ/kg dw soil in adjacent area	Vizard *et al*., 2006
	Chemical manufacture between the 1890s and the 1930s		
Kymijoki River, Finland	Chemical manufacture	1,060 ng I-TEQ/g dw	Verta *et al*., 2009
	Chloralkali	Total historical sum is 29 kg (I-TEQ) with the contribution of 20 kg (I-TEQ) from Ky-5, and total affected sediment volume is $5x10^6$ m^3	
Soils at sawmills, Finland	Utilization of chlorophenol in wood preservation and bleach wood by chlorine	233,300 – 285,100 ug/m^2 of PCDFs were detected in soils at wood-preserving facilities	Kitunen *et al*., 1987
Central and Southern Vietnam	Herbicide usage and storage	366 kg 2,3,7,8-TeCDD in total was sprayed during 10 years from 1961 – 1971	Stellman *et al*., 2003
		Up to 6,820 pg TEQ/g dw (sediment in lakes inside Danang Airport)	Nguyen *et al*., 2009
		Up to 436 pg WHO-TEQ/g dw (Bien Hung lake – adjacent to Bien Hoa Airbase)	Mai *et al*., 2007
		50 – 1,000 pg I-TEQ/g dw (Aluoi Valley)	Dwernychuk *et al*., 2002

Masan Bay, Korea	Open burn and use of pentachlorophenol	Up to 76 pg TEQ/g dw	Im *et al*., 2002
Hyeongsan River, Korea	Various industrial activities	Up to 937 pg TEQ/g dw	Koh *et al*., 2004
Rice paddy soils, Japan	Herbicides used in agriculture (pentachlorophenol, and chlornitrofen)	1.3 – 435 pg TEQ/g dw	Seike *et al*., 2007
Nose, Osaka, Japan	Municipal waste incineration	120,340 pg/g dw (Nose soil)	Kishida *et al*., 2010
HongKong Bay	Industrial activities in within the densely populated area nearby	12.5 ng I-TEQ/kg dw	Müller *et al*., 2002
Guiyu, China	E-waste recycling using old technology	203-1,100 pg WHO-TEQ/g, dry wt (acid mine drainage site) 84.3-174pgWHOTEQ/g dw (combusted residue)	Leung *et al*., 2007
Love Canal, Niagara Falls, NY, USA	Chemical manufacture	0.9 – 312 ng 2,3,7,8-TeCDD/g dw sediment	Smith *et al*., 1983
Passaic River and Newark Bay, Newark, NJ, USA	Chemical manufacture	Up to 21,000 ppt Released 4-8 kg 2,3,7,8-TeCDD into Newark Bay since late 1940s	Bopp *et al*., 1991
Tittabawassee River, Midland, MI, USA	Chemical manufacture	4 – 1,980 pg TEQ/g dw	Hilscherova *et al*., 2003
Times Beach, MO, USA	Chemical manufacture	8 kg of 2,3,7,8-TeCDD was accumulated	Hites, 2011
Lake Ontario, Canada	Niagara river watershed with hazardous chemical facilities	111 – 300 pg TEQ/g dw	Marvin *et al*., 2003
Homebush Bay, Jackson port, Australia	Chemical manufacture	32 – 4,352 pg WHO-TEQ/g dw	Birch *et al*., 2007

Table 1.3. PCDD reductive dechlorinating bacteria and enrichment cultures.

Culture	Phylogenetic Affiliation	Other electron acceptors	Electron donor	Environment	Reference
Enrichment culture	ND	PCDFs, PCBs,	Propionate Butyrate Benzoate	Hudson River sediment (downstream of Fort Edward, NY), USA Creosote-contaminated groundwater aquifer near Pensacola, Florida, USA	Adriaens & Grbić-Galić, 1994 Adriaens *et al*., 1995
Enrichment culture	ND	Chlorobenzenes, PCBs	Lactate	Rhine River sediment, the Netherlands	Beurskens *et al*., 1995
Enrichment culture	ND	ND	Hydrogen Butyrate Benzoate	Lower Passaic River sediment, USA	Barkovskii & Adriaens, 1996 Albrecht *et al*., 1999; Fu *et al*., 2001
Dehalococcoides enrichment culture	*Chloroflexi*	ND	Hydrogen, organic acids	Saale River sediment	Ballerstedt *et al*., 1997, 2004
Estuarine enrichment culture	ND	Bromophenols	ND	Arthur Kill sediment, USA	Vargas *et al*., 2001
Estuarine enrichment culture	ND	PCDFs, chlorobenzenes, chloroanisoles, chlorophenols, bromophenols	Lactate Propionate	Paleta Creek, Graving Dock, Shelter Island and Tuckerton sediments, USA	Ahn *et al*., 2005, 2007
Enrichment cultures		PCDFs		PCDD/F polluted river, Japan	Yoshida *et al*., 2005
Dehalobacter mixed culture	*Firmicutes*	PCBs, 4,5,6,7-tetrachlofophthalide (fthalide)	Hydrogen	Paddy soil, Japan	Yoshida *et al*., 2009a, b

Dehalococcoides mccartyi 195	*Chloroflexi*	PCDFs, PCBs, chloroethenes, chloroethanes, chloronaphthalenes	Hydrogen	Sewage sludge, USA	Maymó-Gatell *et al*., 1997 Fennell *et al*., 2004; Liu & Fennell, 2008; Schmidt *et al*., 2014; Zhen *et al*., 2014
Dehalococcoides mccartyi CBDB1	*Chloroflexi*	PCBs, chlorobenzenes, chlorophenols	Hydrogen	Saale River sediment, Germany	Adrian *et al*., 2000, 2007a, 2009 Bunge *et al*., 2003
Dehalococcoides mccartyi DCMB5	*Chloroflexi*	Chlorobenzenes, chlorophenols, chloroethene (perchloroethene only)	Hydrogen	Spittelwasser Creek sediment, Germany	Pöritz *et al*., 2015; Bunge *et al*., 2007
Dehalococcoides mccartyi BTF08	*Chloroflexi*	Chloroethenes, chloroethanes, chlorobenzenes, lindane, chlorophenols, vinyl bromide	Hydrogen	Chloroethene contaminated aquifer, Bitterfeld, Germany	Cichocka *et al*., 2010; Kaufhold *et al*., 2013
Dehalococoides mixed culture	*Chloroflexi*	PCDFs, chloronitrobenzenes, chlorobenzenes	Lactate Propionate	Kymijoki River sediment, Finland	Liu *et al*., 2013, 2014 Kuokka *et al*., 2014

ND: not determined

Table 1.4. List of characterized reductive dehalogenases.

Organism	Reductive dehalogenase with known function	Substrates	References
Dehalococcoides mccartyi CBDB1	CbrA	1,2,3,4-TeCB 1,2,3-TriCB	Kube *et al*., 2005 Adrian *et al.*, 2007b
Dehalococcoides mccartyi 195	PceA	PCE, 2,3-DCP	Magnuson *et al.*, 1998 Fung *et al.*, 2007
	TceA	TCE, cis-DCE, 1,1-DCE, 1,2-DCA, 1,2-BrCA	Magnuson *et al.*, 2000 Seshadri *et al.*, 2005
Dehalococcoides mccartyi VS	VcrA	VC, DCE	Müller *et al.*, 2004 McMurdie *et al.*, 2009
Dehalococcoides mccartyi BAV1	BvcA		Krajmalnik-Brown *et al.*, 2004 McMurdie *et al.*, 2009
Dehalococcoides mccartyi MB	MbrA	PCE to 1,2-trans DCE	Chow *et al*., 2010 Low *et al.*, 2015
Dehalococcoides mccartyi 11a	VcrA	TCE to DCEs	Low *et al.*, 2015
Dehalococcoides mccartyi JNA	JNA_RD8 JNA_RD11	PCE, PCB	Wang *et al*., 2015
Dehalogenimonas lykanthrorepellans BL-DC-9			Mukherjee *et al*., 2014
Dehalogenimonas sp. WBC-2	TdrA	tDCE to VC	Molenda *et al*., 2016
Dehalobacter restrictus PER-K23	PceA		Maillard *et al*., 2003 Kruse *et al*., 2013

Dehalobacter WL	DcaA (RdhA1)	1,2-DCA	Grostern & Edwards, 2009
Dehalobacter CF	CfrA	Chloroform, 1,1,1-TCA	Tang & Edwards, 2013
Dehalobacter DCA	DcrA		Tang *et al*., 2013
Desulfitobacterium dichloroeliminans DCA-1	DcaA	1,2-DCA	Marzorati *et al*., 2007
Desulfitobacterium PR	CtrA	DCA and chloroform	Ding *et al*., 2014
Desulfitobacterium chlorespirans	CprA	3-chloro 4-hydroxybenzoic acid, hydroxylated PCB (3,3,5,5-tetrachloro-4,4-biphenyldiol), 2,3-dichlorophenol, 2.6-dichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol, trichloroacetate	Krasotkina *et al.*, 2001
Desulfitobacterium hafniense Y51	PceA	PCE, TCE, chloroethanes	Suyama *et al.*, 2002 Nonaka *et al.*, 2006
Desulfitobacterium dehalogenans	CprA	Ortho chlorine from various chlorinated compounds	Van de Pas, 1999 Kruse *et al.*, 2015
Desulfitobacterium frappieri PCP-1	CprA5	3,5-DCP	Thibodeau *et al*., 2004
	CrdA	2,4,6-TCP	Boyer *et al*., 2003
Desulfitobacterium sp. KBC1	PrdA	PCE	Tsukagoshi *et al*., 2006
Sulfurospirillum multivorans	PceA	PCE, TCE, DBrE	Neumann *et al.*, 1998

Figure 1.1. General structure of PCDDs. m,n denote numbers of chlorine on each aromatic ring and vary from 0-4.

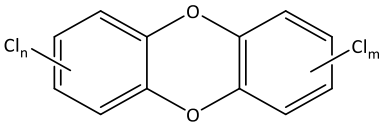


Figure 1.2. Toxicity mechanism of PCDDs: Ahr/Arnt (Adapted from Mandal, 2005).

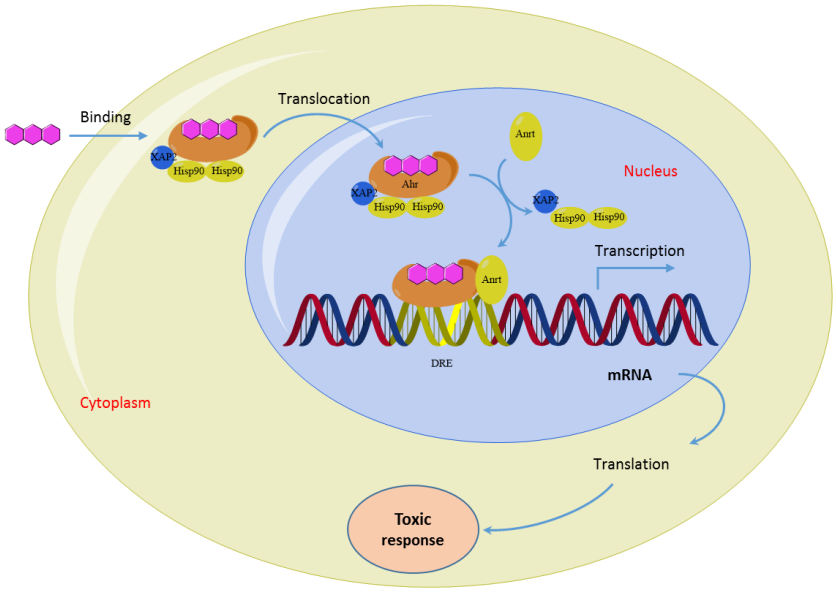


Figure 1.3. Production of 2,3,7,8-TeCDD as the impurity during 2,4,5-Trichlorophenoxyacetic acid production (redrawn from Hites, 2011).

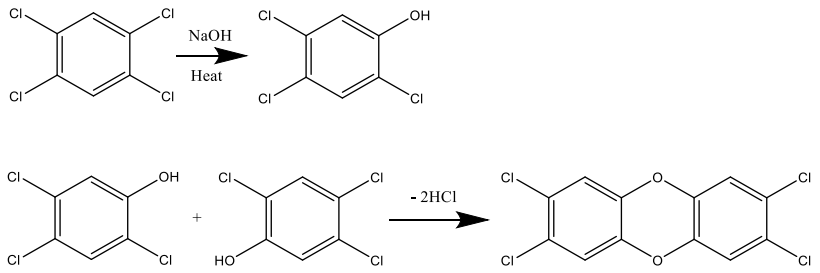


Figure 1.4. Photo-induced formation of chlorinated dibenzo-*p*-dioxins from triclosan (redrawn from Buth *et al*., 2010).

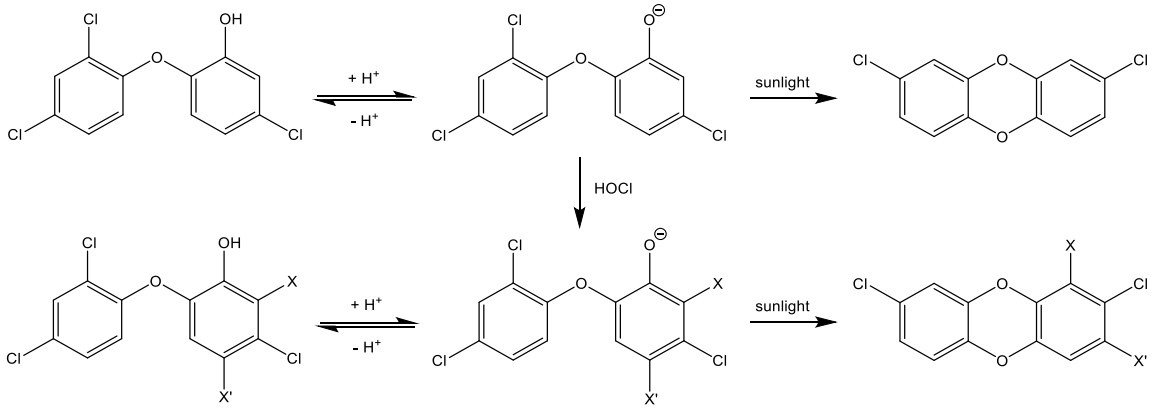


Figure 1.5. Fate of PCDDs in the environment.

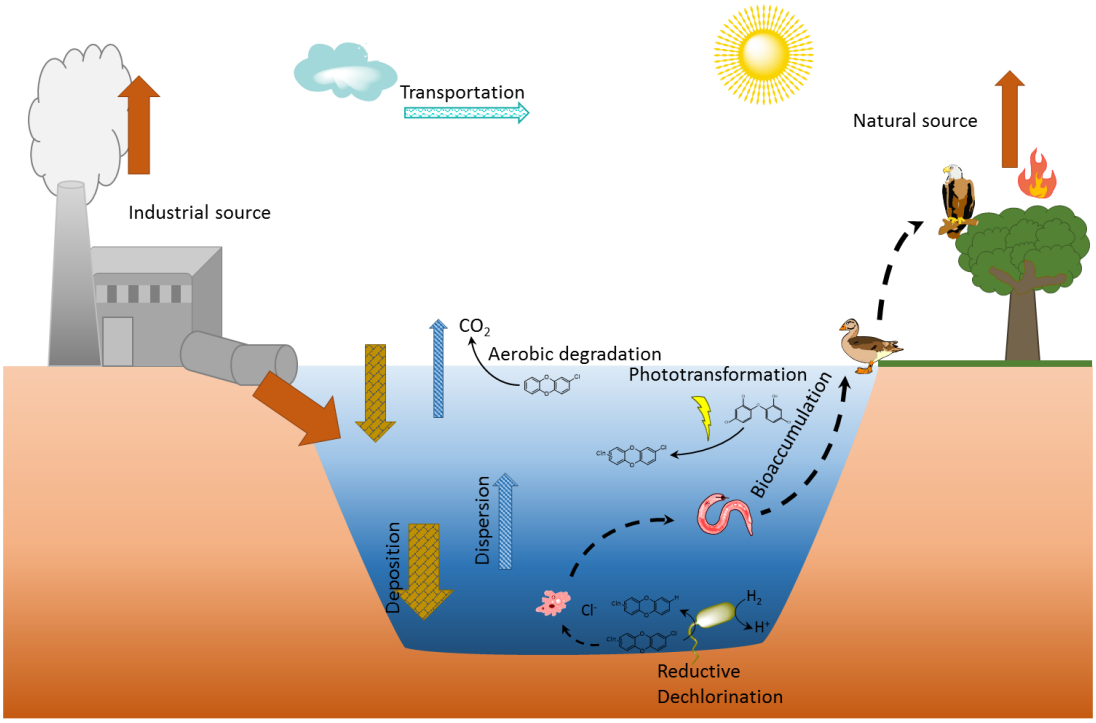


Figure 1.6. Phylogenetic analysis of 16S rRNA gene sequences of known reductive dehalogenating bacteria. 16S rRNA sequences were imported from GenBank, their accession numbers are displayed in parentheses. PCDD reductive dechlorinating bacteria are indicated with a star (*). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-8035.8161) is shown. Bootstrap values (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved in 43 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1070 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

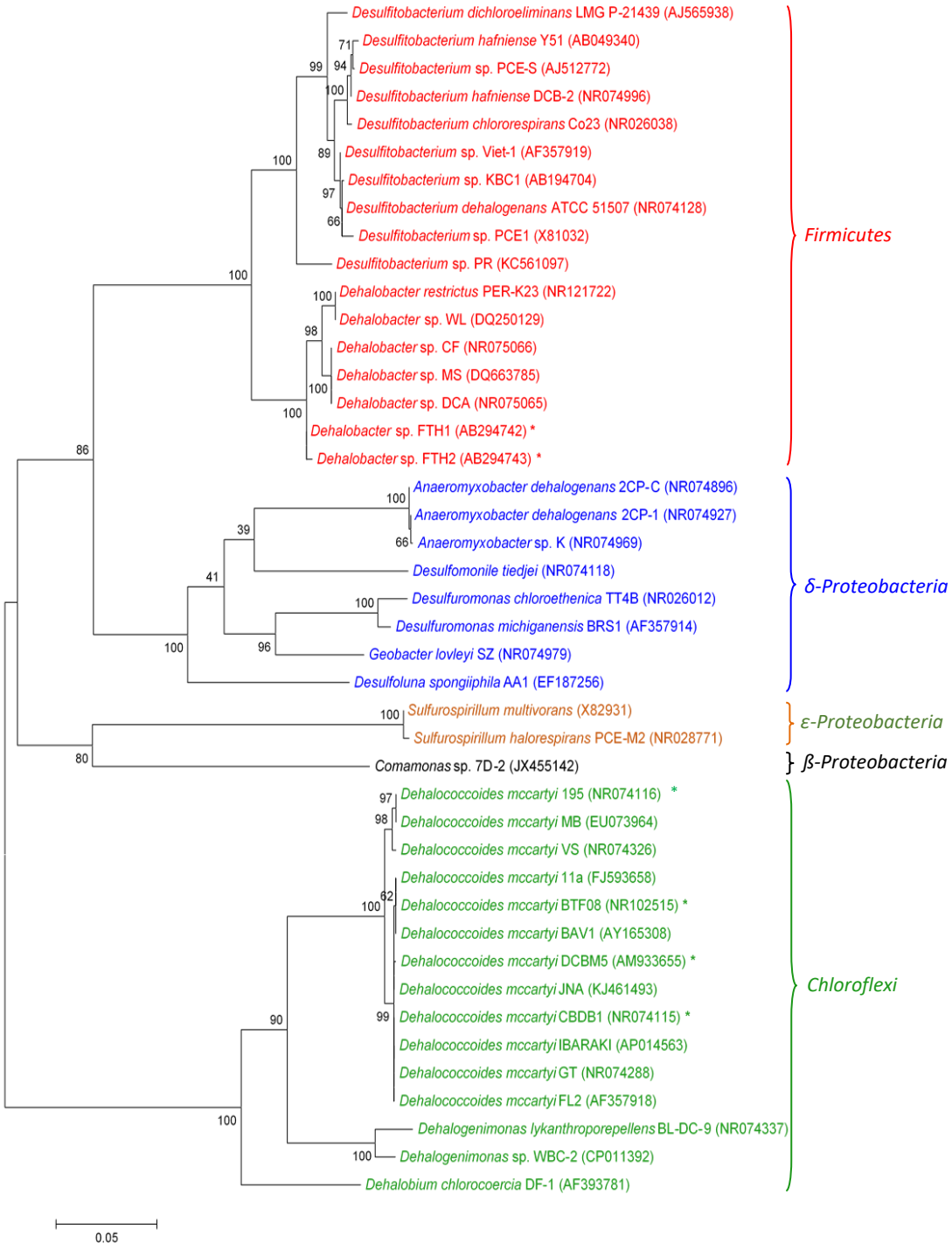

Desulfitobacterium dichloroeliminans LMG P-21439 (AJ565938)
Desulfitobacterium hafniense Y51 (AB049340)
Desulfitobacterium sp. PCE-S (AJ512772)
Desulfitobacterium hafniense DCB-2 (NR074996)
Desulfitobacterium chlororespirans Co23 (NR026038)
Desulfitobacterium sp. Viet-1 (AF357919)
Desulfitobacterium sp. KBC1 (AB194704)
Desulfitobacterium dehalogenans ATCC 51507 (NR074128)
Desulfitobacterium sp. PCE1 (X81032)
Desulfitobacterium sp. PR (KC561097)
Dehalobacter restrictus PER-K23 (NR121722)
Dehalobacter sp. WL (DQ250129)
Dehalobacter sp. CF (NR075066)
Dehalobacter sp. MS (DQ663785)
Dehalobacter sp. DCA (NR075065)
Dehalobacter sp. FTH1 (AB294742) *
Dehalobacter sp. FTH2 (AB294743) *
Firmicutes
Anaeromyxobacter dehalogenans 2CP-C (NR074896)
Anaeromyxobacter dehalogenans 2CP-1 (NR074927)
Anaeromyxobacter sp. K (NR074969)
Desulfomonile tiedjei (NR074118)
Desulfuromonas chloroethenica TT4B (NR026012)
Desulfuromonas michiganensis BRS1 (AF357914)
Geobacter lovleyi SZ (NR074979)
Desulfoluna spongiiphila AA1 (EF187256)
δ-Proteobacteria
Sulfurospirillum multivorans (X82931)
Sulfurospirillum halorespirans PCE-M2 (NR028771)
ε-Proteobacteria
Comamonas sp. 7D-2 (JX455142)
ß-Proteobacteria
Dehalococcoides mccartyi 195 (NR074116) *
Dehalococcoides mccartyi MB (EU073964)
Dehalococcoides mccartyi VS (NR074326)
Dehalococcoides mccartyi 11a (FJ593658)
Dehalococcoides mccartyi BTF08 (NR102515) *
Dehalococcoides mccartyi BAV1 (AY165308)
Dehalococcoides mccartyi DCBM5 (AM933655) *
Dehalococcoides mccartyi JNA (KJ461493)
Dehalococcoides mccartyi CBDB1 (NR074115) *
Dehalococcoides mccartyi IBARAKI (AP014563)
Dehalococcoides mccartyi GT (NR074288)
Dehalococcoides mccartyi FL2 (AF357918)
Dehalogenimonas lykanthroporepellens BL-DC-9 (NR074337)
Dehalogenimonas sp. WBC-2 (CP011392)
Dehalobium chlorocoercia DF-1 (AF393781)
Chloroflexi
0.05

Figure 1.7. Schematic of respiratory reductive dechlorination of PCDDs.

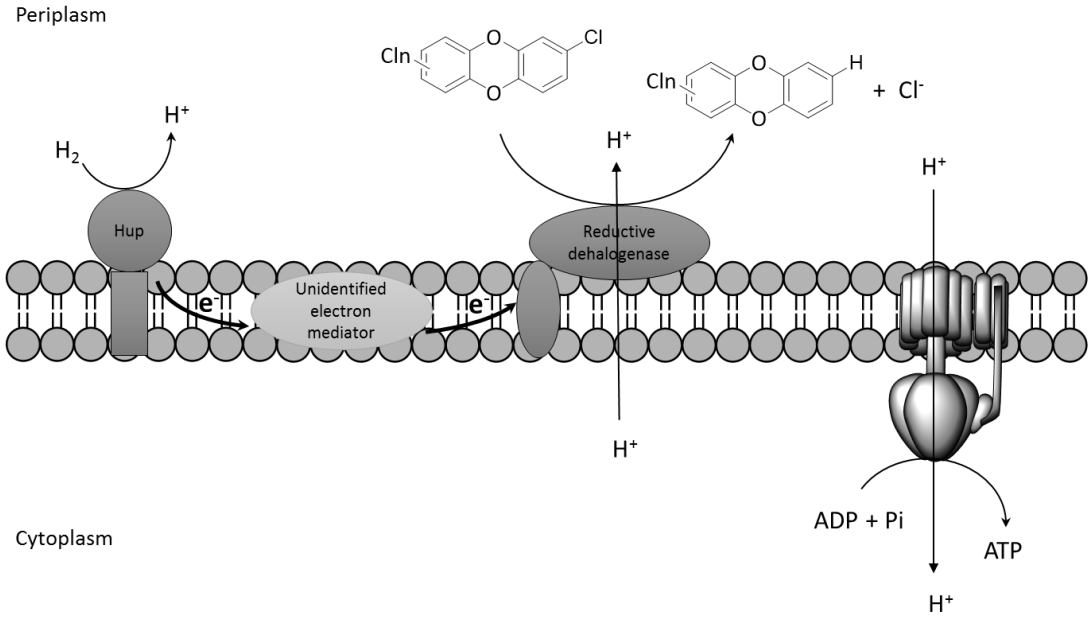


Figure 1.8. Organization of reductive dehalogenase genes.

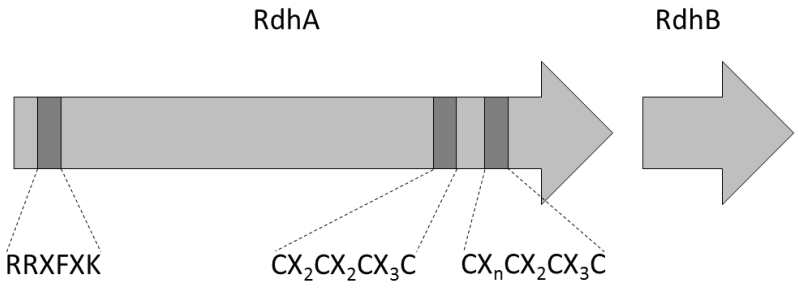


Figure 1.9. Alignment of TAT motif and iron-sulfur clusters in some biochemically characterized reductive dehalogenases. CbrA (*Dehalococcoides mccartyi* CBDB1, GenBank accession number CAI82345), CprA (*Desulfitobacterium dehalogenans* ATCC 51507, GenBank accession number AAD44542), PceA[1] (*Dehalobacter restrictus* PER-K23, GenBank accession number CAD28790), PceA[2] (*Sulfurospirillum multivorans* DSM 12446, GenBank accession number AAC60788), TceA (*Dehalococcoides mccartyi* 195, GenBank accession number AAW39060), and VcrA (*Dehalococcoides mccartyi* VS, GenBank accession number ACZ62391).

CbrA VS**RR**D**F**M**K**A-X_{343}-LITRF**C**AT**C**KK**C**AEM**C**PG-X_{32}-HK**C**RPWAWPPSPNTVGS**C**GV**C**QAV**C**VF

CprA MN**RR**S**F**L**K**V-X_{310}-GLLDF**C**RV**C**KK**C**ADN**C**PN-X_{25}-KK**C**TEFRTTNEEG--SS**C**GT**C**LKV**C**PW

PceA[1] IN**RR**N**F**L**K**A-X_{408}-SVREF**C**RL**C**KK**C**ADA**C**PA-X_{35}-NR**C**GSFWAYNG----SP**C**AN**C**VAV**C**SW

PceA[2] LS**RR**D**F**G**K**L-X_{349}-IVTEF**C**ET**C**KK**C**ARE**C**PS-X_{31}-NK**C**LGYWPESG----GY**C**GV**C**VAV**C**PF

TceA VT**RR**D**F**M**K**R-X_{420}-LIREF**C**KT**C**GI**C**AEH**C**PT-X_{31}-HK**C**IN----------**C**TI**C**EAV**C**PF

VcrA IS**RR**D**F**M**K**G-X_{384}-LMFEF**C**KT**C**YI**C**RDV**C**VS-X_{32}-SG**C**HNQ----------**C**GM**C**QSS**C**PF

TAT motif Fe-S binding motif Fe-S binding motif

Chapter 2

Impact of Estuarine Gradients on Reductive Dechlorination of 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin in River Sediment Enrichment Cultures

Abstract

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are among the most persistent organic pollutants. Although the total input of PCDDs into the environment has decreased substantially over the past four decades, their input via non-point sources is still increasing, especially in estuarine metropolitan areas. Here we report on the microbially mediated reductive dechlorination of PCDDs in anaerobic enrichment cultures established from sediments collected from five locations along the Hackensack River, NJ and investigate the impacts of sediment physicochemical characteristics on dechlorination activity. Dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) and abundance of *Dehalococcoides* spp. negatively correlated with salinity and sulfate concentration in sediments used to establish the cultures. 1,2,3,4-TeCDD was dechlorinated to a lesser extent in cultures established from sediments from the tidally influenced estuarine mouth of the river. In cultures established from low salinity sediments, 1,2,3,4-TeCDD was reductively dechlorinated with the accumulation of 2-monochlorodibenzo-*p*-dioxin as the major product. Sulfate concentrations above 2 mM inhibited 1,2,3,4-TecDD dechlorination activity. Consecutive lateral- and peri- dechlorination took place in enrichment cultures with a minimal accumulation of 2,3-dichlorodibenzo-*p*-dioxin in active cultures. A *Dehalococcoides* spp. community was enriched and accounted for up to 64% of *Chloroflexi* detected in these sediment cultures.

Introduction

Urban estuarine regions support a wide variety of industrial and municipal activities which results in contamination by many pollutants including PCDDs. They are among the most notorious and persistent organic pollutants known as the “dirty dozen” compounds in the initial Stockholm Convention list. Historically, PCDDs were released as by-products from chemical manufacturing, metallurgy, paper and pulp processing, and other industries (Hites, 2011). They accumulate in sediments of estuarine water bodies, such as the lower Passaic River and Newark Bay (Bopp *et al*., 1991; Wenning *et al*., 1993; Huntley *et al*., 1998), Victoria harbor, Hong Kong (Müller *et al*., 2002), Tokyo Bay (Hosomi *et al*., 2003), and Homebush Bay, Australia (Birch *et al*., 2007). PCDDs of industrial origin from the upper rivers contribute to the contamination of the corresponding estuarine and marine water bodies by means of river discharge (Huntley *et al*., 1998; Salo *et al*., 2008; Friedman *et al*., 2012). In addition, uncontrolled anthropogenic and natural combustion events contribute to PCDD deposition level into both metropolitan and pristine estuarine watersheds (Müller *et al*., 2002; Hosomi *et al*., 2003; Sundqvist *et al*., 2009; Dwyer & Themelis, 2015). Due to both human and natural activities, PCCDs are ubiquitous in urban estuaries and contaminated sediments pose risks to both human health and the environment.

PCDDs are notoriously persistent but they have been known to be biodegradable via several different processes (Bunge & Lechner, 2009). Microbial reductive dechlorination under anaerobic conditions is the most important biological process that may transform PCDDs and potentially decrease their toxicity. Reductive dechlorination of PCDDs has been observed in contaminated sediments and soils. Dehalogenating bacteria belong to diverse phylogenetic groups (Maphosa *et al*., 2010), but so far only obligately organohalide-respiring bacteria from the genera *Dehalococcoides* and *Dehalobacter* have been found to dechlorinate PCDDs (Bunge *et al*., 2003; Fennell *et al*., 2004; Yoshida *et al*., 2009b; Pöritz *et al*., 2015). PCDD dechlorination was detected

in various historically contaminated estuarine systems (Albrecht *et al*., 1999; Vargas *et al*., 2001; Ahn *et al*., 2005). However, limited information is available about the bacteria which drive dechlorination in these estuarine and marine environments in which the high salinity and sulfate concentrations shape the microbial communities (Zanaroli *et al*., 2015). Non-methanogenic, non-thermophilic, non-spore-forming microorganisms were implicated to be responsible for dechlorination of PCDDs in estuarine lower Passaic River sediment cultures, however, further characterization of these dechlorinating bacteria was not done (Barkovskii & Adriaens, 1996). Ahn and co-workers (2007) did not find evidence of known dechlorinating bacteria such as *Dehalococcoides* or *Desulfitobacterium* in their active dechlorinating culture established from estuarine sediments. Reductive dechlorination of PCCDs appears to be prevalent yet not fully understood in estuarine sulfate-impacted systems.

The lower Hackensack River, located in the New York/New Jersey Harbor estuary, is within a vibrant metropolitan area and one of the oldest industrial zones in the U.S. The river is contaminated by a wide range of pollutants, including PCDDs from both historical and emerging non-point sources due to industrial and municipal activities (Wenning *et al*., 2004). The objective of this study was to investigate the potential for reductive dechlorination of PCDDs and its relation to sampling locations along the tidal influenced sections of the Hackensack River. We also aimed to relate the relative distribution of dehalogenating bacteria belonging to the *Chloroflexi* to environmental parameters at the sampling sites.

Materials and Methods

Sediment source

Sediments were collected by grab sampling along the Hackensack River at 5 stations (H1, H2, H3, H4 and H5) in August 2012 (See Figure S2.1 for sampling locations). The uppermost station (H1) is located at 19.6 km from the river mouth and the lowermost downstream station (H5) is 2 km from the river mouth. Physicochemical parameters of sediment samples were provided by John Reinfelder and Sarah Janssen, Department of Environmental Sciences, Rutgers University, and are summarized in Table S2.1. Temperature and pH were the same at all locations at values of approximately 28 ^{o}C and 7, respectively. Total organic matter and organic carbon were slightly lower in the sediment samples collected from stations H4 and H5 than in samples taken from the upper stations. However, salinity and sulfate concentration were substantially higher near the river mouth, indicative of the high tidal influence on water and sediments at stations H4 and H5 (Table S2.1).

PCCDs.

Dibenzo-*p*-dioxin (DD) and its chlorinated congeners were purchased from AccuStandard Inc. (New Haven, CT, USA). Chlorinated congeners include 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD), 1,2,3- and 1,2,4-trichlorodibenzo-*p*-dioxins (1,2,3- and 1,2,4-TriCDDs), 1,2-, 1,3-, 1,4-, and 2,3-dichlorodibenzo-*p*-dioxins (1,2-, 1,3-, 1,4-, and 2,3-DiCDDs), 1- and 2-monochlorodibenzo-*p*-dioxins (1- and 2-MoCDDs).

Enrichment culture set up

Anaerobic enrichment cultures (50 mL) were established in triplicate using 10% w/v (5 g) of sediment as inoculum in 60 mL serum bottles sealed with Teflon^{TM} lined gray chlorobutyl-isoprene septa and crimped with aluminum caps. Carbonate buffered mineral salts medium was prepared according to Monsserate and Häggblom (1997) except that sodium chloride concentration was

1.17 g/L. Silicon oxide (silica) was used to deliver electron acceptor to dehalogenating bacteria in the enrichment cultures as described below. 0.5 g of silicon oxide (200 Mesh or finer) was added to a 60 mL serum bottle and spiked with 500 µL of 2 mM 1,2,3,4-TeCDD stock solution in toluene. Toluene was evaporated leaving a layer of 1,2,3,4-TeCDD coated on silicon oxide particles. Anaerobic sediment slurry (50 mL) was added yielding a 20 µM nominal concentration of 1,2,3,4-TeCDD. A mixture of short chain organic acids (acetate, propionate, and lactate) was added at a 150 µM concentration each as electron donors and carbon sources. Enrichment cultures were set up in an anaerobic vinyl glove box (Coy Laboratory Products Inc., Great Lakes, MI, USA) and maintained under strictly anaerobic conditions under a headspace of 3%H_2/97%N_2. Electron donors were re-supplemented every 4 months, cobalamin was supplemented at 100 µg/L after 4 months of incubation to enhance dehalogenation activity and every 4 months during the course of incubation. Killed controls were established in duplicate to determine if abiotic dehalogenation occurs. They were set up the same way as the experimental cultures, but were autoclaved for 1 hour on 3 consecutive days. Enrichment cultures were transferred when 1,2,3,4-TeCDD were depleted to a concentration below 2 µM. Subsets of active enrichment cultures (H1, and H2) were transferred 10% (v/v) into fresh medium amended with 1,2,3,4-TeCDD, 1,2,3-TriCDD, or 1,2,4-TriCDD. Less silica (i.e., 100 mg) was added in the second and third transfers to reduce the amount of inorganic carrier in the cultures.

To examine the role of sulfate, a set of subcultures was established in triplicate by transferring 3 mL of the original H1 enrichment culture into 27 mL fresh mineral medium. 1,2,3,4-TeCDD and the mixture of organic acids were supplemented as electron acceptor and carbon and energy sources, respectively, as described above. Sulfate was amended anaerobically to concentrations of 0, 2, 5, and 10 mM.

Sampling and analytical methods

One mL samples of enrichment cultures were withdrawn using a syringe purged with nitrogen gas. Subsampling was performed every month over the course of 13 months for the first enrichment or 5 months for determination of dehalogenation pathways.

Chlorinated dibenzo-*p*-dioxins were extracted using a 1.5 mL mixture of toluene and acetone (1:1) on a shaker at 60 rpm for 3 h. The toluene phase which contained chlorinated dibenzo-*p*-dioxins was transferred to a glass vial and analyzed using an Agilent 6890 series Gas Chromatograph coupled with an Agilent 5973 series Mass Spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a HP-5MS capillary column (60 m x 0.25 mm, 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA). The oven temperature program was as follows: hold at 50 ^{o}C for 2 min, increase by 12 ^{o}C/min to 150 ^{o}C, then increase by 8 ^{o}C/min to 300 ^{o}C, and hold at 300 ^{o}C for 1 min. Chlorinated dibenzo-*p*-dioxins were identified based on their retention times and their most dominant molecular ions in comparison to authentic standards listed above. Quantification was performed using an external standard four-point calibration curve of mixture of 1,2,3,4-TeCDD, 1,2,3-TriCDD, 1,3-DiCDD, 1-MoCDD, and DD with concentration ranging from 0.2 µM to 20 µM each. The mol% of each dibenzo-*p*-dioxin congener or the sum of all congeners belonging to the same group was calculated and presented with the assumption that no transformation other than reductive dechlorination occured under the experimental conditions.

Putative dehalogenating Chloroflexi community

Enrichment cultures were sampled (2-3 mL) at time 0 and after 399 days of incubation and stored at -80 ^{o}C until further analysis. Genomic DNA of cultures was isolated using PowerSoil® DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). The 16S rRNA gene of putative dehalogenating bacteria belonging to the *Chloroflexi* was amplified using nested PCR with primer pairs 338F – Chl1101R and 341F-GC – 534R as described (Krumins *et al*., 2009; Park *et al*., 2011).

PCR products were analyzed on an 8% polyacrylamide gel with a denaturing gradient from 40% to 60% on a DCode™ Universal Mutation Detection System. 100% denaturant contained 7 M urea and 40% formamide. The electrophoresis was performed at 40 V for 17 h. The gel was then stained in 1 µg/mL ethidium bromide solution for 20 min and visualized under UV light. Gel pictures were taken with a PhotoDoc-It™ Imaging System (UVP, LLC., Upland, CA, USA). DGGE banding patterns were analyzed by Bionumerics 7.0 (Applied Maths Inc., Austin, TX, USA). Band matching was carried out based on the relative location of each band when compared to reference bands. The number and intensity of bands on each lane represent number and relative abundance of 16S rRNA gene sequences of the *Chloroflexi* community present in enrichment cultures, respectively. A distance matrix of all bands present in normalized gels was calculated using Pierson correlation. Cluster analysis was performed using the UPGMA algorithm and was visualized in a dendrogram.

16S rRNA gene clone library and identification of DGGE bands

We used a clone library approach in order to obtain representative clones for members of the *Chloroflexi* community with sufficiently long reads enabling a more accurate phylogenetic analysis. Approximately 750 bp of the 16S rRNA genes were amplified from DNA isolated from enrichment cultures H1-1, and H5-2 using the 338F and Chl1101R primer pair. PCR products were ligated into the pGEM®-T Easy Vector system and transformed into JM109 High Efficiency Competent Cells (Promega Corporation, Madison, MI, USA). Plasmid DNA of clones in the library were isolated using the Zyppy™ Plasmid Miniprep kit (Zymo Research Corporation, Irvine, CA, USA) and screened by nested PCR – DGGE as described above. DGGE banding patterns of clones in the library were compared to that of mixed community. Plasmid DNAs of the desired clones were sent for Sanger sequencing at Genewiz® (South Plainfield, NJ, USA).

Phylogenetic analysis of the 16S rRNA genes were conducted using MEGA version 6 (Tamura *et al*., 2013). These included sequences of the inserted 16S rRNA genes of 7 clones in our clone

library and 14 reference sequences imported from GenBank. Alignment was done using the built-in ClustalX program in MEGA. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model with 500 bootstrap replications. The tree with the highest likelihood was shown. The partial 16S rRNA gene sequences were deposited in GenBank under accession numbers KX831893 to KX831893.

Quantitative PCR (qPCR)

Dehalococcoides 16S rRNA genes were quantified using primers DhcF (5'-GGA GCG TGT GGT TTA ATT CGA TGC-3') and DhcR (5'-GCC CAA GAT ATA AAG GCC ATG CTG-3') (Adrian *et al*., 2007a). The qPCR reactions were carried out on an iCycler iQ™ Real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA) using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA). A five-point standard curve was produced from 10-fold serial dilutions using plasmid containing a Dehalococcoides 16S rRNA gene from clone H1-C8 in the clone library.

Reductive dehalogenase gene profiles

Reductive dehalogenase homologue A (*rdhA*) genes were amplified using 12 primer pairs which were designed to amplify a set a *rdhA* genes common in *Dehalococcoides mccartyi* strains 195 and CBDB1. Primer sequences and PCR conditions were described in Park *et al*. (2011). Seven microliters of PCR products were analyzed on 1% agarose gel and visualized on a UV transilluminator. Pictures were taken and band intensity was measured and analyzed using the ImageJ software (Rasband, 1997). A Student's t-test was performed on Microsoft® Excel to determine the difference in band intensity of rdhA gene profiles of different cultures.

Results

Dechlorination of chlorinated dibenzo-p-dioxins in Hackensack River sediment enrichment cultures

Dechlorination of 1,2,3,4-TeCDD was observed in all five sets of enrichment cultures established with sediments from the Hackensack River. The rate and extent of dechlorination, however, differed depending on the sediment source and tidal influence. Dechlorination was most rapid and complete in the H1 and H2 cultures established from the uppermost stations. After 399 days of incubation, 90% of spiked 1,2,3,4-TeCDD in H1 cultures was dechlorinated with 2-MoCDD accumulating as the end-product (Figure 2.1A). Cultures H4 and H5, which were established using sediments collected from the lower section of the river, showed accumulation of trichlorinated dibenzo-*p*-dioxin congeners at 3.5 and 16 mol%, respectively.

No dehalogenation products were detected in killed controls during the course of 399 days (Figure 2.1A), indicating that abiotic processes did not contribute to dechlorination of 1,2,3,4-TeCDD. However, the concentration of 1,2,3,4-TeCDD in killed controls decreased over time (to 16.7 ± 2.3 mM at the end of 502 day incubation period). The loss of 1,2,3,4-TeCDD can be explained by its hydrophobicity and its tendency to absorb onto rubber septa that were exposed after the septa were punctured for sampling. A reduction in the total PCDD concentration in experimental cultures was also observed. Nonetheless, it is noteworthy that dechlorination was microbially mediated, as evidenced by the substantial decrease in relative concentration of 1,2,3,4-TeCDD and concurrent increase in that of its daughter products (i.e., 1,2,3- and 1,2,4-TriCDD, 1,3- and 2,3-DiCDD, and 2-MoCDD).

The difference in the 1,2,3,4-TeCDD dechlorination activity of H1 and H5 cultures is illustrated from the time course (Figures 2.1B and 2.1C). Dechlorination in H1 enrichment cultures had a relatively short lag phase of 77 days, followed by a rapid decrease in the mole percentage of

1,2,3,4-TeCDD, with sequential accumulation of 1,2,3- and 1,2,4-TriCDD, 1,3- and 2,3-DiCDD and 2-MoCDD (Figure 2.1B). In contrast, dechlorination in H5 enrichment cultures started after 213 days and dechlorination proceeded to a lesser extent (Figure 2.1C). Overall, these results indicate that H1 enrichment cultures exhibited faster dechlorination than H5 cultures and they were successfully maintained over several transfers by sub-culturing (data not shown).

In order to determine whether sulfate concentration influenced dechlorination by the Hackensack River sediment microbial community, a set of subcultures was established using the H1 enrichment culture. Sulfate was amended to concentrations from 0 to 10 mM and the extent of 1,2,3,4-TecDD dechlorination was recorded after 76 days of incubation. Without sulfate amendment, 1,2,3,4-TeCDD was dechlorinated with accumulation of 55.6 ± 17.6 mol% MoCDD. Dechlorination activity was inhibited in subcultures amended with sulfate (2 – 10 mM): 70-73 mol% of 1,2,3,4-TeCDD remained in these subcultures, with accumulations of 15.7 – 16.9 mol% DCDD and 8.3 – 10.3 mol% MoCDD (Figure 2.2).

Dechlorination pathway of 1,2,3,4-TeCDD in H1 enrichment cultures

In our original enrichment cultures, 1,2,3- and 1,2,4-TriCDDs were detected as transient dechlorination products which were quickly transformed to less chlorinated dibenzo-*p*-dioxins (Figures 2.1B and 2.1C). This causes difficulties in inferring the dechlorination pathway directly from the 1,2,3,4-TeCDD amended culture. In order to deduce the pathway of 1,2,3,4-TeCDD dechlorination, three sets of subcultures were established in which 1,2,3,4-TeCDD and its intermediate products, 1,2,3- and 1,2,4-TriCDD, were amended individually. Dechlorination of chlorinated substrates in H1 sub-cultures was monitored over the course of 138 days. The extent and rate of 1,2,4-TriCDD dechlorination was greater than that of 1,2,3-TriCDD and 1,2,3,4-TeCDD dechlorination. There was a steep decrease in the relative concentration of amended 1,2,4-TriCDD by 51 days after cultures were established (Figure 2.3C). Different dechlorinated dibenzo-*p*-dioxin

congeners accumulated in cultures amended with the different TriCDD substrates. In 1,2,4-TriCDD amended cultures, 1,3-DiCDD reached its peak after 51 days of incubation, followed by a rapid decrease, and remained at a low relative concentration while the concentration of 2-MoCDD increased steadily (Figure 2.3C). In 1,2,3-TriCDD spiked cultures, 2,3-DiCDD was produced but was not dechlorinated further. On day 138, 2,3-DiCDD was detected at 53.9 ± 16.5 mol% of total chlorinated dibenzo-*p*-dioxins detected (Figure 2.3B). In contrast, 2,3-DiCDD was not detected at a substantial level in either 1,2,4-TriCDD or 1,2,3,4-TeCDD fed cultures. The accumulation of 2-MoCDD and the lack of 2,3-DiCDD in 1,2,4-TriCDD and 1,2,3,4-TeCDD fed cultures demonstrate that dechlorination of 1,2,3,4-TeCDD was predominantly via 1,2,4-TriCDD.

Analysis of the Chloroflexi community

The bacterial communities of the dechlorinating cultures were examined, with a focus on putative dechlorinating *Chloroflexi*. The *Chloroflexi* communities of the five sets of enrichment cultures at time 0 and after 399 days were investigated by PCR-DGGE (Figure 2.4). Cluster analysis of the *Chloroflexi* communities in enrichment cultures at time 0 and after 399 days of incubation revealed 2 distinct groups (Figure S2.2). The first group consisted of enrichment culture H5 at 399 days of incubation and all 5 sets of sediment cultures at time 0. The second group corresponded to enrichment cultures established with sediments from sites H1 to H4 after 399 days of incubation. The exception was the H4-1 enrichment culture which grouped within group 1 (Figure S2.2). However, only 16% of 1,2,3,4-TeCDD was dechlorinated in the H4-1 culture, while in other replicates the extent of 1,2,3,4-TeCDD dechlorination was 56% and 42%. The discrepancy within replicates of H4 cultures may be explained by a low abundance of targeted *Chloroflexi* (or other dehalogenating bacteria) in sediments when the sample was collected, which in turn, could lead to their disproportional distribution into triplicate cultures. The *Chloroflexi* community in the Hackensack River sediments, represented in enrichment cultures at time 0, clustered into 3

subgroups based on their locations of the river, i.e., subgroups H1 – H2, H3 – H4, and H5. This implies that there was a subtle difference in the structure of the sediment *Chloroflexi* community along the river. The enrichment cultures from H5, established using sediment taken from the most tidally influenced estuarine section at the mouth of the Hackensack River, were distinctly different from the other sites and the *Chloroflexi* community structure did not substantially change during the course of incubation. DGGE banding patterns of the enrichment cultures H5 after 399 days were almost similar to that of sediment collected at location H5 (Figure 2.4A). In contrast, enrichment on 1,2,3,4-TeCDD as the terminal electron acceptor drastically shifted the *Chloroflexi* community structure in H1-H4 enrichment cultures.

In order to assign phylogenetic identity to each DGGE gel band, two clone libraries of bacterial 16S rRNA genes (750 bp) of one replicate of enrichment cultures H1 and H5 (i.e., H1-1 and H5-2) were constructed. 40 clones of each library were screened using the same PCR-DGGE procedure applied to enrichment cultures. The banding pattern of each clone was compared to those of their corresponding parent cultures. Seven representative clones were selected for sequence analysis of the inserted 16S rRNA genes (Figure 2.4). The 16S rRNA gene clone H1-C8, which produced the same PCR-DGGE pattern as indicated in Figure 2.4A, had 99% sequence similarity to the 16S rRNA gene sequence of *Dehalococcoides mccartyi* strains CBDB1 and DCMB5, with only one base pair difference. *D. mccartyi* strains CBDB1 and DCMB5 are known to mediate respiratory dechlorination of PCDDs (Bunge *et al*., 2003; Pöritz *et al*., 2015). The 16S rRNA gene sequence of clone H1-C8 was distinct from that of *D. mccartyi* strain 195 with 8 bp differences. Other clones that originated from the H5-2 cultures showed closest similarity to non-dechlorinating bacteria belonging to the *Chloroflexi* phylum (Figure 2.4B). *D. mccartyi* became the most dominant bacterium within the *Chloroflexi* phylum in the upstream enrichment cultures which accounted for 41 to 64% of *Chloroflexi* community according to the intensity of the corresponding DGGE

band H1-C8 (Figure 2.4A; Table S2.2). Quantitative PCR of *Dehalococcoides* 16S rRNA genes revealed that H1 enrichment cultures contained 4.3 x 10^5 gene copy number/ng DNA isolated, which was about an order of magnitude higher than in H5 enrichment cultures ($p < 0.001$) (Figure 2.5). The *Dehalococcoides* 16S rRNA gene copy number was higher when more 1,2,3,4-TeCDD was dechlorinated indicating that growth of the *Dehalococcoides* population in our enrichment cultures was supported by reductive dechlorination of 1,2,3,4-TeCDD.

Reductive dehalogenase gene amplification and band intensity analysis

16S rRNA gene sequences are conserved among *Dehalococcoides mccartyi* and do not usually reflect their ability to dechlorinate a specific organohalide (Kube *et al*., 2005; McMurdie *et al*., 2009; Hug *et al*., 2013). Therefore, we examined the *Dehalococcoides* spp. populations in our enrichment cultures by analyzing the reductive dehalogenase gene (*rdhA*) profile (Figure 2.6). The relative abundance of a set of 12 *rdhA* genes (designated here as *rdhA1* to *rdhA12* based on the PCR primer set numbering of Park *et al*. (2011)) differed in the enrichment cultures established from different sites along the Hackensack River. The 12 *rdhA* genes in H5 enrichment cultures were barely detected on agarose gels, which matched with the low abundance of *Dehalococcoides* spp. 16S rRNA genes based on DGGE analyses. The relative abundance of six *rdhA* genes (*rdhA3, rdhA6, rdhA7, rdhA9, rdhA11,* and *rdhA12*), which are associated within reductive dehalogenase orthologue groups 22, 30, 31, 33, 13, and 15, respectively, according to the classification system of reductive dehalogenase genes proposed by Hug *et al*. (2013), were higher in cultures in which more 2-MoCDD accumulated. The relative abundances of these six *rdhA* genes were at least 2 times higher in H1 than in H5 enrichment cultures ($p < 0.001$). Interestingly, the *rdhA12* band intensity in H1 cultures was 7.2 times higher than in H5 cultures ($p < 0.001$) (Figure 2.6). The relative abundance of *rdhA4, rdhA5, rdhA8,* and *rdhA10*, which belongs to orthologue groups 36, 20, 32 and 34, did not substantially vary among the five sets of enrichment cultures and their

relative abundances did not exceed 1.8 times compared to that of the H5 cultures. The relative abundance of *rdhA1* and *rdhA2* (orthologue groups 29 & 47, and 22) did not correspond to dechlorinating activity. Their band intensities were low in both cultures with lowest and highest dechlorinating activity and highest in H2 and H3 cultures with mediocre dechlorination activity (Figure 2.6).

Discussion

Dechlorination of 1,2,3,4-TeCDD in enrichment cultures of estuarine sediments

Dechlorination of 1,2,3,4-TeCDD was observed in all anaerobic enrichment cultures established with sediments collected along the tidally affected area of the Hackensack River. The extent of 1,2,3,4-TeCDD dechlorination differed among the 5 sets of enrichment cultures and tended to decrease when salinity and sulfate concentration of the site increased. A similar effect was also observed for microbially mediated reductive dechlorination of other chlorinated aromatic compounds including chlorobenzenes, bromobenzenes and pentachloroaniline established with samples from the same sites of the Hackensack River (Sohn & Häggblom, 2016). Sulfate concentrations of 2 mM or higher inhibited dechlorination of 1,2,3,4-TeCDD by the microbial community. Effects of salinity and sulfate concentration on dechlorination have been studied but the results are not consistent (Zanaroli *et al*., 2015). Dechlorination has been observed to be generally inhibited by the presence of high sulfate concentrations. In a previous study, the lag phase of PCDD dechlorination in sulfate reducing enrichment cultures was substantially longer than in those maintained under methanogenic conditions (Ahn *et al*., 2005). Similarly, dechlorination of tetrachloroethene occurred at a lower rate and to a lesser extent in cultures at high sulfate concentrations (Berggren *et al*., 2013). It is postulated that dechlorination is strongly inhibited because of competition between sulfate reducing bacteria and dehalogenating bacteria

for hydrogen as well as by high levels of sulfide accumulated under sulfidogenic conditions (Ahn *et al*., 2005; Berggren *et al*., 2013; Panagiotakis *et al*., 2014). In contrast, Fu *et al*. (2001) observed an increase in dechlorination rate and extent as salinity increased in enrichment cultures from the Passaic River. Moreover, different dechlorination pathways observed under different sulfate concentrations imply the involvement of different microorganisms in dechlorination (Berkaw *et al*., 1996; Fu *et al*., 2001).

The deduced pathway of 1,2,3,4-TeCDD dechlorination by H1 enrichment cultures is shown in Figure 2.7. Dechlorination of 1,2,3,4-TeCDD in the Hackensack River cultures had sequential peri- and lateral- dechlorination steps which resembles the pathway observed in *D. mccartyi* CBDB1 cultures (Bunge *et al*., 2003) and 195 cultures (Fennell *et al*. 2004), as well as in enrichment cultures established from freshwater sediments (Ballerstedt *et al*., 1997; Bunge *et al*., 2001; Liu *et al*., 2014). However, dechlorination of 1,2,3,4-TeCDD by Hackensack River enrichment cultures slightly differs from other cultures which contain *Dehalococcoides* spp. The extended lag phase for 1,2,3,4-TeCDD dechlorination and the accumulation of 2,3-DiCDD in 1,2,3-TriCDD fed cultures suggests that lateral-dechlorination is the rate-limiting step during dechlorination of 1,2,3,4-TeCDD (Figure 2.3). Dechlorination via 1,2,4-TriCDD is likely to be the major route in the Hackensack River cultures, whereas dechlorination via 1,2,3-TriCDD is the more dominant route by *D. mccartyi* strains CBDB1 and DCMB5 (Bunge *et al*., 2003; Pöritz *et al*., 2015). Kuokka *et al*. (2014) also observed a similar dechlorination pathway with 1,2,3,4-tetrachlorodibenzofuran. The lack of 2,3-substituted products is assumed to reduce the toxicity of chlorinated dibenzo-*p*-dioxins and other chlorinated dioxin-like compounds which have the same toxicity mechanism (Schecter *et al*., 2006). Most of the peri-dechlorination steps in the pathway (i.e., removal of peri-chlorine from 1,2,3-TriCDD and 1,2,4-TriCDD) are thermodynamically less favorable compared to lateral-dechlorination from the same parent chlorinated dibenzo-*p*-dioxins (Huang *et al*., 1996). The

preference to dechlorinate chlorinated dibenzo-*p*-dioxins via peri-dechlorination in the Hackensack River cultures might be explained by the higher accessibility of the chlorine-carbon linkage at the lateral position or the adaptation of dechlorinating bacteria (Ballerstedt *et al*., 1997). Vargas *et al*. (2001) and Ahn *et al*. (2005) also observed the dechlorination of 1,2,3,4-TeCDD via 1,2,4-TriCDD in their estuarine enrichment cultures. However, 1,2,3-TriCDD and its dechlorination products were not found in their enrichment cultures and no further dechlorination of 1,3-DiCDD was detected. Dechlorination of 1,2,3,4-TeCDD by a mixed culture of *D. mccartyi* 195 proceeded via the same pathway (Fennell *et al*., 2004) as for the estuarine enrichment cultures, suggesting that it has a limited ability to dechlorinate 1,2,3,4-TeCDD compared to dehalogenating bacterial population in the Hackensack River enrichment cultures.

Influence of environmental factors on dehalogenating community

Enrichment on 1,2,3,4-TeCDD drastically changed the *Chloroflexi* community in actively dechlorinating cultures. A *Dehalococcoides* spp. was the most dominant OTU accounting for up to 64% of *Chloroflexi* OTUs detected by PCR-DGGE in cultures in which 1,2,3,4-TeCDD was dechlorinated to 2-MoCDD. The variance in the relative abundance of a set of 12 *rdhA* genes implied that there might be more than one *Dehalococcoides* species with distinct *rdhA* gene profiles in the Hackensack enrichment cultures. *D. mccartyi* is known to respire a wide range of organohalides and is the only bacterial species so far that can dechlorinate PCDDs or polychlorinated dibenzofurans with more than 4 chlorines (Liu & Fennell, 2008; Yoshida *et al*., 2009b; Zhen *et al*., 2014). Although *Dehalococcoides* spp. are widespread in sediment environments, they are typically found at low abundance and are often outcompeted by faster growing organisms such as methanogens in freshwater systems or sulfate reducing bacteria in marine environments (Yang & McCarty, 1998; Wasmund *et al*., 2014). The presence of *Dehalococcoides* spp. is usually associated with organohalide contamination and its abundance

increases during organohalide dechlorination (Krzmarzick *et al*., 2012). Most PCDD dechlorinating *Dehalococcoides* spp. have been enriched from freshwater sediments (Adrian *et al*., 1998; Bunge *et al*., 2003; Pöritz *et al*., 2015). The abundance of the *Dehalococcoides* population in the Hackensack River sediments had a strong negative correlation with salinity and sulfate concentration in sediments, and were found only in sediments with sulfate levels less than 650 mg/L (6.7 mM). *Dehalococcoides* spp. have been found in a few estuarine sediments and actively dechlorinate spiked organohalide contaminants. Berggren *et al*. (2013) observed a decrease in PCE dechlorination rate but did not see the decrease in the ratio of *Dehalococcoides* to total bacteria in cultures which contained 1 mM sulfate. Interestingly, they observed a shift in the community from *Dehalococcoides* belonging to Pinellas to Cornell groups, which likely contributed to the rate reduction of PCE dechlorination. This finding suggests that some *D. mccart*yi are tolerant of high salinity and sulfate concentrations. Some dehalogenating *Chloroflexi* species are also found in estuarine and marine environments, however, they are distantly related to *Dehalococcoides* (Fagervold *et al*., 2005; Yan *et al*., 2006; Cutter *et al*., 2001; May *et al*., 2008). In our study, *Dehalococcoides* spp. was enriched and probably responsible for 1,2,3,4-TeCDD dechlorination in enrichment cultures in which sulfate concentration of the inoculum was less than 650 mg/l or 6.7 mM.

Conclusions

Here we demonstrate dechlorination of 1,2,3,4-TeCDD in enrichment cultures of estuarine river sediment. Reductive dechlorination and abundance of *Dehalococcoides* spp. were found to have strong negative correlation with salinity and sulfate concentration in the tidally affected river. This is one of the few studies that has detected the presence of a community in which *Dehalococcoides* spp. are dominant and active in an estuarine system. Reductive dechlorination

is the only process that transforms highly chlorinated dibenzo-*p*-dioxins under reducing conditions such as in soils or sediments and plays an important role in bioremediation. Products of PCDD dechlorination are more susceptible to aerobic degradation leading to complete mineralization, implying that the activity of dechlorinating bacteria can play a major role in bioremediation of PCDD contaminated estuarine sediments located in highly urbanized areas.

Acknowledgements

We thank Drs. Sarah Janssen and John Reinfelder (Department of Environmental Sciences, Rutgers University) for providing us with Hackensack River sediments and information on the physiochemical properties of samples. This study was supported in part by the New Jersey Water Resource Research Institute and the New Jersey Agricultural Experiment Station. Hang Dam was a recipient of a Vietnam International Education Development (VIED) fellowship.

Figure 2.1. 1,2,3,4-TeCDD dechlorination of Hackensack enrichment cultures. (A) Relative mole distribution of different chlorinated dibenzo-*p*-dioxin (CDD) congeners after 399 days of incubation. Abiotic control culture (HK) was autoclaved for one hour on three consecutive days. (B, C) Time course of 1,2,3,4-TeCDD dechlorination in enrichment cultures H1 and H5, respectively. The H1 cultures were respiked with 1,2,3,4-TeCDD on day 410 (denoted by arrow). The respike of 1,2,3,4-TeCDD caused the decline in mole percentage of the residual MoCDD. Data points represent the mean and standard deviations of triplicate cultures.

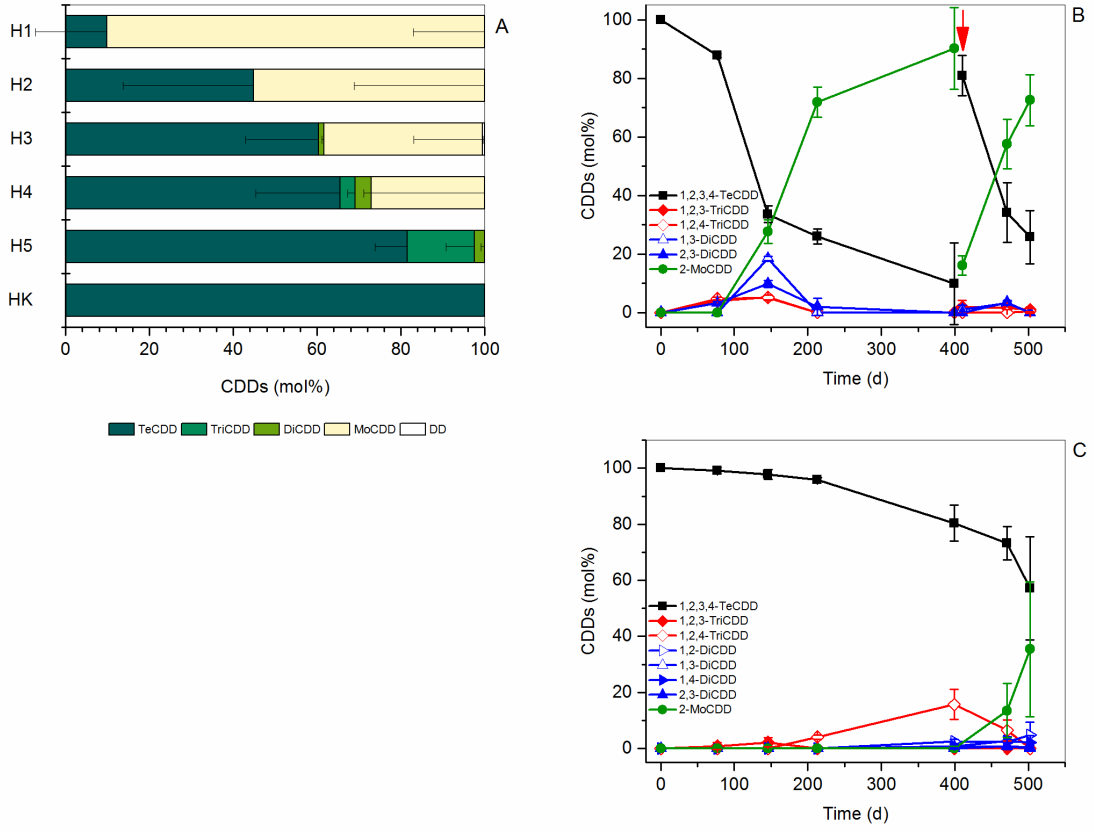


Figure 2.2. Dechlorination of 1,2,3,4-TeCDD by H1 subcultures supplemented with 0 – 10 mM sulfate after 76 days of incubation. Data points represent mean and standard deviation of the sum of all congeners that have the same number of chlorines.

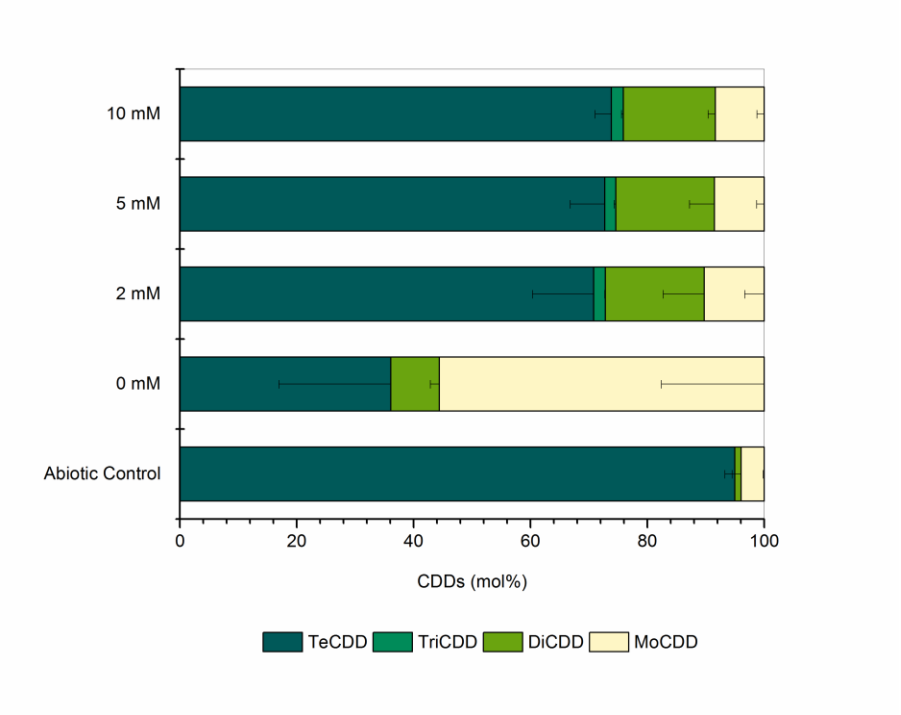


Figure 2.3. Dechlorination of 1,2,3,4-TeCDD (A), 1,2,3-TriCDD (B), and 1,2,4-TriCDD (C) in subcultures established by 10% (v/v) transfer from the initial enrichment culture H1. Mean values and standard deviations of mole percentage of chlorinated dibenzo-*p*-dioxins of triplicatecultures are shown.

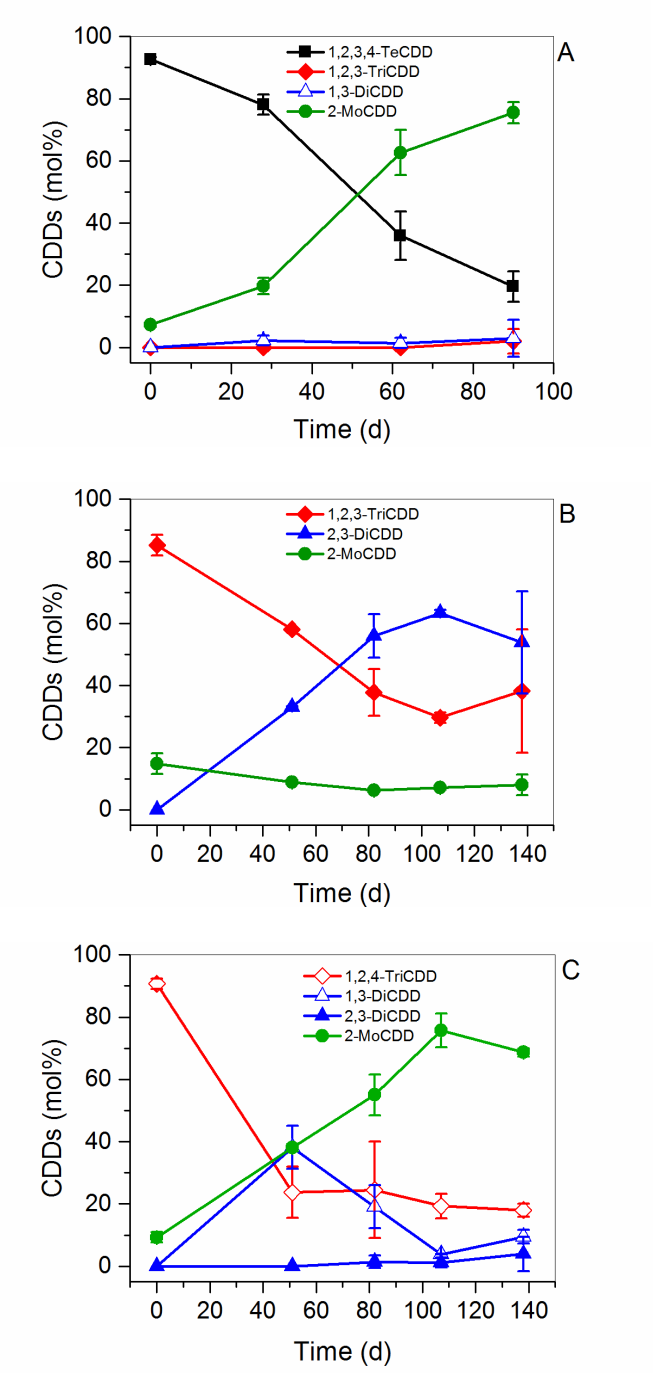


Figure 2.4. Analysis of *Chloroflexi* community in Hackensack River sediment enrichment cultures. (A) Nested PCR – DGGE analysis. DNA was isolated and analyzed from cultures at time 0 and from triplicate cultures after 399 days of incubation. The marked bands possessed the same migration behavior as the insert of the corresponding clones. (B) Phylogenetic tree of 16S rRNA gene sequences of clones corresponding to marked DGGE bands and selected sequences from GenBank. Bootstrap values (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 794 positions in the final dataset.

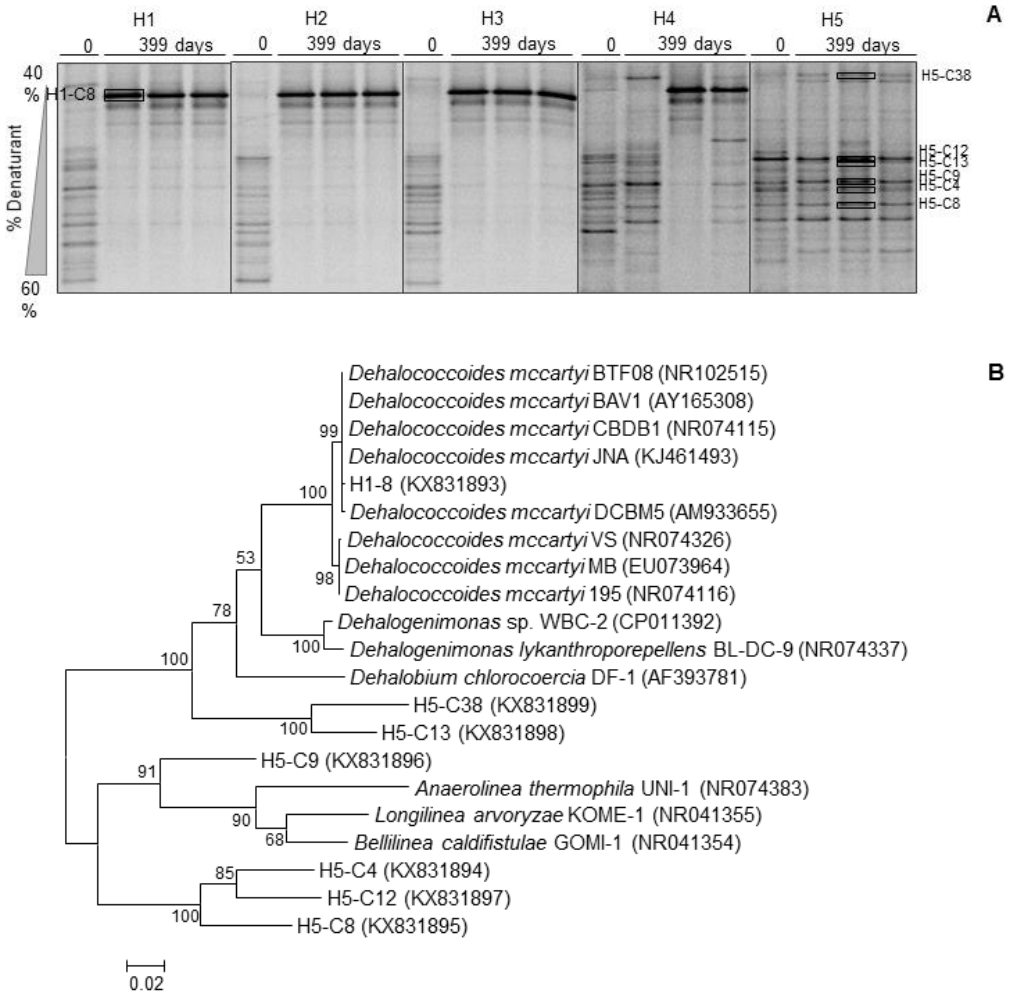


Figure 2.5. Correlation between *Dehalococcoides mccartyi* 16S rRNA gene copy number and percentage of 1,2,3,4-TeCDD dechlorinated in Hackensack enrichment cultures after 399 days of incubation. Mean values and standard deviations are shown. The line represents linear regression of data points.

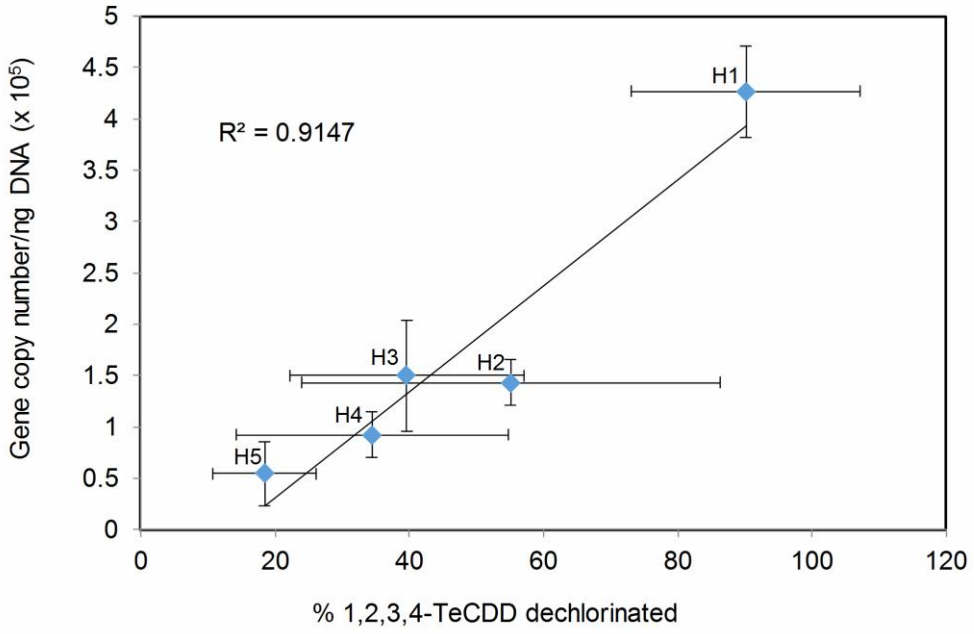


Figure 2.6. Relative PCR band intensity of 12 putative reductive dehalogenase (*rdh*) genes (mean ± standard deviation) amplified from Hackensack enrichment cultures after 399 days of incubation.

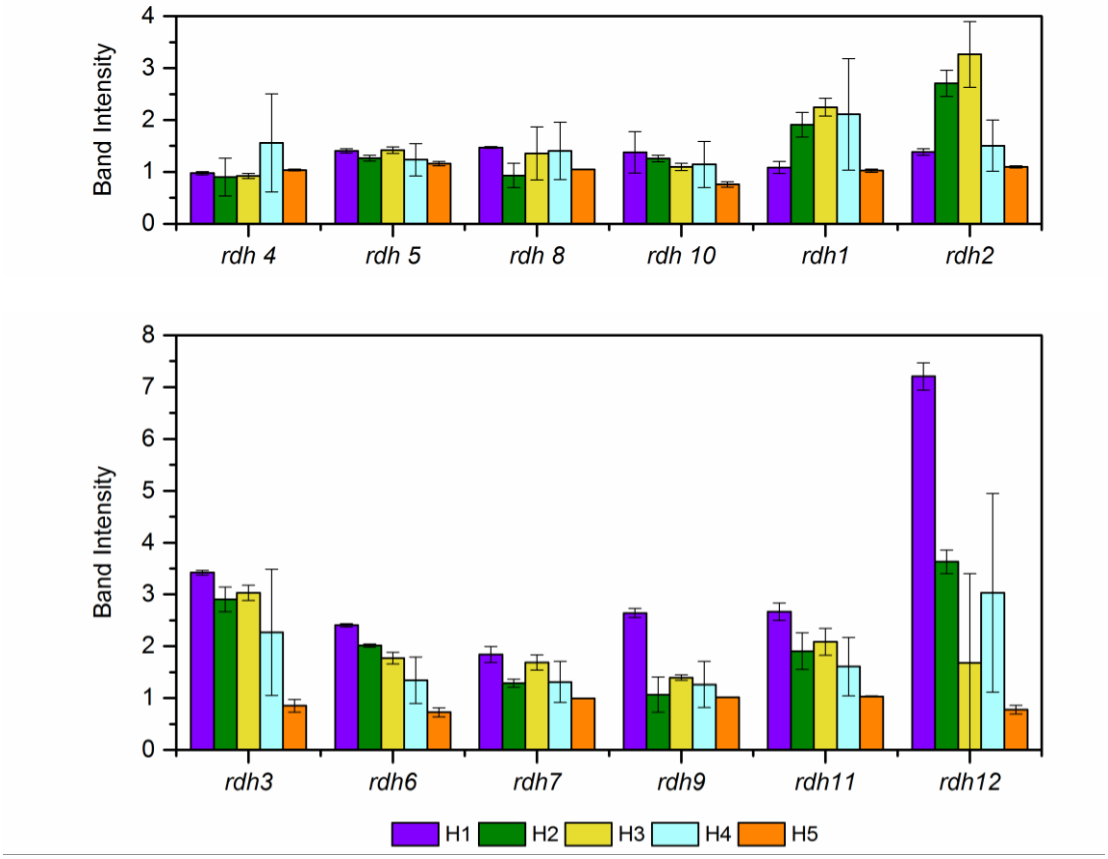
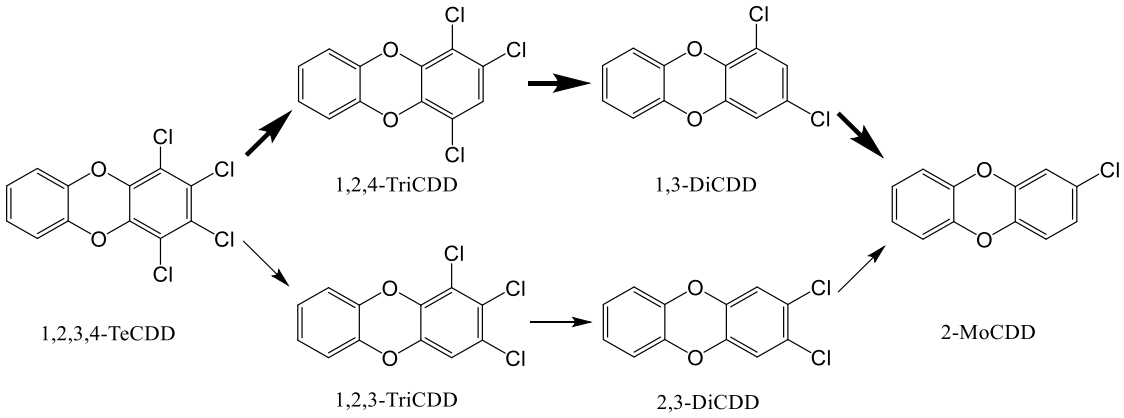


Figure 2.7. Dechlorination pathway of 1,2,3,4-TeCDD in H1 enrichment culture. The major dechlorination route is illustrated by bold arrows.



SUPPLEMENTARY DATA

Figure S2.1. Sampling location on the Hackensack River, New Jersey, USA.

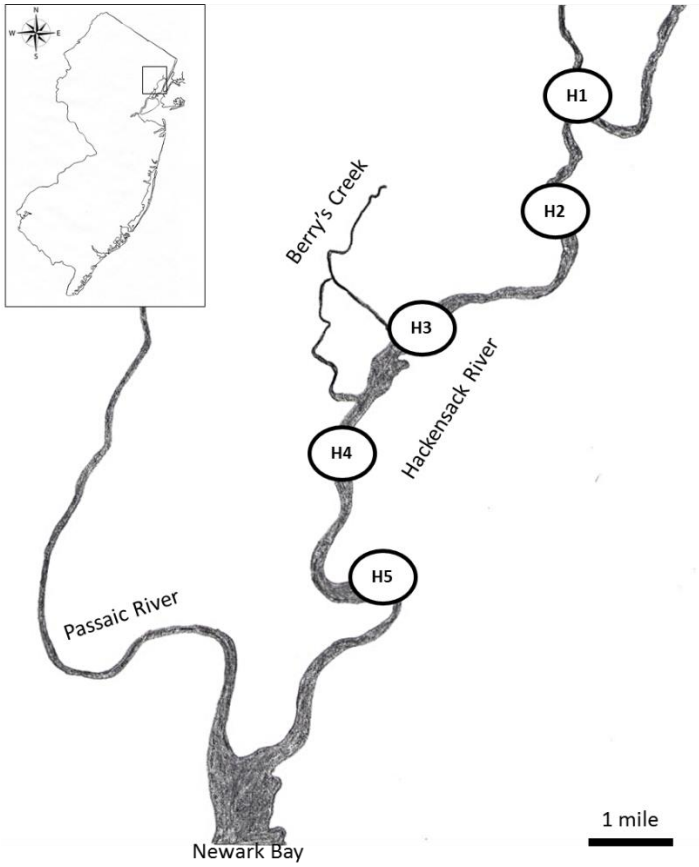


Figure S2.2. Cluster analysis of putative dehalogenating *Chloroflexi* in Hackensack enrichment cultures. DNA was isolated and PCR-DGGE was implemented from five sets of enrichment cultures (H1 to H5) at the 0 day time-point (designated as H1-0, H2-0, etc.) and three distinct replicates at 399 days (designated as H1-1, H1-2, and H1-3, etc.).

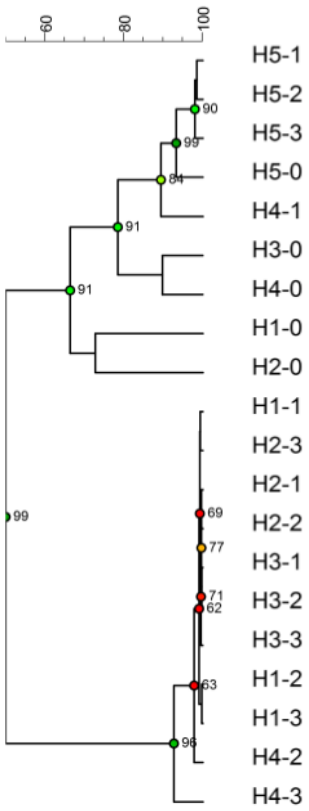


Table S2.1. Physicochemical parameters of sediments collected from the Hackensack River in Summer, 2012.

Sampling station	Latitude	Longitude	Temp (°C)	pH	Organic matter (%)	Organic carbon (%)	Salinity (ppt)	Sulfate (mg/L)
H1	40.849446	-74.030282	28	7.5	12.1	4.8	4.2	348
H2	40.824721	-74.03552	28	7.7	13.8	5.5	5.8	445
H3	40.802262	-74.065859	28	7.7	10.2	4.4	8.5	647
H4	40.777879	-74.090422	28	7.5	8.6	3.8	12	1130
H5	40.74179	-74.079431	28	7.4	9.4	3.9	17.1	1609

Janssen SE, Johnson MW, Blum JD, Barkay T, Reinfelder, JR, 2015. Separation of monomethylmercury from estuarine sediments for mercury isotope analysis. Chemical Geology 411, 19–25.
SE Janssen and JR Reinfelder, personal communication

Table S2.2. Relative abundance of OTUs in *Chloroflexi* community in enrichment cultures.

Band location	H1-0	H1-1	H1-2	H1-3	H2-0	H2-1	H2-2	H2-3	H3-0	H3-1	H3-2	H3-3	H4-0	H4-1	H4-2	H4-3	H5-0	H5-1	H5-2	H5-3
19.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.9	0	0	0	0
26.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.18	0	0	0	0
28.9	0	3.57	0	0	1.05	0	3.09	0	0.8	2.72	0	0	0	0	0	0	0	0	0	0
29.9	0	0	0	0	0.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
38.4	2.53	0	4.63	0	0	0	0	0	0	0	0	4.58	0	0	0	0	0	0	1.26	1.18
40.4	0.92	0	0	0	0	0	0	0	0	4.27	0	0	0	0	0	0	0	0	0	0
42.3	7.57	0	0	0	2.44	0	0	0	4.18	0	0	0	0	0	0	7.26	2.07	5.61	5.87	5.46
43.2	0	0	0	0	0	0	0	0	4.23	0	0	0	3.66	11.7	0	0	2.33	4.34	4.88	4.85
44.6	**4.72**	**57.4**	**49.2**	**48.8**	**4.19**	**54.7**	**53.4**	**63.7**	**0**	**52.3**	**58**	**52.9**	**3.15**	**0**	**57.9**	**40.2**	**0**	**0**	**1.66**	**1.49**
46.5	2.19	20.5	20	21.3	0	21.5	19.8	21.9	1.84	20.2	21.1	19.9	0	0	20.8	10.5	0	0	0	0
48.7	0	0	0	0	2.41	0	0	0	0.96	0	0	0	0.95	2.59	0	0	0	0	1.66	2.09
49.9	1.27	8.43	9.54	11.5	1.71	10.8	10.1	9.6	1.81	9.28	10.5	8.66	0	0	10.2	4.68	2.38	1.9	2.5	2.4
51.6	0	4.37	5.75	6.39	0	5.8	5.25	4.77	0	5.01	5.92	5.99	1.2	0	5.56	0	0	0	0	0
54.5	4.09	0	2.7	3.21	2.76	0	0	0	2.63	0	0	0	3.15	5.79	0	10.9	3.42	3.77	4.6	3
56.4	5.13	0	0	0	12.2	1.8	2.21	0	6.89	0	0	0	7.31	5.5	0	0	7.06	3.96	4.46	4.97
57.5	8.24	0	3.47	0	0	0	0	0	0	1.55	0	0	7.96	0	0	3.99	17.8	17.5	14.8	15.4
58.2	0	2.23	0	3.21	0	1.8	1.77	0	0	0	1.38	2.91	0	8.9	0	0	0	0	0	0
59.3	0	0	0	0	0	0	0	0	0	0	0	0	5.59	6.57	0	0	6.82	6.63	5.44	0
60.2	3.77	0	0	0	0	0	0	0	5.03	0	0	0	0	0	0	0	3.52	0	0	0
61.5	9.72	1.78	3.09	3.62	6.25	1.35	0	0	0	1.94	1.38	2.91	11.2	18.4	2.4	6.86	8.37	14.6	12.6	13.5
62.6	6.24	0	0	0	0	0	2.21	0	10.5	0	0	0	8.21	6.57	0	2.9	9.15	8.85	7.48	9.67
63.9	0	0	0	0	6.49	0	0	0	8.97	0	0	0	7.06	5.15	1.2	0	5.02	0	4.39	0
65	0	0	0	0	3.8	0	0	0	7.1	0	0	0	0	0	0.8	0	6.37	9.51	7.55	10.9
65.7	0	0	0	0	0	0	0	0	5.3	1.17	0.92	0.83	6.33	8.26	0	3.63	0	0	0	0

66.9	6.02	0	0	0	4.5	0	0	0	0	0	0	0	4.06	0	0	0	0	0	0	10.9
68.3	8.84	1.78	1.54	2.01	10.4	2.25	0	0	4.23	1.55	0.92	1.25	6.67	11	1.2	3.99	7.58	13	10.1	2.09
69.7	2.82	0	0	0	8.34	0	2.21	0	8.49	0	0	0	12.9	2.56	0	0	3.3	1.55	1.87	0
71.3	9.19	0	0	0	0	0	0	0	9.55	0	0	0	2.96	0	0	0	2.83	1.59	1.79	1.52
73.2	0	0	0	0	7.68	0	0	0	2.29	0	0	0	1.22	1.26	0	0	2.12	3.77	2.5	4.85
74.1	0	0	0	0	0	0	0	0	1.54	0	0	0	0	1.91	0	0	0	0	0	0
75.3	3.23	0	0	0	4.19	0	0	0	2.24	0	0	0	2.27	0	0	0	3.04	0	0.84	0.91
76.7	2.53	0	0	0	0	0	0	0	0	0	0	0	0	1.29	0	0	0	0	0	0
78.6	5.35	0	0	0	11.9	0	0	0	0.8	0	0	0	0	0	0	0	0	0	0	0
80.6	0	0	0	0	0	0	0	0	5.06	0	0	0	2.2	0.97	0	0	1.28	0	0	0
81.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.07	0	1.05	1.52
82.9	0	0	0	0	1.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
84.5	0	0	0	0	0	0	0	0	0.77	0	0	0	0	0	0	0	0	0	0	0
86.3	0	0	0	0	0	0	0	0	1.33	0	0	0	1.95	1.62	0	0	2.85	2.54	1.51	3.34
87.9	2.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.64	0.92	1.24	0
89.9	3.14	0	0	0	3.8	0	0	0	1.6	0	0	0	0	0	0	0	0	0	0	0
91.7	0	0	0	0	2.79	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
93.1	0	0	0	0	0.66	0	0	0	0.8	0	0	0	0	0	0	0	0	0	0	0
99.1	0	0	0	0	0.7	0	0	0	1.06	0	0	0	0	0	0	0	0	0	0	0

DGGE band corresponding to *Dehalococcoides* species is presented in bold

Chapter 3

Identification of a Chlorodibenzo-*p*-dioxin Dechlorinating *Dehalococcoides* spp. by Stable Isotope Probing and Pyrosequencing of 16S rRNA

Abstract

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are released into the environment from a variety of both anthropogenic and natural sources. Highly chlorinated dibenzo-*p*-dioxins are persistent to under aerobic conditions, however, in anaerobic environments, they can be reductively dechlorinated to less chlorinated dibenzo-*p*-dioxin compounds that are more accessible to subsequent aerobic degradation. In this study, we used a DNA-stable isotope probing (SIP) approach to identify the bacteria active in dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) in river sediments. A set of operational taxonomic units (OTUs) responded positively to the addition of 1,2,3,4-TeCDD in SIP culture. An OTU corresponding to *Dehalococcoides* spp. accounted for a substantial greater abundance in cultures with 1,2,3,4-TeCDD than in culture without 1,2,3,4-TeCDD, as revealed in TRFLP profiles of the ^{13}C labeled heavy DNA fraction. This implies the involvement of this *Dehalococcoides* strain in reductive dechlorination of 1,2,3,4-TeCDD, and suggests the applicability of SIP for a robust assessment of bioremediation potential of organohalide contaminated sites. Bacterial community analyses using a combination of DNA-SIP and pyrosequencing of 16S rRNA genes suggested an active cycling of sulfur in the Hackensack River sediments enrichment culture mediated by a *Sulfuricurvum* spp. A member of the genus *Azonexus* was thought to be involved in oxygen scavenging to create strictly anaerobic conditions for *Dehalococcoides*. *Syntrophobacter* spp. are assumed to play an

important role in fermentation of propionate to provide hydrogen for *Dehalococcoides* spp. in the PCDD dechlorinating culture.

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are produced in both anthropogenic and natural processes. Historical deposition of PCDDs in the environment is associated with large scale production, storage, utilization, and disposal of chlorinated herbicides and pesticides in which PCDDs are present as impurities (Hites, 2011). In addition, combustion of municipal solid waste using old technology contributes to the overall input of PCDDs into the environment (Kishida *et al*., 2010; Takeda & Takaoda, 2013). PCDDs are also produced naturally in forest fires and by microbially mediated polymerization of chlorophenols (Sheffield, 1985; Öberg & Rappe, 1992; Dwyer & Themelis, 2015). They are highly persistent which can impede the carbon and chlorine cycles (Hites, 2011). PCDDs are present at substantially high concentrations in soil and sediment environments long after chemical production, which formed them, has been banned or after municipal waste combustion technology has been improved (Götz *et al*., 2007; Salo *et al*., 2008; Takeda & Takaoda, 2013). PCDDs are highly hydrophobic, they bioaccumulate and biomagnify through the food chain, which can pose negative effects on animals of the upper levels of the food chain, including humans (Shiu *et al*., 1988; Isosaari *et al*., 2004). Five decades after the peak of PCDD production (Götz *et al*., 1998), they are still problematic to the environment and humans are still trying to find efficient methods to remediate PCDD contamination.

PCDDs have the tendency to bind to organic matter and accumulate in anoxic environments, which become their ultimate sinks. In sediments, PCDDs can undergo reductive dechlorination, which is the only biological process known to transform PCDDs in anaerobic environments (Bunge & Lechner, 2009; Fennell *et al*. 2011). Dechlorination of PCCDs has been demonstrated in pure

and mixed cultures established from various contaminated sediments (Bunge *et al*., 2003; Yoshida *et al*., 2009b; Pöritz *et al*., 2015). Products of PCDD reductive dechlorination contain fewer chlorines and are more prone to aerobic degradation and eventually aromatic ring cleavage. Hence, reductive dechlorination is an important biological process for bioremediation of PCDD contaminated environments.

Reductive dechlorination is carried out by a group of phylogenetically diverse anaerobic bacteria (Maphosa *et al*., 2010; Hug *et al*., 2013). In many cases, reductive dechlorination is also a respiratory process by which microorganisms gain energy while removing chlorines from chlorinated compounds (Holliger *et al*., 1998b). While many dehalogenating bacteria have been isolated from terrestrial environments, sewage sludge, or river sediments (Scholz-Muramatsu *et al*., 1995; Maymó-Gatell *et al*., 1997; Adrian *et al*., 2000), reductive dehalogenase gene sequences are also found in marine environments (Futagami *et al*., 2009), indicating that dehalogenating bacteria are widely distributed in the environment. However, they are typically resistant to cultivation and outcompeted by other microorganisms since dechlorination of highly hydrophobic compounds such as PCDDs yields a limited amount of energy. Dehalogenating bacteria are often underrepresented in the microbial community and it is difficult to exactly determine which phylogenetic groups are responsible for dechlorination using conventional DNA-based molecular techniques and 16S rRNA genes as marker genes. Stable isotope probing followed by a microbial fingerprinting technique is a sophisticated method used to identify metabolically active members of the community (Boschker *et al*., 1998; Radajewski *et al*., 2000). It has successfully been applied to identify bacteria involved in degradation of various contaminants (Tillman *et al*., 2005; Liu *et al*., 2016; Sun *et al*., 2015).

Our previous study revealed that that an indigenous microbial community dechlorinated 1,2,3,4-TeCDD in sediments of the Hackensack River, NJ (Chapter 2). The objective of this study

was to identify the dehalogenating bacteria capable of reductive dechlorination of PCDDs. Reductive dechlorination of PCDDs is a dissimilatory process, therefore the substrate of dechlorination is not assimilated into the cell biomass. However, many dechlorinating bacteria use acetate as a carbon source (for review, see Maphosa *et al*., 2010). Therefore, we used the combination of a DNA-SIP approach in which ^{13}C labeled acetate was amended in the medium with 1,2,3,4-TeCDD as the electron acceptor to identify organohalide-respiring bacteria, and pyrosequencing of the 16S rRNA genes to reveal the active bacterial community in the dechlorinating culture.

Materials and methods

Initial anaerobic enrichment culture setup

Fifty milliliter (50 mL) anaerobic enrichment cultures were established in triplicate in bicarbonate buffered medium with 10% Hackensack River sediments. 1,2,3,4-TeCDD was amended at a nominal concentration of 20 µM as the only terminal electron acceptor for bacterial respiration. A mixture of short chain organic acids (acetate, propionate, and lactate) was provided at a 150 µM concentration each as electron donor and carbon source. Cultures were maintained under strictly anaerobic conditions with a mixture of 3%H_2/97%N_2 gas in the headspace. Sediments used to establish enrichment cultures for this experiment (sites H1 and H5) were collected by grab sampling at 19.6 km and 2 km from the river mouth, respectively. Control cultures included autoclaved cultures and no electron acceptor cultures. For detailed description on the procedure for enrichment culture setup and sediment sampling locations see Chapter 2.

Stable isotope probing culture setup

SIP cultures (8 mL) were established in 10 mL serum bottles in an anaerobic vinyl glove box (Coy Laboratories Products Inc., Great Lakes, MC, USA) with a H_2/N_2 (3/97 %) headspace. One

milliliter of the initial enrichment culture H1 was used as inoculum at a time point when 90% of the spiked 1,2,3,4-TeCDD had been dechlorinated. Experimental cultures established in triplicate were spiked with 1,2,3,4-TeCDD at a nominal concentration of 20 µM as terminal the electron acceptor, lactate and propionate at a concentration of 150 µM each as electron donors, and ^{13}C labeled acetate (Sigma Aldrich Inc., Saint Louis, MO, USA) at a concentration of 30 µM or 150 µM as the carbon source. The no-electron acceptor control cultures received ^{13}C labeled acetate but no 1,2,3,4-TeCDD. ^{12}C-acetate and 1,2,3,4-TeCDD amended cultures were also established as technical controls.

Sampling and analytical methods

Samples were taken using strict anaerobic technique with a syringe purged with nitrogen gas. Residual 1,2,3,4-TeCDD and its dechlorination products were extracted from a 1 mL sample into toluene phase using solid-liquid extraction method as described previously (Chapter 2). The concentration of chlorinated dibenzo-*p*-dioxins were determined by gas chromatography/mass spectrometry (GC/MS) (Agilent Technologies Inc., Santa Clara, CA, USA) using a five-point standard curve of a mixture of authentic chlorinated dibenzo-*p*-dioxin congeners as described previously (Chapter 2).

SIP Molecular Analyses

The entire SIP cultures were harvested for DNA extraction after approximately 80% of 1,2,3,4-TeCDD had been dechlorinated. Biomass and residual sediment were collected for DNA extraction by centrifugation at 16,300 x g for 10 minutes. Total genomic DNA of SIP cultures was extracted immediately using the PowerSoil®DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) with some modifications to increase DNA yield. Isolated DNA was visualized and quantified on an agarose gel using a lambda DNA ladder of known concentration (Sigma Aldrich Inc., Saint Louis, MO, USA). ^{13}C-labeled DNA was separated from ^{12}C-labeled DNA by subjecting the extracted DNA

to cesium chloride (CsCl) gradient ultracentrifugation (225,000 x g for 48 hours) using an Optima MAX-TL Ultracentrifuge System with a TLA-110 rotor (Beckman Coulter Inc., Indianapolis, IN). Due to the low DNA yield, light and heavy DNA was visualized by adding ^{12}C- and ^{13}C-labeled archaeal DNA isolated from a pure culture of *Halobacterium salinarum* into the extracted DNA before ultracentrifugation. The light (^{12}C) and heavy (^{13}C) DNA fractions were subjected to dialysis to remove CsCl from the ultracentrifugation step and then subsequently used as template in a 2-step PCR reaction to amplify bacterial 16S rRNA genes. The primer pairs 27F (5'-AGAGTTTGATCCTGGCTCAG-3') labeled with carboxyfluorescine at 5' end) and 1100R (5'-AGGGTTGCGCTCGTTG-3') were used (Lane, 1991; Reysenbach & Pace, 1995). The thermocycle conditions for PCR with 27F and 1101R primer pair was as follows: 94 ^{o}C for 5 min, followed by 28 cyles of 94 ^{o}C for 30 s, 57 ^{o}C for 30 s, and 72 ^{o}C for 1 min and 10 s. A final extention step was carried out at 72 ^{o}C for 10 min and samples were then stored at 4 ^{o}C. PCR products were subjected to restriction digestion by endonuclease *MnlI* (New England Biolabs Inc., Ipswich, MA, USA) for 6 hours at 37 ^{o}C. Precipitation of digested DNA was carried out as described previously (Tuorto *et al*., 2014) before being analyzed on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, USA) for the purpose of community fingerprinting by Terminal Restriction Length Polymorphism (TRFLP).

Clone library and identification of Terminal Restriction Fragments (TRFs)

The heavy (^{13}C) DNA fraction extracted from the SIP cultures amended with 1,2,3,4-TeCDD and 30 µM ^{13}C labeled acetate was used to establish a clone library to identify TRFs. The bacterial 16S rRNA genes of the community were amplified using the 27F and 1101R primer pair and ligated into the pGEM®-T Easy Vector system and transformed into JM109 High Efficiency Competent Cells (Promega Corporation, Madison, MC, USA). Plasmid DNA from the clone library was isolated using Zyppy™ Plasmid Miniprep kit (Zymo Research Corporation, Irvine, CA, USA) and screened

by comparing the TRFLP profile of each clone to that of the parent culture. Clones whose TRFs matched those of the TFRLP profile of the parent culture were sent for Sanger sequencing at Genewiz® (South Plainfield, NJ, USA). Sequences from the clone library were compared to reference sequences using the Basic Local Alignment Search Tool (BLAST) on NCBI for taxonomic identification. A maximum likelihood phylogenetic tree was constructed using MEGA version 6.0 (Tamura *et al*., 2013) with 16S rRNA gene sequences of clones of interest and their corresponding matches from the BLAST searches.

Pyrosequencing

Samples from enrichment cultures H1 and H5, and their control cultures without electron acceptor after 18 months of incubation, were stored at -80 °C until further analysis. The RNA extraction protocol was adapted from Porebski *et al*. (1997) followed by DNase treatment and RNA purification. The complete protocol was as follows: 4 mL of culture was centrifuged to remove supernatant. Then, 100 µL extraction buffer (100 mM Tris-Cl, 20 mM ethylenediaminetetraacetic acid (EDTA), 4% cetyl trimethylammonium bromide (CTAB), 1.12 M NaCl, and 0.3% β-mercaptoethanol) was added to the remaining sediment and biomass and the mixture underwent five freeze-thaw steps before being incubated with 100 µL lysozyme (100 mg/mL) and 400 µL extraction buffer. The mixture was then extracted twice with a 800 µL mixture of phenol:chloroform:isoamyl alcohol (25:24:1). Nucleic acids were precipitated with ethanol and eluted in deionized water. DNA was removed by incubating with Turbo™ DNase (ThermoFisher Scientific Inc.). RNA was further purified on a RNeasy Mini Spin column (QIAGEN Inc., Valencia, CA, USA) to remove any remaining impurities. Reverse transcription was carried out using iScript™ Reverse Transcription Supermix kit for RT-qPCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA). 16S rRNA genes were amplified from cDNA using the 27F and 519R primer pair. Barcoded amplicon sequencing was performed using Roche 454 FLX titanium instruments and reagents

(Molecular Research LP, Shallowater, TX). Data generated from pyrosequencing was processed using a proprietary analysis pipeline (Molecular Research LP, Shallowater, TX) as briefly described as follows: Barcodes, primers, short sequence of less than 200 bp, sequences with ambiguous base calls, and sequences with homopolymer exceeding 6 bp were removed. Sequences were then denoised and operational taxonomic units (OTUs) defined clustering at 97% similarity. Singleton sequences and chimeras were removed. Final OTUs (from an amplicon length of approximately 490 bp) were taxonomically assigned using BLASTn against a curated database derived from GreenGenes (http://greengenes.lbl.gov), NCBI (www.ncbi.nlm.nih.gov), and the Ribosomal Database Project (http://rdp.cme.msu.edu).

Statistical analysis

All experiments were set up in triplicate or duplicate. Results were recorded as mean and standard deviation. A Student's test was performed to determine whether treatments resulted in significant differences. A p value of less than 0.05 was considered to be statistically different.

Results

Dechlorination activity of original enrichment cultures and SIP cultures

Anaerobic enrichment cultures established from the Hackensack River sediments showed dechlorination activity towards 1,2,3,4-TeCDD which was spiked as the only terminal electron acceptor (Chapter 2). Enrichment cultures were re-spiked with 1,2,3,4-TeCDD when its concentration decreased to below 2 µM. All enrichment cultures showed dechlorination activity towards 1,2,3,4-TeCDD at a 18 month time-point after inoculation (data not shown). Two of the cultures (established from sites H1 and H5), and their corresponding no-electron acceptor control cultures were harvested to isolate RNA for pyrosequencing.

Cultures for SIP analysis were then established in triplicate by transferring 10% of enrichment culture H1 into fresh medium. ^{13}C-labeled acetate was amended at two concentrations, 30 µM (low) and 150 µM (high). After 3 months, more than 80% of 1,2,3,4-TeCDD was dechlorinated to 2-MoCDD (Figure 3.1). The dechlorination rates in SIP cultures were relatively higher than those observed in the original Hackensack River enrichment cultures (Chapter 2) as well as in comparison to pure cultures and mixed cultures from other studies (Vargas *et al*., 2001; Bunge *et al*., 2001, 2003; Fennell *et al*., 2004).

Active bacterial community revealed by stable isotope probing

SIP-TRFLP profiles generated from light and heavy DNA fractions represent the resident and active bacterial community, respectively. Representative SIP-TRFLP profiles of each set of SIP cultures are illustrated in Figure 3.2. Concentrations of genomic DNA isolated from SIP cultures were relatively low, ranging from 0.7 – 3 ng/µL. Visualization of ^{12}C and ^{13}C DNA fractions during CsCl gradient separation was therefore enhanced by adding both ^{12}C- and ^{13}C-archaeal DNA (Tuorto *et al*., 2014). The presence of archaeal DNA did not affect the outcome of bacterial community fingerprint from the use of bacteria-specific primers. A total of 109 distinct TRFs, each accounting for more than 0.5% of total bacterial community, were detected in TRFLP profiles of the ^{13}C DNA fraction isolated from three sets of triplicate SIP cultures (i.e., cultures with 1,2,3,4-TeCDD at low and high concentrations of ^{13}C labeled acetate and cultures without 1,2,3,4-TeCDD) (Table S3.1). There was no difference in the number of TRFs resolved in the TRFLP profiles of ^{13}C-labeled DNA between three different cultures, suggesting that the presence of 1,2,3,4-TeCDD does not cause an obvious toxic effect to the overall bacterial community. Each treatment contained 41-48 resolved TRFs (Table S3.1). The heavy DNA fraction of control cultures amended with ^{12}C labeled acetate produced a flat TRFLP profile.

Four TRFs, i.e., TRFs 111, 166, 237, and 279, were taxonomically identified from the clone library generated from the heavy DNA fraction of the SIP cultures amended with 20 µM 1,2,3,4-TeCDD and 30 µM of ^{13}C-acetate. These predominant TRFs made up a total of 42.5 ± 1.7% of the overall TRFLP peak area in the low acetate culture (Figure 3.3). The addition of 1,2,3,4-TeCDD as the terminal electron acceptor resulted in a higher relative abundance of TRF 111 than in the control cultures without 1,2,3,4-TeCDD ($p < 0.05$). The relative abundance of TRF 111 in the SIP-TRFLP profiles of the ^{13}C DNA fraction of experimental cultures amended with 30 µM and 150 µM are similar, at 12.1 ± 2.5% and 11.7 ± 2.9%, respectively. In the no-electron acceptor control culture, TRF 111 constituted only 2.3 ± 0.5% of the active bacterial community (Figure 3.3). TRF 111 was phylogenetically affiliated with *Dehalococcoides* spp. and grouped with the Pinellas subgroup in the phylogenetic tree (Figure 3.4). Other TRFs (113, 196, 271, 323, and 419 bp) showed significant differences between SIP cultures with and without 1,2,3,4-TeCDD ($p < 0.05$, except for TRF 271 in culture amended with 30 µM acetate and 1,2,3,4-TeCDD compared to that of culture without 1,2,3,4-TeCDD) (Figure 3.3). However, their taxonomic affiliations were not determined.

One set of the clones exhibited both TRFs 166 and 169 in their TRFLP profiles. The relative abundances of peaks 166 and 169 were not in the same proportion among different clones and they appear to be formed from a peculiar restriction digest by the endonuclease *MnlI*. Therefore, we summed the peak areas of these two TRFs and considered them one OTU for data analysis. A large variation in the relative abundance of TRF 166 was observed among replicates of the same treatment. Relative abundances of TRF 166 differed by up to 200% within one set of SIP cultures (Table S3.1). TRFs 237 and 279 were found in all three SIP cultures. Their relative abundance in the heavy DNA fraction of three sets of SIP cultures were similar, and were 13.1 ± 2.3 and 6.4 ± 1.1% of the total electropherogram, respectively (Figure 3.3). Both sequences corresponding to

TRFs 237 and 279 grouped with *Sulfuricurvum* spp. (98% similar to *Sulfuricurvum kujiense* DSM 16994) (Figure 3.4).

Bacterial community revealed by pyrosequencing of 16S rRNA

Bacterial community analysis of Hackensack River enrichment cultures H1 and H5 by pyrosequencing of 16S rRNA genes showed no substantial differences at the phylum level between the 1,2,3,4-TeCDD dechlorinating cultures and the corresponding no-electron acceptor controls. Interestingly, in most cultures, *Chloroflexi* accounted for approximately 20% of the total bacterial community, and was the second most abundant phylum after *Proteobacteria*, except for replicate 1 of the H5 culture without 1,2,3,4-TecDD (Figure 3.5).

A total of 14 genera of the *Chloroflexi* phylum were found in the Hackensack River sediment cultures. Notably, two obligate organohalide-respiring bacteria, i.e., *Dehalococcoides* spp. and *Dehalogenimonas* spp., accounted for more than 41% of the *Chloroflexi* phylum members (Figure 3.6). They were found among the four most dominant genera in all Hackensack River enrichment cultures with the total number of reads at 3144 and 1523, respectively (Table S3.2). *Dehalococcoides* spp. responded positively to the addition of 1,2,3,4-TeCDD while *Dehalogenimonas* spp. did not. *Dehalogenimonas* spp. were present at a greater abundance in H1 enrichment culture without 1,2,3,4-TeCDD as the terminal electron acceptor. No significant difference in their relative abundance were observed in H5 enrichment cultures in the presence or absence of 1,2,3,4-TeCDD (Figure 3.7). However, *Dehalococcoides* spp. unexplainably constituted 98% of total number of RNA-derived 16S rRNA sequence reads in one of H5 culture without 1,2,3,4-TeCDD. The two other dominant genera of the *Chloroflexi* phylum were *Bellilinea* and *Anaerolinea*. The former was found to be more dominant in H1 cultures, which was established from low salinity sediments, whereas *Anaerolinea* made up a larger portion in the

Chloroflexi phylum in H5 enrichment cultures which was established from estuarine river sediments closest to Newark Bay (Figure 3.6).

Desulfobacterium spp. belonging to the *Proteobacteria* phylum was the third most predominant genus with 1746 reads. *Desulfobacterium* spp. represented a 3-fold higher abundance in cultures without 1,2,3,4-TeCDD ($p < 0.05$) (Figure 3.7, Table S3.2). *Sulfuricurvum* spp. was not detected in H5 enrichment cultures or in the H1 cultures without 1,2,3,4-TeCDD. The *Sulfuricurvum* spp. was present at a relatively low abundance of approximately 2% in two replicates of the H1 cultures amended with 1,2,3,4-TeCDD, but it was found at a strikingly high abundance (approximately 55%) in the third replicate culture (Table S3.2). In addition to *Dehalococcoides* spp., *Syntrophobacter* spp. was shown to increase in relative abundance in two of three replicate H1 enrichment cultures amended with 1,2,3,4-TeCDD, compared to H1 cultures without chlorinated electron acceptor. Some genera were found to be more dominant in cultures without an external source of chlorinated dibenzo-*p*-dioxin compound (Figure 3.7). They included *Bellilinea* spp. (*Chloroflexi*), *Desulfobacterium*, *Syntrophus* spp. and *Geobacter* spp. (*Proteobacteria*); *Clostridium* spp. (*Firmicutes*) and *Acidobacteria* spp. (*Acidobacteria*) (Figure 3.7). In enrichment culture H5, different genera seemed to dominate the bacterial communities (i.e., *Sulfurospirillum* and *Thioalbus*) in which *Sulfurospirillum* spp. was significantly more active in the presence of 1,2,3,4-TeCDD. Its relative abundance of RNA-derived 16S rRNA gene sequence was 11.5 ± 4.5% in the presence of 1,2,3,4-TeCDD but none was found without 1,2,3,4-TeCDD (Table S3.2).

Discussion

SIP analysis allows for linking microbial activity and phylogeny in complex environmental samples. SIP incubations are usually followed by DNA fingerprinting to determine the phylogenetic affiliation of organisms that assimilate the amended stable isotope labeled substrate and is commonly used to assess the bioremediation potential of a contaminated site. In many cases, the ^{13}C labeled substrates are also environmental pollutants, such as benzene, toluene, aniline, methyl tert-butyl ether (MTBE). Assimilation of the ^{13}C substrate into cell biomass at an early stage of incubation usually indicates directly the ability of the specific microorganism to transform the labeled substrate and assimilate it into biomass (e.g., Oka *et al*., 2008; Sun & Cupples, 2012; Liu *et al*., 2016). In contrast, when contaminants take part in dissimilatory processes such as dissimilatory respiration of oxidized nitrogen and metal species or reductive dechlorination, detection of microorganisms that are actively involved in the process depends on the assimilation ^{13}C labeled carbon sources such as acetate, ethanol or lactate (Lear *et al*., 2007; Hori *et al*., 2010; Akob *et al*., 2011; Ishii *et al*., 2011; Héry *et al*., 2015). In this experiment, we used ^{13}C-acetate to trace the activity of dehalogenating bacteria, since it has been shown to be used as a carbon source by many organohalide-respiring bacteria (Utkin *et al*., 1994; Maymó-Gatell *et al*., 1997; Holliger *et al*., 1998a; Adrian *et al*., 2000). However, acetate can also be used by a wide variety of anaerobic bacteria which use different substrates for respiration other than chlorinated aromatic compounds. We therefore established a control culture without addition of 1,2,3,4-TeCDD in order to deduce the involvement of bacteria that are only involved in reductive dechlorination.

Identification of Dehalococcoides spp. as the PCDD dehalogenating bacterium

Dehalococcoides spp. were found to be present at a significantly higher relative abundance in 1,2,3,4-TeCDD dechlorinating cultures than in the controls without the amendment. The low

relative abundance of TRF 111 (corresponding to *Dehalococcoides* spp.) in the no-electron acceptor controls at approximately 2% of the electropherogram may result from respiration of the remnant 1,2,3,4-TeCDD that was present in the culture inoculum. *Dehalococcoides* spp. are known to be obligate organohalide-respiring bacteria. Among a wide variety of chlorinated substrates, highly complex and hydrophobic compounds, such as PCDDs, are also used as sources of energy for this group of bacteria (Bunge *et al*., 2000; Pöritz *et al*., 2015). There have been only a few studies using a SIP approach to identify members of bacterial community that are involved in anaerobic dechlorination. In most of these cases, the carbon skeleton of the chlorinated compounds was assimilated into cell biomass (Borodina *et al*., 2005; Sul *et al*., 2009; Martinez-Lavanchy *et al*., 2011). Only one previous study has used SIP analysis to identify orgabohalide-respiring bacteria (Kittelmann & Friedrich 2008), suggesting the applicability of this approach to detect and identify novel dehalogenating bacterial strains. A *Dehalococcoides* spp. was found to be dominant in PCE dechlorinating river sediment culture at 20 ^{o}C, whereas a novel bacterial species affiliated within the *Chloroflexi* phylum (i.e., Lahn cluster) was determined to coupled dechlorination of PCE to cis-DCE to oxidation of acetate at 15 ^{o}C (Kittelmann & Friedrich, 2008).

Dehalogenating bacteria are slow growers and the energy they obtain from respiratory dehalogenation is limited because chlorinated compounds used as terminal electron acceptors tend to have low water solubilities and low bioavailabilities. A high concentration of acetate can lead to the overgrowth of competitors of dehalogenating bacteria and therefore results may not reflect the community of slow growing organisms. However, in this study, TeCDD dechlorination activity was found to be similar between cultures incubated at low and high concentration of acetate (Figure 3.1). This concentration of acetate between 30 and 150 µM is within the range which sufficiently supports the growth of organohalide-respiring bacteria during dechlorination

of 0.16 micromole 1,2,3,4-TeCDD, this amount of acetate is not high enough to cause the overgrowth of other fast growing competitors that can inhibit dehalogenating bacteria.

The high abundance of *Dehalococcoides* spp. in the dechlorinating H1 culture, based on 16S rRNA pyrosequence data, confirmed their role in TeCDD dechlorination as shown in SIP experiments. *Dehalococcoides* spp. responded positively to the addition of chlorinated dibenzo-*p*-dioxins and correlated with dechlorination activity (Chapter 2). *Dehalogenimonas* was also detected at a lower relative abundance, but was not retrieved in the SIP clone library analysis. *Dehalogenimonas* spp. did not responded to amendment with chlorinated dibenzo-*p*-dioxins, suggesting that chlorinated dibenzo-*p*-dioxins were not used as terminal electron acceptors. The proportion of sediment in enrichment cultures H1 and H5 was still high (10%), and it is therefore possible that the cultures contained a variety of naturally or anthropogenically produced organohalides, which may have served as an energy source for other dehalogenating bacteria belonging to the phylum *Chloroflexi* (Krzmarzick *et al*., 2012, 2013).

Potential roles of other members of the bacterial community

Three dominant TRFs (166, 237, and 279) were found in the heavy DNA fragment of all three treatments with and without 1,2,3,4-TeCDD. These three TRFs presumably consume acetate or its degradation products for growth. TRFs 237 and 279 were taxonomically closest to *Sulfuricurvum* spp. (98% similarity over 1000 bp). *Sulfuricurvum* spp. belongs to the *Epsilonproteobacteria*. The type species, *Sulfuricurvum kujiense*, is an obligate chemolithotroph, which couples oxidation of reduced sulfur species such as sulfide, elemental sulfur, and thiosulfate to nitrate reduction, and can also grow microaerophilically (Kodama & Watanabe, 2004; Handley *et al*., 2014). *Sulfuricurvum* cannot use hydrocarbons or organic acids including acetate as carbon sources. The detection of *Sulfuricurvum* in the heavy DNA fraction may result from its utilization of $H^{13}CO_3^-$ produced from acetate. Growth of *Sulfuricurvum* may also have been supported by other possible

terminal electron acceptors, such as nitrate, iron (III) and especially sulfate present at sufficient concentrations in the cultures, since the sediments used to establish these enrichment cultures are impacted by marine water from Newark Bay. Furthermore, sulfide was added to the culture medium to remove trace amounts of oxygen to maintain strictly anaerobic conditions. The presence of sulfide oxidizing bacteria, which indicates active sulfur cycling in the anaerobic dehalogenating culture, is not unexpected.

TRF 166 is closely related to *Azonexus fungiphilus* (99% similarity over 1090 bp) and distantly related to *Dechloromonas* spp. (96% similarity over 1110 bp). *Azonexus* spp. are microaerophilic and strictly require oxygen for respiration (Hurek *et al*., 1997; Reinhold-Hurek & Hurek, 2000), suggesting its role as an oxygen scavenger in our dehalogenating cultures. Kittelmann and Friedrich (2008) also found a predominant TRF in the ^{13}C labeled DNA of their SIP experiments that was closely related to *Dechloromonas* spp., suggesting the involvement of *Dechloromonas* in reductive dechlorination of PCE, in addition to its ability to respire perchlorate, nitrate or ferric iron oxide as reported by Zhang *et al*. (2002) and Hori *et al*. (2010).

The 16S rRNA pyrosequencing data aided in resolving the bacterial community when phylogenetic affiliations of many TRFs could not be retrieved from the clone library. Based on 16S rRNA gene pyrosequence data, the phylum *Chloroflexi* was the second most abundant phylum, in both sets of Hackensack enrichment cultures, in the presence or absence of chlorinated dibenzo-*p*-dioxins. Members of the phylum *Chloroflexi* are widely distributed in various environments, including river sediments (Hanada *et al*., 2014). In addition to *Dehalococcoides* and *Dehalogenimonas*, two other members of the *Chloroflexi* (*Bellilinea* spp. and *Anaerollinea* spp.) were found. Their 16S rRNA genes have been detected in a wide variety of habitats which indicates that the two organisms are ubiquitous and contribute significantly to biological processes in the environment. A few studies on cultivated strains of *Bellilinea* and *Anaerolinea*

revealed their strictly anaerobic and fermentative lifestyles, they enhanced growth while exhibiting syntrophic relationship with hydrogenotrophic methanogens (Sekiguichi *et al*., 2001; Yamada *et al*., 2005, 2006, 2007). This suggested that in our enrichment cultures, these organisms grew fermentatively using organic materials in the remaining sediments and might provide hydrogen for the community. The different distribution of *Bellilinea* spp. and *Anaerolinea* in enrichment cultures H1 and H5 indicates that they inhabit different niches.

The dehalogenating *Dehalococcoides* and *Dehalogenimonas* spp. found in our enrichment cultures depend solely on hydrogen as electron donor, they couple oxidation of hydrogen to reductive dechlorination of organohalides in order to obtain energy for growth (Moe *et al*., 2009; Hug *et al*., 2013). The original sediment cultures were amended with lactate and propionate (in addition to acetate) as substrates for fermentative bacteria, which would yield hydrogen for respiratory dehalogenation. Fermentative bacteria that were found in these enrichment cultures belonged to phylogenetically diverse groups including *Firmicutes*, *Proteobacteria* and *Chloroflexi*. In enrichment culture H1, which showed high 1,2,3,4-TeCDD dechlorination activity, *Syntrophobacter* spp. was detected at a greater abundance than in culture H5 which had lower dechlorination activity or in cultures without 1,2,3,4-TeCDD. *Syntrophobacter* spp. exhibit a fermentative mode of metabolism in which fermentation of propionate becomes thermodynamically feasible when hydrogen is consumed by a hydrogen consuming bacterium (Wallrabenstein *et al*., 1995). The presence of *Syntrophobacter* spp. in our actively dechlorinating cultures suggests that they actively served as hydrogen producers from fermentation of organic acids.

Conclusions

Stable isotope probing and sequence analysis of 16S rRNA allowed us to conclusively identify that a *Dehalococcoides* spp. mediated the reductive dechlorination of PCDDs while assimilating acetate into its biomass. This study demonstrated that the SIP approach can be used to identify the responsible organisms and possibly novel dehalogenating bacteria in anaerobic sediments. This method allows for quick assessment of the potential of bioremediation at PCDD contaminated sites.

Figure 3.1. Dechlorination of 1,2,3,4-TeCDD in SIP cultures amended with ^{13}C-acetate at concentrations of 30 or 150 µM after 3 months of incubation. Average and standard deviation of relative concentrations of chlorinated dibenzo-*p*-dioxin congeners from triplicate cultures are presented.

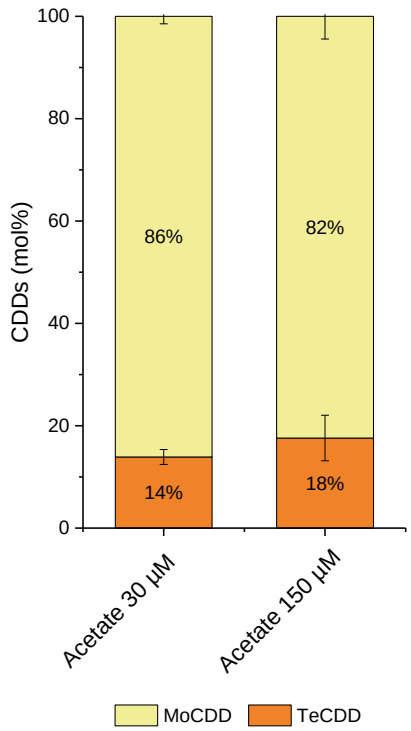


Figure 3.2. Representative 16S rRNA gene TRFLP profiles of light ^{12}C-DNA (blue) and heavy ^{13}C-DNA (red) bands, representing active vs. resident community. No ^{13}C-labeled TRFs were detected in the ^{12}C acetate fed culture.

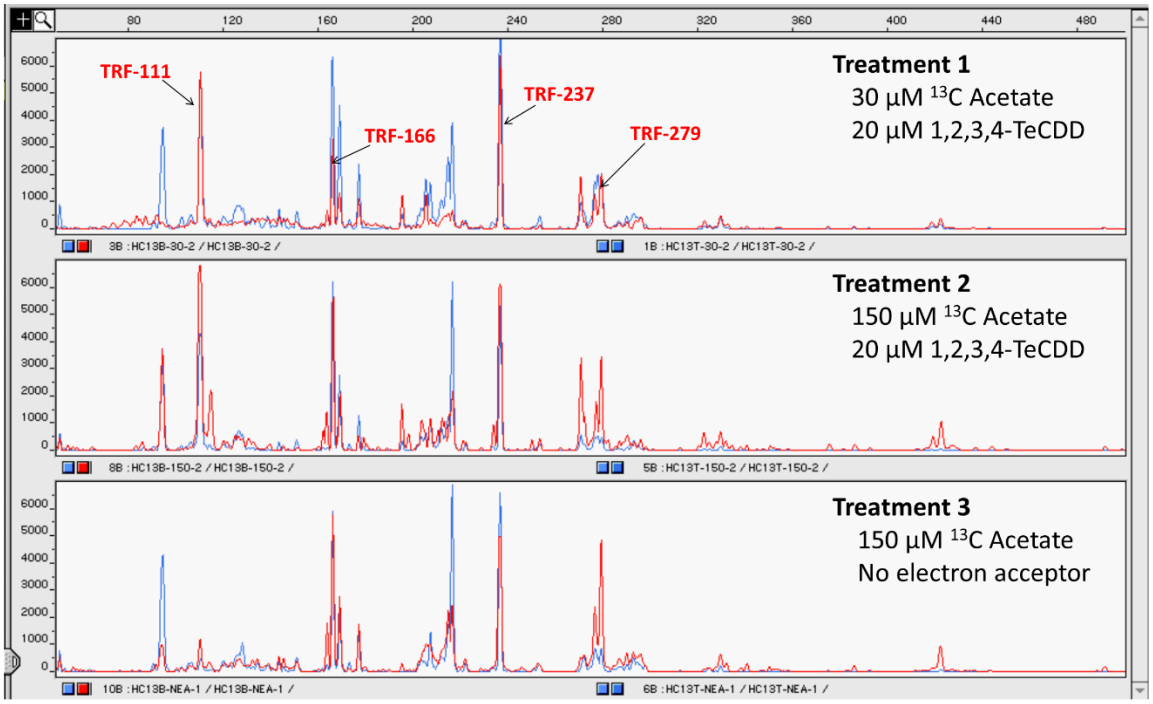


Figure 3.3. Relative abundance of representative TRFs in SIP-TRFLP profile of ^{13}C labeled DNA fraction of SIP cultures.

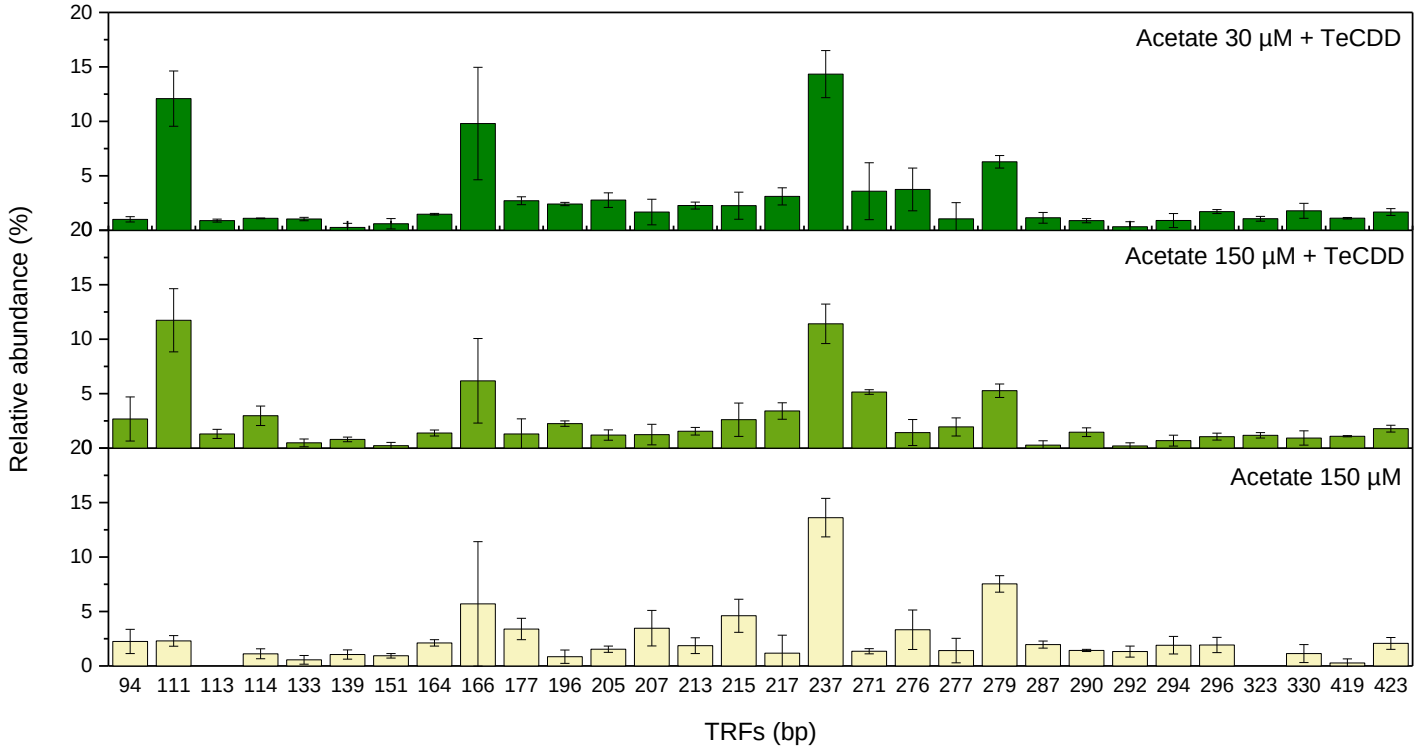


Figure 3.4. Maximum likelihood phylogenetic tree constructed from the inserted 16S rRNA gene sequences of the four most abundant clones derived from dehalogenating enrichment culture amended with 20 µM 1,2,3,4-TeCDD and 30 µM ^{13}C-acetate, and reference 16S rRNA gene sequences imported from GenBank. Bootstrap values (500 replicates) are shown next to the branch nodes. The tree is drawn to scale, with branch lengths measured in the number of substitution per site. Approximately 1000 bp of unambiguously aligned positions was used for tree re-construction.

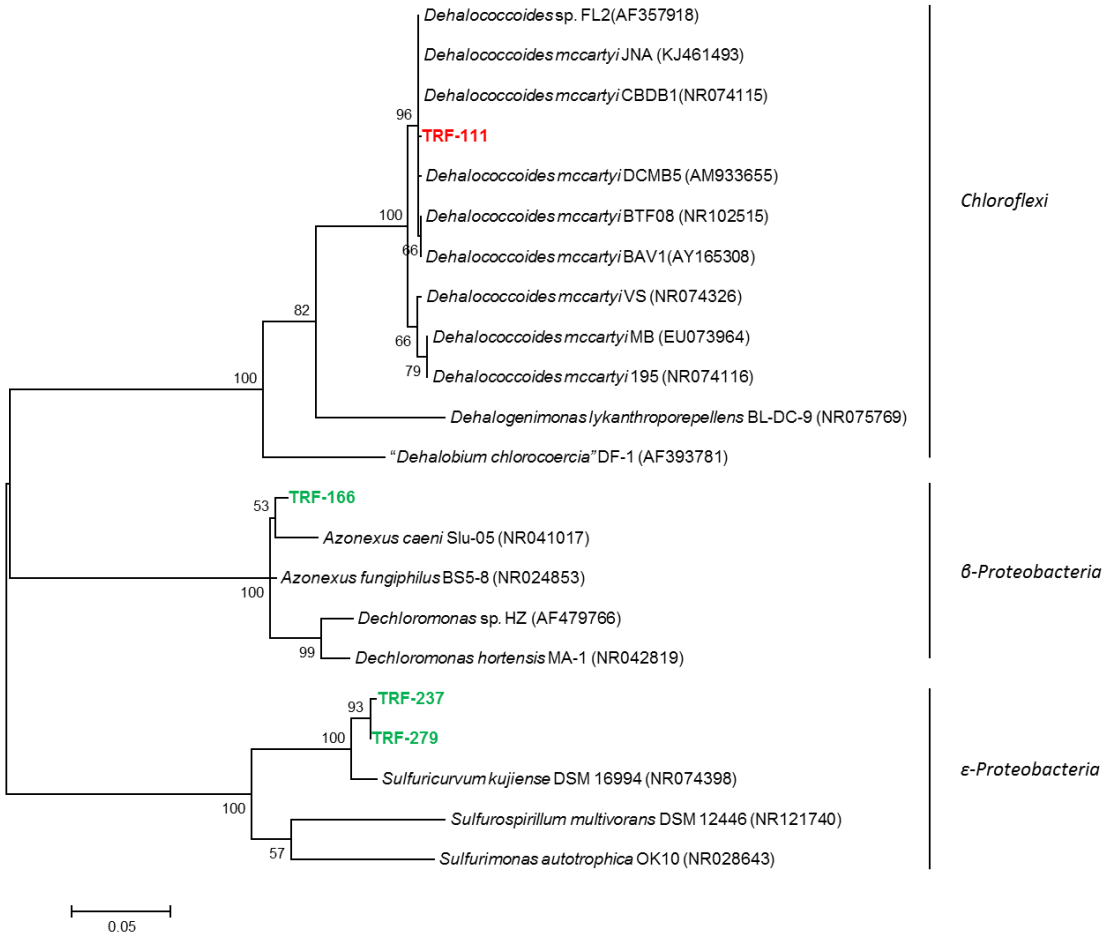


Figure 3.5. Relative abundance of bacterial OTUs (in %) at the phylum level identified by 16S rRNA gene pyrosequencing of reverse transcribed rRNA of Hackensack enrichment cultures H1 and H5. Data of individual replicate cultures with and without 1,2,3,4-TeCDD are presented.

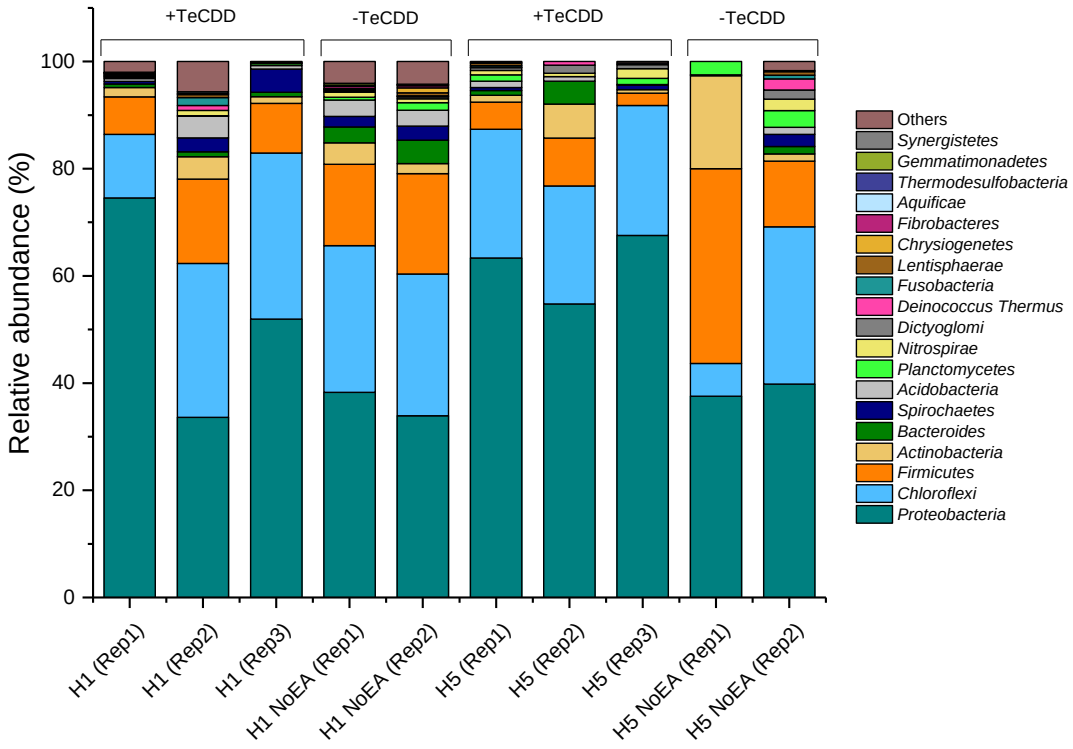


Figure 3.6. Relative abundance of members of the phylum *Chloroflexi* in Hackensack dehalogenating enrichment cultures H1 and H5 and their corresponding no-electron acceptor controls as revealed by 16S rRNA gene pyrosequencing of reverse transcribed rRNA. Data for individual replicate cultures are presented.

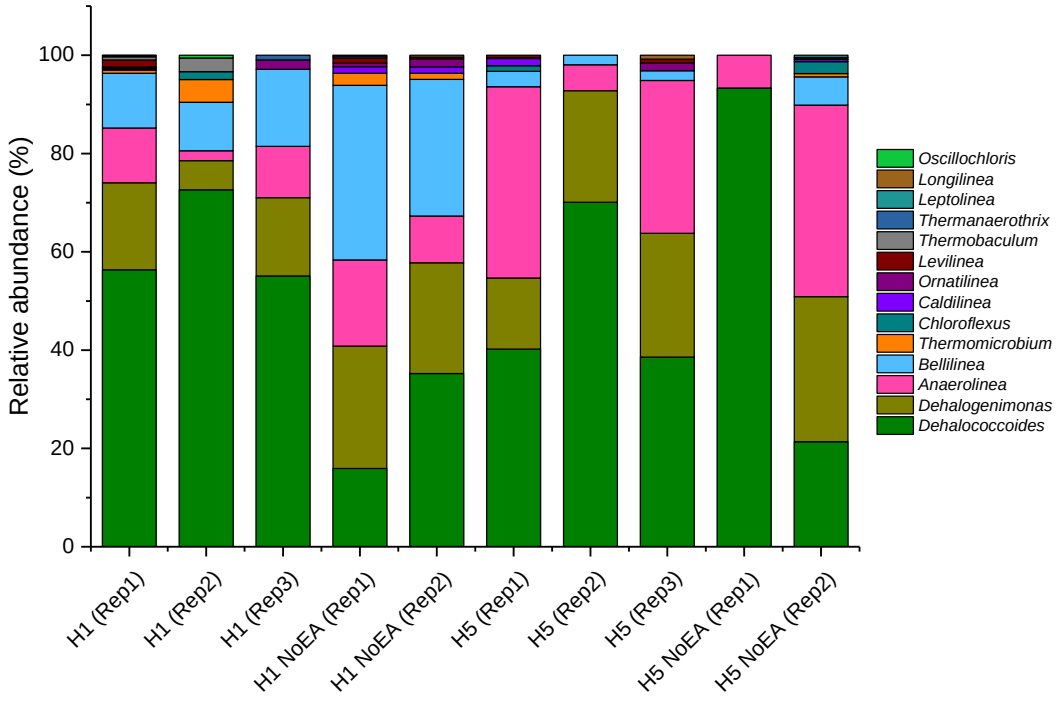
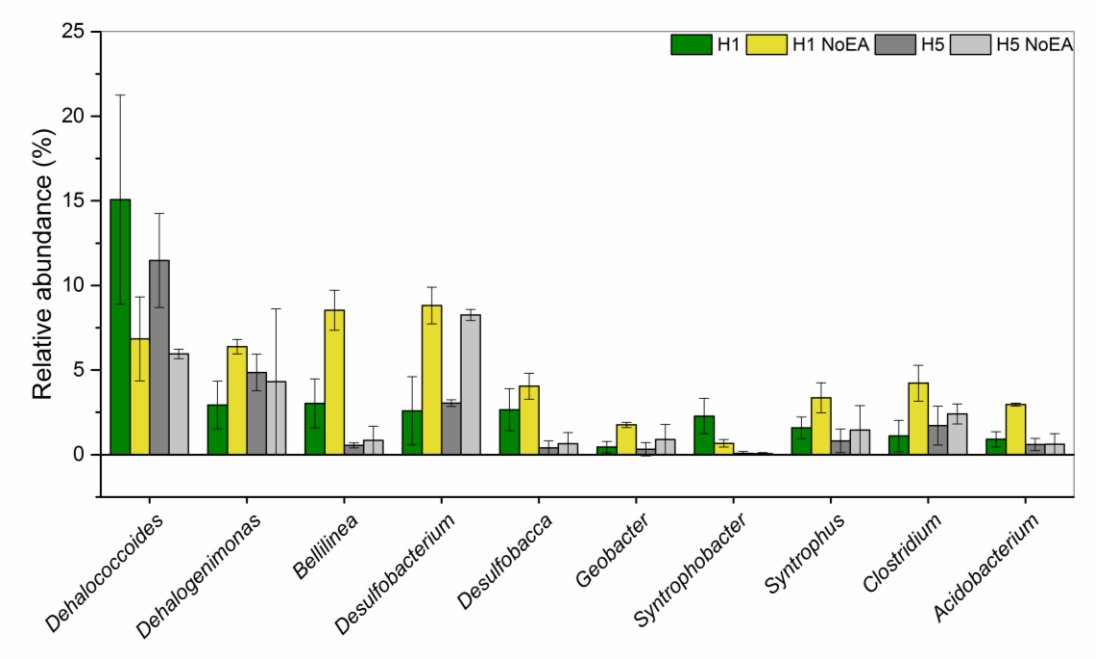


Figure 3.7. Relative abundance of representative genera in the active bacterial community as determined by 16S rRNA gene pyrosequencing of reverse transcribed rRNA. Average and standard deviation of three replicate cultures are shown.



SUPPLEMENTARY DATA

Table S3.1. Relative abundance of TRFs accounted for more than 0.5% of the total bacterial community in the ^{13}C DNA fraction of SIP enrichment cultures.

TRF	Acetate 30 µM + TeCDD			Acetate 150 µM + TeCDD			Acetate 150 µM - TeCDD		
(bp)	R1	R2	R3	R1	R2	R3	R1	R2	R3
50	0	0	0	0	0	0	0	0.9	0.0
51	0	0	0	0	0	0	0.8	0	0
63	0	0	0	0.7	0	0	0	0	0
65	0	0	0	1.1	0	0	0	0	0
67	0	0	0	0.8	0	0	0	0	0
70	0	0	0	4.6	0	0	0	0	0
72	0	0	0	1.7	0	0	0	0	0
73	0	0	0	1.7	0	0	0	0	0
74	0	0.9	0	0	0	0	0	0	0
77	0	0	0	1.4	0	0	0	0	0
78	0	1.5	0	1.9	0	0	0	0	0
80	0	0	0	2.0	0	0	0	0.9	0.0
82	0	0	0	8.9	0	0	0	1.2	0.0
84	0	0.9	0	0.0	0	0	0	0.0	0.0
85	0	0.7	0	0.8	0	0	0	0.0	0.0
88	0	1.1	0	0	0	0	0	0.0	0.0
89	0	0	0	0	0	0	0	1.3	0.0
91	0	0	0	0	0	0	0	1.8	0.0
92	0	1.4	0	0	0	0	0	1.5	0.0
94	1.0	0.7	1.3	0	4.9	3.1	1.9	3.8	1.1
98	0	0	0	0	0	0	0	0.6	0
100	0	0	0	0	0	0	0	0.8	0
102	0	0	0	0	0	0	0	2.3	0
105	0	0	0	0	0	0	0	0.7	0
107	0	1.0	0	0	0	0	0	0.8	0
111	15.6	11.0	9.6	7.8	12.7	14.7	1.7	2.9	2.3
113	0.8	0.8	1.1	0.9	1.1	1.9	0	0	0
114	1.1	1.1	1.1	1.7	3.6	3.6	0.8	1.8	0.8
120	0	0	0.7	0	0	0.8	0	1.1	1.2
121	0	0	0	0	0	0	0.9	0	0
123	0	0	0	0.7	0	0	0	0	0
125	0	0.7	0.7	0	0.7	0	0.7	0	0
127	0.8	0	0	2.6	0.9	0	1.2	0	0
128	0.0	1.3	0.8	0	0.7	0	0	1.4	1.0
130	0.0	0	0	1.4	0.0	0	0	0	0
131	0.0	0	0	1.2	0.7	0	0	0	0
133	0.9	1.2	0.9	0.8	0.6	0	0.8	0.9	0
135	0.0	0.9	1.0	1.0	0	0	0.7	1.5	0.8
136	0.0	1.7	0	0.7	0	0	0	0	0
139	0.0	0.8	0	1.1	0.6	0.7	0.6	1.6	0.9
141	0.0	1.1	0	2.5	0	0	0	0.8	0

144	0.0	1.1	0	0.7	0	0	0.8	0.7	0
145	0.0	1.2	0.7	0	0	0	0.8	0.8	0
146	0.0	0.0	0	0	0	0	0	0.9	0
148	0.0	0.9	0	0	0	0	0	1.9	0
151	0.0	1.2	0.6	0	0	0.7	0.8	1.2	0.8
152	0.0	0	0	0	0	0	0	0.8	0
154	0.0	0	0	0	0	0	0	1.4	0
156	0.0	0	0	0	0	0	0	0.7	0
159	0.0	0	0	0	0	0	0	0.8	0
162	0.0	0	0	0	0.9	1.1	0	0	0
164	1.5	1.4	1.6	1.5	1.7	1.0	2.4	1.7	2.2
166	3.6	9.6	16.2	0.9	7.6	10.1	13.7	1.1	2.3
170	0.0	0	0	0	2.7	0	0	0	0
177	3.2	2.5	2.4	3.2	0.7	0	2.6	4.8	2.8
179	0.0	1.1	0	0	0	0	0	0	0
181	0.0	1.0	0	0	0	0	0	0	0
186	0.0	0	0	0.8	0	0	0	0.8	0
196	2.2	2.5	2.5	2.6	2.2	2.0	0	1.4	1.2
198	0.0	0	0	0	0.9	0	0	0	0
202	0.7	0	0	0	0	0	0.9	0	1.5
203	0.7	0	0	0	2.4	1.8	0.0	0	0
205	1.9	3.5	3.0	1.9	0.9	0.8	1.9	1.2	1.5
206	0.0	0	0	0	0	1.3	1.7	0	2.6
207	3.3	1.0	0.7	1.4	2.3	0	1.5	3.4	5.5
208	0.0	0	0	0	0	0	1.8	0	0
209	0.9	0	0	0	0	0	1.1	0	0
210	0.0	0	0	0	1.2	0	0.9	0	0
212	1.1	1.0	0	0	1.8	1.4	2.5	0	2.5
213	2.1	2.0	2.7	1.1	1.9	1.6	1.0	2.8	1.7
215	4.0	1.5	1.2	1.3	1.8	4.8	5.4	2.5	6.0
216	0.0	0	1.9	2.5	0	0	4.8	0	4.6
217	3.7	2.0	3.6	2.6	3.2	4.4	0	3.5	0
221	0.0	0.8	0	0	0	0	0	0	0.9
222	0.8	0	0.7	0.7	0	0.9	1.0	0	1.4
233	0	0	0	0	1.3	0	0	0	0.7
237	17.4	12.9	12.7	8.9	13.0	12.4	13.6	15.8	11.4
254	0.6	0	0.8	0.9	0	0	0	0.7	1.2
270	0	0	6.4	0	0	0.8	0	0	0.0
271	6.2	4.6	0	4.9	5.4	5.2	1.1	1.7	1.3
272	0	0	0	0	2.2	1.7	1.6	1.1	1.0
275	0	0	0	0	0	0	0.0	0.6	1.5
276	1.6	3.4	6.3	0	1.4	2.9	5.9	1.7	2.4
277	3.1	0	0	0.9	3.0	2.0	1.5	0	2.8
278	0	0	0	0	0	0	0	0.7	0.0
279	6.7	6.7	5.5	6.1	4.9	4.7	8.3	6.5	7.8
281	0	0	0	0.7	0	0.0	0	0	0.9
282	0.7	0	0	0	0	0.6	0	0	0.0
285	0.7	0	0	0	0	1.2	0	0	0.9
287	1.8	1.0	0.6	0.8	0	0	1.8	1.6	2.4
288	0.7	0	0	0	0	0	0	0	0
290	1.1	0.7	0.8	2.0	1.3	1.1	1.3	1.5	1.5
292	1.0	0	0	0	0	0.6	0.6	1.8	1.5
293	1.3	0	0.9	0	0	1.0	1.4	0	1.6

294	0	1.2	1.5	0.9	0	1.2	1.1	3.0	1.6
296	2.0	1.6	1.6	1.5	0.8	0.9	2.0	1.0	2.8
298	0	0	0	0	0	0	0	0	0.7
323	0.9	0.9	1.4	0.8	1.3	1.4	0	0	0
325	0	0	0	0	0.7	0	0	0	0
328	0	0	0	0	0.7	0	0	0	0
330	1.1	1.6	2.7	0	1.4	1.4	1.9	0	1.5
332	0	0	0.8	0	0.8	1.6	0	0	1.0
339	0	0	0	0	0	0	0	0	0.8
341	0	0	0	0	0	0	0	0	0.9
348	0	0	0	0	0	0	0	0	0.9
354	0	0	0	0	0	0	0	0	0.9
419	1.2	1.0	1.1	1.0	1.1	1.2	0	0	0.8
423	2.1	1.3	1.6	1.5	2.2	1.6	2.1	1.4	2.7
427	0	0	0	0	0	0	0	0	0.7

Table S3.2. Relative abundance of the 50 most dominant genera in Hackensack enrichment cultures H1 and H5 based on pyrosequencing of 16S rRNA genes

Genus	H1 (R1)	H1 (R2)	H1 (R3)	H1 NoEA (R1)	H1 NoEA (R2)	H5 (R1)	H5 (R2)	H5 (R3)	H5 NoEA (R1)	H5 NoEA (R2)	Total number of reads
Sulfuricurvum	55.4	2.6	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7466
Dehalococcoides	6.7	21.4	17.1	4.4	9.3	9.7	15.4	9.4	5.7	6.2	3144
Desulfobacterium	2.4	5.1	0.2	9.9	7.7	2.8	3.3	3.1	8.6	7.9	1746
Dehalogenimonas	2.1	1.8	4.9	6.8	6.0	3.5	5.0	6.1	0.0	8.6	1523
Anaerolinea	1.3	0.6	3.2	4.8	2.5	9.3	1.2	7.5	0.4	11.4	1435
Moorella	2.5	9.8	0.0	6.8	7.2	2.0	0.0	1.0	0.0	6.0	1430
Thioalbus	0.2	0.8	0.0	0.0	0.3	16.8	2.5	20.2	0.0	12.2	1404
Bellilinea	1.3	2.9	4.9	9.7	7.4	0.7	0.4	0.5	0.0	1.7	991
Desulfobacca	2.6	1.2	4.2	3.3	4.8	0.9	0.0	0.3	0.0	1.3	814
Sulfurospirillum	0.1	3.2	0.0	0.0	0.0	17.8	7.8	8.9	0.0	0.0	764.0
Clostridium	1.0	0.0	2.3	3.2	5.3	1.3	3.3	0.6	1.8	3.0	751
Syntrophus	1.2	2.5	1.1	4.2	2.5	1.7	0.0	0.8	0.0	2.9	654
Smithella	1.6	1.3	0.0	3.8	2.1	0.6	0.0	0.0	0.0	1.0	507
Pseudomonas	0.5	1.9	8.9	1.5	1.3	0.4	0.2	0.0	8.2	0.6	494
Syntrophobacter	2.7	0.9	3.3	0.9	0.4	0.2	0.0	0.0	0.0	0.1	478
Spirochaeta	0.4	2.6	4.3	1.9	2.6	0.6	0.0	0.0	0.0	1.7	440
Acidobacterium	0.5	1.5	0.7	3.1	2.9	0.9	0.9	0.1	0.0	1.2	418
Desulfitobacterium	2.1	0.0	6.7	0.2	0.3	0.4	0.0	0.2	0.0	0.0	392
Caldithrix	1.1	2.8	0.0	2.4	1.8	0.1	0.0	0.0	0.0	0.6	379
Geobacter	0.5	0.0	0.8	1.6	1.9	0.9	0.0	0.1	0.0	1.8	325
Geopsychrobacter	0.4	0.0	0.0	1.3	1.1	0.2	0.0	0.4	0.0	3.3	319
Desulfobulbus	0.4	3.3	0.0	0.3	0.3	5.1	0.0	1.8	0.0	0.1	303
Thiobacillus	0.0	0.0	15.9	0.0	2.0	0.0	0.0	0.0	0.0	0.0	302
Sphingomonas	0.0	0.0	0.0	0.0	0.0	0.0	16.4	0.0	0.0	0.3	285

Methylobacter	0.0	0.0	0.0	0.0	0.0	0.9	2.2	20.8	0.0	0.0	277
Eubacterium	0.2	3.7	0.0	0.8	0.7	0.2	5.6	0.0	0.0	0.1	263
Desulfatibacillum	0.2	0.8	0.0	0.2	0.1	3.1	5.1	3.4	0.0	0.0	257
Acidaminococcus	0.2	0.0	0.0	0.0	0.2	0.4	0.1	0.1	8.6	1.4	248
Desulfosarcina	0.3	0.8	0.0	0.7	0.4	0.5	0.0	1.3	0.0	2.2	247
Candidatus *Methylomirabilis*	0.7	2.0	0.0	1.4	1.5	0.0	0.0	0.0	0.0	0.0	238
Nitrospira	0.2	0.2	0.1	0.9	0.3	0.8	0.6	1.8	0.0	2.1	228
Leadbetterella	0.1	0.0	0.0	1.3	1.0	0.4	4.0	0.0	0.1	0.3	183
Dictyoglomus	0.1	0.0	0.0	0.1	0.4	0.5	1.5	0.8	0.0	1.7	172
Thermus	0.1	0.9	0.1	0.3	0.1	0.1	0.2	0.0	0.0	2.0	165
Tetrasphaera	0.9	0.6	0.0	0.5	0.5	0.0	0.0	0.0	0.0	0.0	161
Desulforhabdus	0.7	1.5	0.0	0.4	0.3	0.3	0.0	0.0	0.0	0.0	156
Candidatus *Magnetomorum*	0.1	0.0	0.0	1.2	0.5	1.8	1.0	2.2	0.0	0.1	151
Desulfovibrio	0.3	0.0	0.0	0.2	0.4	0.1	0.5	0.8	3.6	0.3	148
Afipia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.1	0.2	145
Methylocystis	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	144
Ralstonia	0.0	0.3	0.0	0.0	0.0	0.1	0.0	0.0	7.7	0.1	134
Cupriavidus	0.0	0.0	8.5	0.0	0.2	0.1	0.0	0.0	0.0	0.0	131
Thermolithobacter	0.2	0.2	0.0	1.5	1.2	0.2	0.0	0.0	0.0	0.1	131
Thermoanaerobacter	0.3	1.3	0.0	0.9	0.9	0.0	0.0	0.0	0.0	0.0	129
Leucothrix	0.0	0.0	0.0	0.0	0.0	0.0	7.8	0.0	0.0	0.0	128
Bacillus	0.1	0.0	0.1	0.0	0.4	0.0	0.0	0.0	6.4	0.0	126
Streptococcus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.4	0.0	124
Rothia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.3	0.0	108
Corynebacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.3	0.0	108
Azonexus	0.3	2.8	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	107

Chapter 4

Comparative Metagenomics Analysis of Two *Dehalococcoides* spp. Containing Enrichment Cultures Grown on 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin

Abstract

Polychlorinated dibenzo-*p*-dioxin (PCDD) dechlorinating anaerobic enrichment cultures, designated H1-3-2 and KKB3, were established using sediments collected from the Hackensack and Kymijoki rivers, respectively. They showed robust reductive dechlorination towards 1,2,3,4-tetrachlorodibenzo-*p*-dioxin. Here we report the genome reconstruction of the two predominant *Dehalococcoides* spp. (H1-3-2.001 and KKB3.003) from metagenomes of Hackensack River and Kymijoki River sediment enrichment cultures, respectively. Distinct suites of reductive dehalogenase homologous (*rdhA*) genes of the two *Dehalococcoides* strains were identified. Both assembled genomes contained a full length *rdhA* gene (HK_rdhA3 and KKB3_rdhA2) with high sequence similarity to that of *cbrA* in the genome of *Dehalococcoides mccartyi* strain CBDB1 and dcmb_86 in the genome of *Dehalococcoides mccartyi* strain DCMB5. Our metagenome analysis suggests that HK_rdhA3 and KKB3_rdhA2 might be responsible for reductive dechlorination of chlorinated dibenzo-*p*-dioxin compounds and that there is a possibility to use this *rdhA* gene as a biomarker to determine potential for bioremediation of PCDD contaminated sediments.

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are among the most notoriously persistent aromatic contaminants in the environment. “Hot spots” with high concentrations of PCDDs and other co-existing pollutants, such as polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), chlorobenzenes and chlorophenols, are still problematic to the environment and human health. The New York - New Jersey Harbor estuary (USA) and the Kymijoki River (Finland) are two regions which are highly impacted by anthropogenic activities and contaminated with a vast array of environmental pollutants. The Kymojoki River has a high concentration of PCDD/Fs in its sediments from production and utilization of chlorophenol-based wood preservatives (Verta *et al*., 2009). The Hackensack River is located in the highly urbanized area within the great metropolitan of New York and New Jersey, and was contaminated with not only PCDDs, but also heavy metals, dichlorodiphenyltrichloroethane (DDT) and polycyclic aromatic compounds (PAHs) (Wenning *et al*., 2004). In previous studies, we established PCDD dehalogenating cultures from anoxic sediments collected from these two rivers. These cultures showed dechlorination activities towards 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) and *Dehalococcoides* spp. were enriched to a considerably high relative abundance.

Dechlorination of highly chlorinated contaminants is the pivotal bioremediation approach. It is potentially cost effective, environmentally friendly and for some compounds fast (Edwards, 2014). Stepwise removal of chlorine substituents from the organic carbon skeleton in dechlorination is often coupled with energy conservation in organohalide-respiring bacteria, such as *Dehalococcoides* and some closely related *Chloroflexi*, *Dehalobacter*, and *Desulfitobacterium* species. This process has been shown to play an important role in bioremediation of soils and sediments contaminated with chlorinated solvents (tetrachloroethene, trichloethene) and PCBs

(Bedard, 2008; Furukawa, 2003), and therefore, there is hope for exploiting dechlorinating bacteria to bioremediate PCDD contaminated sites.

Reductive dehalogenases are terminal reductases that facilitate the last step of the electron transfer in organohalide-respiring bacteria to external electron acceptors (i.e., chlorinated compounds). This leads to the removal of chlorine from their organic backbone. Members of *Dehalococcoides* have been shown to possess a high number of reductive dehalogenase homologous genes (*rdhA*) encoding for the catalytic subunit A of putative reductive dehalogenases (RdhA), with up to 36 *rdhAs* found in the genome of *Dehalococcoides mccartyi* strain VS (McMurdie *et al*., 2009). However, the function of the majority of RdhAs are still unknown. The slow growth rate of *Dehalococcoides* spp. as well as the high sensitivity to oxygen of RdhAs hinders success in studying the substrate specificity of RdhAs using conventional biochemical approaches (Suyama *et al*., 2002; Maillard *et al*., 2003; Hölscher *et al*., 2003). So far, only a very few RdhAs of *Dehalococcoides* have been purified or partially purified and subjected to biochemical characterization (Magnuson *et al.*, 1998, 2000; Müller *et al*., 2004; Adrian *et al*., 2007b). An alternative approach using transcriptional analysis has been employed to assign substrate specificity to RdhAs (Krajmalnik-Brown *et al*., 2004; Fung *et al*., 2007; Chow *et al*., 2010; Wang *et al*., 2015).

Most studies on the substrate specificity of reductive dehalogenases have focused on reductive dechlorination of chlorinated compounds that are less complex and more water soluble, such as chloroethenes, chloroethanes, and chlorophenols. A recent study aimed to determine the substrate specificity in reductive dechlorination of PCBs (Wang *et al*., 2015). Little is known about reductive dehalogenases involved in dechlorination of PCDDs. Wagner *et al*. (2013) demonstrated that 2,3-DiCDD induced transcription of almost all *rdhAs* in *D. mccartyi* strain CBDB1, in which four genes (*cbrA*, cbdbA1453, cbdbA1624, and cbdbA1588) were transcribed to a higher level than the

others. So far, no other study has successfully determined which RdhAs are responsible for reductive dechlorination of PCDDs.

In this study, we reconstructed partial genomes of two 1,2,3,4-TeCDD dechlorinating *Dehalococcoides mccartyi* strains from metagenomes of dehalogenating enrichment cultures established from sediments of the Hackensack and Kymijoki rivers. The objective of this study was to reconstruct a collection of reductive dehalogenase homologous genes and determine which ones are likely to be responsible for reductive dechlorination of chlorinated dibenzo-*p*-dioxin compounds.

Materials and methods

Culture history

The original Hackensack enrichment culture was established in December 2012 using 10% sediment collected from sampling site H1 on the Hackensack River. The exact location and details of culture set up are described in Chapter 2. This enrichment culture was maintained under anaerobic conditions with 1,2,3,4-TeCDD as electron acceptor, acetate as carbon source, and lactate and propionate as electron donors. 1,2,3,4-TeCDD was kept at a concentration above 2 µM in the original culture as well as in the subsequent transfers. After 18 months of incubation, 10% of the original culture was used to establish a subculture to reduce the amount of sediment as well as dechlorination products. The first subculture was then split equally into two subcultures after only 6 months. Dechlorination activity was monitored periodically as described in Chapter 2. One of the second subculture (culture H1-3-2 with an estimated 0.5% vol/vol sediment) was harvested for DNA isolation for metagenome sequencing after it had been incubated for 7 months.

Kymijoki enrichment cultures were established in July 2013 in triplicate using 20% sediment from the Kymijoki River as inoculum. The Kymijoki River sediment was collected during a sampling trip in June 2013, and was kept at 4 ^{o}C until being further processed. Triplicate cultures, designated KKBs, were established using sediments with finer particles and gray in color. Details of the culture setup are described in Chapter 5. One of the three identical triplicate (KKB3) was harvested for DNA isolation for metagenome sequencing after 2 years of incubation.

DNA isolation, metagenome sequencing, assembly, and annotation

Genomic DNA was isolated from H1-3-2 and KKB3 enrichment cultures using the PowerSoil®DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). Isolated DNA was visualized and quantified on an agarose gel using a lambda DNA ladder of known concentration (Sigma Aldrich Inc., Saint Louis, MO, USA) and sent to the Leibniz Institute, DSMZ – German Collection of Microorganisms and Cell Cultures, for metagenome sequencing as described below. DNA was prepared for paired end sequencing on an Illumina Miseq using NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs Inc., Ipswich, MA, USA). The metagenomes were assembled with Megahit (Li *et al*., 2015). All resulting contigs which were smaller than 1,500 base pairs were combined and reassembled using Newbler (Life Technologies, Carlsbad, CA, USA) to maximize the number of contigs which can be binned as nucleotide-composition based binning is only feasible at contig length of more than 1 kb. All original contigs of more than 1,500 bp and Newbler-reassembled contigs of more than 1,000 bp were combined and binned using Maxbin to reconstruct single pan-genomes (Wu *et al*., 2014). The bin-assessments were done using the CheckM suite (Parks *et al*., 2015). Phylogenetic assignment was carried out using lineage specific marker-genes which were selected individually for each bin, after a preliminary assignment to the most closely related bacterial or archaeal lineages based on 43 universal single-copy marker genes.

The assembled genomes of the two *Dehalococcoides* strains were annotated using the rapid annotation using subsystem technology (RAST) server (Aziz *et al*., 2008), results were manually inspected and corrected when needed. Open reading frames (ORF) that were not called automatically by the RAST server were identified by Geneious (version 9.0). *rdhA* genes were identified by comparing with a customized database of more than 600 RdhA amino acid sequences from pure cultures and well-characterized mixed cultures. The comparison was carried out using the BLAST tool with an e-value threshold of 10^{-5} (Altschul *et al*., 1990, 1998).

Construction of phylogenetic trees

A maximum likelihood phylogenetic tree of 16S rRNA gene sequences of members of the phylum *Chloroflexi* retrieved from metagenomics data was reconstructed using MEGA software package (version 6.0) (Tamura *et al*., 2013). Prior to phylogenetic tree reconstruction, 16S rRNA gene sequences of *Chloroflexi* bacteria in the two metagenomes and selected corresponding matches from BLAST searches were aligned using ClustalW.

Alignment of reductive dehalogenase, subunit A (RdhA), of the two *Dehalococcoides* strains in H1-3-2 and KKB3 enrichment cultures and a selected database of 159 *rdhA* genes were performed using ClustalW on the MEGA software package as described above, and was manually edited if needed. Only RdhA sequences of 300 amino acids and above were included in the analysis. A Maximum likelihood phylogenetic tree with 500 bootstrap iterations was then reconstructed. The phylogenetic tree with a circular conformation was visualized and edited using Figtree program (version 1.4.2) (http://tree.bio.ed.ac.uk/software/figtree/).

Results and Discussion

General features of metagenomes and microbial community

The metagenome of Hackensack River enrichment culture H1-3-2 consisted of 131 Mbp, and was assembled into 63,515 contigs with an average length of 2,063 bp. Kymijoki River enrichment culture KKB3 had a metagenome size of 170 Mbp, it was assembled into 59,034 contigs with a mean contig length of 2,886 base pairs. The numbers of predicted protein coding genes of cultures H1-3-2 and KKB3 were 118,444 and 135,079, respectively. The general features of metagenomes of the two enrichment cultures are presented in Table 4.1.

In the Hackensack River culture, the microbial community was drastically simplified after two transfers and long-term incubation under anaerobic conditions with 1,2,3,4-TeCDD as the terminal electron acceptor. Three bacterial species belonging to the class *Dehalococcoidia* of the phylum *Chloroflexi* were found, totaling 58.6% of the total microbial community (Figure 4.1). Among these three *Dehalococcoidia* species, one binned genome of a bacterium closest to *Dehalococcoides mccartyi* was reconstructed with a completeness of 92% (Table 4.2). This strain, accounting for 56% of the community, is referred to as *Dehalococcoides* sp. H1-3-2.001. No other non-*Dehalococcoidia* members of the phylum *Chloroflexi* was found. In the Kymijoki River KKB3 enrichment culture, two bacteria belonging to the class *Dehalococcoidia* were found, which only made up 12% of total microbial community (Figure 4.1). One strain, referred to as *Dehalococcoides* sp. KKB3.003, had a binned genome with 98% completion (Table 4.2). This strain was more abundant than the other *Dehalococcoidia*, representing 11% of the microbial community in the KKB3 culture. Four non-*Dehalococcoidia* members of *Chloroflexi* were found in the KKB3 culture, that combined accounted for 5.4% of the community, their further phylogenetic affiliation was not resolved (Figure 4.1).

Members of *Deltaproteobacteria* made up only 14% of microbial community in the H1-3-2 enrichment culture but dominated (53%) the KKB3 culture (Figure 4.1), which had not been transferred and still contained a large proportion of sediment. Members of *Deltaproteobacteria* can grow on sulfate as well as by fermentation of short chain organic acids which were amended in our enrichment cultures as electron donors for dehalogenating bacteria, or residual organic materials present in sediment (Hug *et al*., 2012). Archaea accounted for a small proportion of the total microbial community under dehalogenating conditions. They were present at 9% in H1-3-2 and only 1.7% in the KKB3 enrichment cultures (Figure 4.1). Many archaea in the H1-3-2 enrichment culture are methanogens, which grow on the same source of electron donor, acetate, as *Dehalococcoides* spp.

General features of assembled genomes of the two dominant* Dehalococcoides *strains:

The two dominant *Dehalococcoides* strains in the H1-3-2 and KKB3 enrichment cultures (H1-3-2.001 and KKB3.003) had similar estimated assembled genome sizes of 1.34 and 1.45 Mbp, respectively. Their 16S rRNA gene sequences were identical (100% similarity over 881 bp; from the metagenome data 1374 bp of the 16S rRNA gene of H1-3-2.001 and 1009 bp of the 16S rRNA gene KKB3.003 was obtained; Figure 4.2). In the H1-3-2 enrichment culture, *Dehalococcoides* reads were assembled into 486 contigs, ranging in size from 1,000 to 15,989 bp, with an average length of 2,753 bp. The genome encoded for 1,531 proteins, in which 1,061 were functionally predicted. The KKB3.003 assembled genome had a total of 435 contigs, which ranged in size from 1,000 to 24,374 bp. The average length of contigs was 3,333 bp. The genome of *Dehalococcoides* sp. KKB3.003 had 1,505 protein encoding sequences, and the functions of 1,047 protein encoding sequences were assigned. Figure 4.3 summarizes functional categories of the two *Dehalococcoides* strains based on annotation made by the RAST server. Both *Dehalococcoides* genomes were binned using Maxbin tool and still had many contigs. However, at this moment,

with the focus on exploring features of genes that are involved in respiratory reductive dechlorination, we did not attempt to close the genomes of these two *Dehalococcoides* strains.

Reductive dehalogenase genes

Reductive dehalogenase homologous genes (*rdhAs*) were identified based on sequence similarity as well as the presence of a twin-arginine signal motif at the 5' end and two iron-sulfur clusters binding motifs at the 3' end. The *rdhA* gene annotation was implemented using the RAST server and open reading frame finding function of Geneious. Overall, *Dehalococcoides* sp. H1-3-2.001 harbored 31 putative *rdhA* genes encoding for proteins of more than 300 amino acids, in which 17 *rdhA* genes had a full length of approximately 1,500 bp, with complete 2 iron-sulfur cluster binding motifs and a twin-arginine signal motif. Seven *rdhA* genes were likely full length, but their sequences were truncated at either the 5' end or 3' end. *Dehalococcoides* sp. KKB3.003 had a total of 26 putative *rdhA* genes corresponding to proteins of more than 300 amino acids, 18 of which were full length and 5 genes which were missing the twin-arginine motif or the two iron-sulfur cluster binding sites. However, similar to what was observed for *Dehalococcoides* sp. H1-3-2.001, it is very likely that their sequences are truncated at the 5' or 3' end. Sequence analysis revealed that the majority of *rdhA* genes of the two strains shared high similarity with those of known *Dehalococcoides mccartyi* strains. Compared to different *D. mccartyi* strains belonging to three subgroups (Pinellas, Cornell, and Victoria), the majority of *rdhA* genes (24 of 31 *rdhA* genes in H1-3-2.001 and 24 of 26 *rdhA* genes in KKB3.003) shared more than 90% similarity at the amino acid level with *rdhA* genes previously found in other *D. mccartyi* strains (Tables 4.3 and 4.4). 19 *rdhA* genes in H1-3-2.001 and 20 *rdhA* genes in KKB3.003 belong to distinct ortholog groups based on the new naming system proposed by Hug *et al*. (2013) using the 90% pairwise amino acid identity cutoff. Although the two strains have different suites of *rdhA* genes, they shared 13

ortholog pairs of which the amino acid sequence similarity ranged from 95.4 to 100%. (Table 4.3; Figure 4.4).

The reconctructed genomes of the two *Dehalococcoides* strains obtained from the metagenomics data consisted of many short contigs. In many cases when *rdhA* genes were located on such short contigs of approximately 1,500 bp, part of their sequences were truncated. *rdhA* associated genes, therefore, were also difficult to determine. In order to obtain more information about *rdhA* genes, the genomes of the two *Dehalococcoides* strains H1-3-2.001 and KKB3.003 were aligned against the closely related *Dehalococcoides mccartyi* CBDB1. A total of 448 (92%) and 386 (89%) contigs of these two genomes were assembled using *D. mccartyi CBDB1* as a reference genome. Similar to genomes of other known *D. mccartyi* strains, *rdhA* genes appeared to cluster within two regions of the genome. In the genome of *Dehalococcoides* sp. H1-3-2.001, 5 *rdhA* genes clustered in the first region and the 18 other *rdhA* genes clustered in the second. The total proportion of *rdhA* genes took up 8.9% and 13.7% of total sequence length of the two abovementioned regions. Six contigs that contained 6 *rdhA* genes (HK_rdhA26, HK_rdhA27, HK_rdhA28, HK_rdhA29, HK_rdhA30, and HK_rdhA31) were not aligned with any regions within the genome of *D. mccartyi* strain CBDB1. This is not unexpected, since *rdhA* genes belong to a group of strain-specific genes of *Dehalococcoides* species (McMurdie *et al*., 2009). Sequences of these 6 *rdhA* genes and *rdhA* genes of *Dehalococcoides mccartyi* CBDB1 share less than 60% amino acid identity, except for HK_rdhA28 which shared 87.7% similar to protein sequence encoded by cbdbA1535. Clustering of *rdhA* genes in the genome of *Dehalococcoides* KKB3.003 was similar to that of strain H1-3-2.001: 3 *rdhA* genes were located in the one region and 12 *rdhA* genes were clustered in the other region, 7 *rdhA* genes (KKB3_rdhA20, KKB3_rdhA21, KKB3_rdhA22, KKB3_rdhA23, KKB3_rdhA24, KKB3_rdhA25, KKB3_rdhA26) which shared less than 60% amino

acid similarity to *rdhA* genes of strain CBDB1, were not aligned with genome of strain CBDB1 as the reference genome.

Reductive dehalogenase associated genes

The *rdhB* genes, which encode for small hydrophobic proteins – presumably serve as membrane anchoring proteins for reductive dehalogenases, were found only in close proximity to *rdhAs*. A total number of 19 and 21 *rdhB* gene sequences was present in the assembled genomes of H1-3-2.001 and KKB3.003, respectively. The length of RdhB sequences was found to be between 90 – 115 amino acids, except for one RdhB with only 44 amino acids, possibly because it was truncated at the 5' end. No lone *rdhB* genes were found elsewhere in their genomes as in genomes of *Dehalogenimonas* spp. (Siddaramappa *et al*., 2012; Molenda *et al*., 2016). Genes that are associated with *rdhAB* clusters are mostly MarR-type transcriptional regulator and two-component regulatory system which consists of a histidine kinase and a DNA binding response regulator, as found in the other *D. mccartyi* genomes (Kube *et al*., 2005; Wagner *et al*., 2013), even though the exact numbers could not be determined, likely because of the small contigs.

Substrate specificity of reductive dehalogenases

The two *Dehalococcoides* strains from the Hackensack River and Kymijoki River sediments have identical 16S rRNA genes, but they have distinct suites of reductive dehalogenase genes, suggesting that they are divergent and might have different chlorinated substrate ranges. Thirteen *rdhA* genes of the two *Dehalococcoides* strains shared more than 90% amino acid sequence similarity. Interestingly, HK_rdhA3 and KKB3_rdhA2 shared 100% and 99.8% similarity at the amino acid level to *cbrA* of *D. mccartyi* CBDB1, respectively (Tables 4.3 and 4.4). The *rdhB* gene sequences associated with HK_rdhA3 and KKB3_rdhA2 also shared 100% similarity to *cbrB* that is associated with *cbrA* of strain CBDB1, suggesting that the acquisition of these two genes by the two *Dehalococcoides* strains in this study occurred simultaneously.

The *cbrA* gene of *D. mccartyi* CBDB1 encodes for the only known reductive dehalogenase active in dechlorination of chlorobenzenes coupled to energy conservation and growth (Adrian *et al*., 2000, 2007b). In addition to reductive dechlorination of chlorobenzene, a recent study by Wagner *et al* (2013) showed that expression of *cbrA* was induced to the highest level of all four tested *rdhA* genes in the presence of 2,3-DiCDD. The growth of strain CBDB1 was also supported by reductive dechlorination of chlorinated dibenzo-*p*-dioxins (Bunge *et al*., 2003). Genome sequence analysis of another chlorinated dibenzo-*p*-dioxin-reductive dechlorinating bacterium (*D. mccartyi* DCMB5) revealed that this strain also possesses an ortholog of the *cbrA* gene (dcmb_86 which shared 99% amino acid similarity to *cbrA*) (Bunge *et al*., 2008; Pöritz *et al*., 2013, 2015). No other *rdhA* genes have more than 60% similarity at the amino acid level to *cbrA* according to the basic BLAST search on NCBI. Figure 4.5 illustrates the evolutionary relationships between orthologs of *cbrA* and other characterized RdhAs of *Dehalococcoides* species.

D. mccartyi 195 also exhibited dechlorination activity towards several PCDD congeners (1,2,3,4-TeCDD; 1,2,3,7,8-PeCDD; and 1,2,3,4,7,8-HxCDD). However, this strain does not possess an ortholog of the *cbrA* gene and has not been shown to couple dechlorination of chlorinated dibenzo-*p*-dioxin congeners to growth (Fennell *et al*., 2004; Liu & Fennell, 2008; Zhen *et al*., 2014). Therefore, it can be postulated that that HK_rdhA3 in *Dehalococcoides* sp. H1-3-2.001 and KKB3_rdhA2 in *Dehalococcoides* sp. KKB3.003 may be involved in reductive dechlorination of not only chlorobenzene, but also chlorinated dibenzo-*p*-dioxin in a metabolic manner.

In addition to the presence of a *cbrA* ortholog, the genomes of *Dehalococcoides* strains H1-3-2.001 and KKB3.003 also contained an ortholog of *mbrA* (98% and 98.6% amino acid similarity, respectively) (Tables 4.3 and 4.4). Transcripts of *mbrA* in *D. mccartyi* MB cultures fed with tetrachloroethene (PCE) increased concurrently with the production of trans-dichloroethene (trans-DCE) as the end-product (Chow *et al*., 2010). This suggests the potential PCE to trans-DCE

dechlorination capacity of these two strains. The genome of strain KKB3.003 also contained a sequence with high similarity to *pceA* of *D. mccartyi* 195 (92.5% amino acid similarity) and cbdbA1588 of *D. mccartyi* CBDB1 (98.4% amino acid similarity) (Table 4.4). PceA is responsible for reductive dechlorination of PCE as well as 2,3-dichlorophenol in strain 195 (Magnuson *et al*., 2000; Fung *et al*., 2007), its ortholog in the genome of strain CBDB1 (cbdbA1588) was found to increase in expression when grown in chlorophenol fed medium (Morris *et al*., 2007). No orthologs of *tceA* (*D. mccartyi* 195), *vcrA* (*D. mccartyi* VS), or *bvcA* (*D. mccartyi* BAV1) were found in genomes of either strain. This may indicate that these strains have distinct substrate spectra and dechlorination capacities compared to the three abovementioned chloroethene dechlorinating strains. However, further experiments need to be conducted to confirm these hypotheses.

Conclusions

Genomes of the two *Dehalococcoides* strains H1-3-2.001 and KKB3.003, which accounted for 56% and 11% of the community, respectively, were reconstructed from metagenomes of enrichment cultures established from the Hackensack and Kymojoki rivers. Even though the two genomes are not complete (92 and 98% completeness), with the focus on investigating reductive dehalogenases (a group of *Dehalococcoides* strain-specific genes), we were able to observe differences between the two *Dehalococcoides* genomes. HK_RdhA3 and KKB3_RdhA2 sequences shared high similarity and might contribute to respiratory reductive dechlorination of 1,2,3,4-TeCDD. Elucidating the substrate specificity of reductive dehalogenases of slow growing *Dehalococcoides* spp., especially on highly hydrophobic compounds such as chlorobenzene, PCBs and PCDDs, is challenging. Comparative genome analysis allowed speculation on the substrate specificities of reductive dehalogenases of *Dehalococcoides* spp. H1-3-2.001 and KKB3.003 for

further study at transcriptional or protein level, and suggested that *cbrA* might be a good biomarker for respiratory reductive dechlorination of PCDDs in the environment.

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Table 4.1. General features of metagenomes of enrichment cultures H1-3-2 and KKB3 determined from the Metagenomics RAST annotation tool.

Features	H1-3-2	KKB3
Size (bp)	131,046,768	170,427,173
Sequence count	63,515	59,034
Mean sequence length (bp)	2,063	2,886
Mean GC content (%)	53	56
Predicted protein features	118,444	135,079
Predicted rRNA features	3,929	3,317
Identified protein features	81,218	99,082
Identified rRNA features	412	296
Identified functional categories	69,542	83,048

Table 4.2. Overview of genomes of the two dominant *Dehalococcoides* spp. in Hackensack River H1-3-2 and Kymijoki River KKB3 enrichment cultures.

Features	*Dehalococcoides* sp. H1-3-2.001	*Dehalococcoides* sp. KKB.003
Size (base pair, bp)	1,338,413	1,450,001
Completeness (%)	91.9	98.3
# scaffold	486	435
N50 (scaffold)	3,240	3,814
Mean scaffold length (bp)	2,753	3,333
Longest scaffold (bp)	15,989	24,374
G+C content (%)	47	47.1
Coding density (%)	88.9	89.6
Protein coding genes	1,531	1,505
# of RNAs	43	51
Protein coding genes with predicted function	1,061	1,047
Protein coding genes without predicted function	470	458
# reductive dehalogenase genes (coding for protein of > 300 aa)	31	26

Table 4.3. Comparison between RdhAs in *Dehalococcoides* sp. H1-3-2.001 and those found in *Dehalococcoides* sp. KKB3.003 and representative RdhAs of *Dehalococcoides* spp. belonging to the Pinellas, Cornell and Victoria subgroups. RdhA of more than 90% similarity at the amino acid level are shown. Pairwise amino acid identity is presented in parentheses. Assigned ortholog group of the corresponding RdhA of *Dehalococcoides* sp. H1-3-2.001 is presented in the last column.

		Pinellas			Cornell		Victoria	
H1.3-2.001	**KKB3.003**	***D. mccatyi* CBDB1**	***D. mccartyi* DCMB5**	***D. mccartyi* BTF08**	***D. mccartyi* MB**	***D. mccartyi* 195**	***D. mccartyi* VS**	**Assigned ortholog group[(a)]**
HK_RdhA2	KKB3_RdhA1 (99.4)	cbdbA80 (99)	dcmb_81 (99.6)		MB_mbrA (98)			17
HK_RdhA3	KKB_RdhA2 (99.8)	cbdbA84 (100)	dcmb_86 (98.8)					52
HK_RdhA4	KKB3_RdhA3 (99.6)	cbdbA88 (99.8)	dcmb_91 (99.8)			DET0311 (93.4)		20
HK_RdhA5		cbdbA96 (99.2)						21
HK_RdhA6	KKB_RdhA4 (100)	cbdbA187 (99.7)	dcmb_184 (100)	btf_121 (100)		DET0180 (94.5)	8657123VS (95.5)	23
HK_RdhA7	KKB3_RdhA5 (99.6)	cbdbA238 (99.80	dcmb_235 (99.8)			DET0302 (95.30	8658278VS (95.5)	22
HK_RdhA8	KKB_RdhA8 (99.8)	cbdbA1455 (99.8)	dcmb_1341 (100)		MB_rdhA5 (90.8)			12
HK_RdhA9		cbdbA1503 (99.8)						
HK_RdhA11		cbdbA1539 (100)						
HK_RdhA12				btf_1409 (100)			8658289VS (98.2)	55
HK_RdhA13		cbdbA1546 (97.1)		btf_1436 (97.6)				54

	KKB3_RdhA12	cbdbA1550				8658303VS	
HK_RdhA14	(99.4)	(99.4)				(95.9)	38
	KKB3_RdhA11	cbdbA1563	dcmb_1385				
HK_RdhA15	(100)	(100)	(100)				39
		cbdbA1570			DET1522		
HK_RdhA16		(99.6)			(93.7)		33
	KKB3_RdhA13	cbdbA1575					
HK_RdhA17	(99.3)	(99.6)		btf_1440 (99.8)	DET1519 (91)		32
		cbdbA1578				8658308VS	
HK_RdhA18		(99.4)		btf_1443 (99.4)		(94.3)	19
		cbdbA1582				8658312VS	
HK_RdhA19		(98.5)		btf_1449 (97)		(95.5)	40
	KKB3_RdhA15	cbdbA1595				8658324VS	
HK_RdhA22	(97.8)	(98.1)		btf_1460 (97.9)		(94.9)	36
	KKB3_RdhA17	cbdbA1618				8658346VS	
HK_RdhA23	(95.4)	(95.9)		btf_1481 (95.9)		(91.5)	10
	KKB3_RdhA18	cbdbA1627	dcmb_1438			8658355VS	
HK_RdhA24	(99.8)	(99.8)	(99.8)	btf_1491 (100)		(97.6)	13
	KKB3_RdhA19	cbdbA1638	dcmb_1444		DET1545	8658361VS	
HK_RdhA25	(99.7)	(99.7)	(99.7)	btf_1497 (100)	(93.4)	(97.6)	15
HK_RdhA27				btf_1420 (99.8)			

[(a)]Reductive dehalogenase ortholog groups (RD_OG) were assigned based on the naming system proposed by Hug et al. (2013) and were last updated in May 2015.

Table 4.4. Comparison between RdhAs in *Dehalococcoides* sp. KKB3.003 and representative RdhAs of *Dehalococcoides* spp. belonging to the Pinellas, Cornell and Victoria subgroups. RdhAs of more than 90% similarity at the amino acid level are shown. Pairwise amino acid identity is presented in parentheses. Assigned ortholog group of the corresponding RdhA of *Dehalococcoides* sp. KKB3.003 is presented in the last column.

	Pinellas			Cornell		Victoria	
KKB3.003	***D. mccatyi* CBDB1**	***D. mccatyi* DCMB5**	***D. mccatyi* BTF08**	***D. mccartyi* MB**	***D. mccartyi* 195**	***D. mccartyi* VS**	**Assigned ortholog group[(a)]**
KKB3_RdhA1	cbdbA80 (99.2)	dcmb_81 (99.8)		MB_mbrA (98.6)			17
KKB3_RdhA2	cbdbA84 (99.8)	dcmb_86 (98.6)					52
KKB3_RdhA3	cbdbA88 (99.8)	dcmb_91 (99.8)			DET0311 (93.8)		20
KKB3_RdhA4	cbdbA187 (99.8)	dcmb_184 (100)	btf_121 (100)		DET0180 (95.6)	8657123VS (96)	23
KKB3_RdhA5	cbdbA238 (99.8)	dcmb_235 (99.8)			DET0302 (95.5)	8658278VS (95.9)	22
KKB3_RdhA6	cbdbA1092 (100)	dcmb_1041 (100)	btf_1057 (100)		DET1171 (94.9)	8658285VS (96.1)	31
KKB3_RdhA7	cbdbA1453 (98.2)	dcmb_1339 (97.6)		MB_dceA1 (90.8)		8658352VS (91)	17
KKB3_RdhA8	cbdbA1455 (100)	dcmb_1341 (99.8)		MB_rdhA5 (91)			12
KKB3_RdhA9		dcmb_120 (99.8)			DET0173 (93.9)		48
KKB3_RdhA10	cbdbA1560 (100)	dcmb_1383 (100)					14
KKB3_RdhA11	cbdbA1563 (100)	dcmb_1385 (100)					39
KKB3_RdhA12	cbdbA1550 (100)					8658303VS (95.8)	38
KKB3_RdhA13	cbdbA1575 (99.8)		btf_1440 (99.6)		DET1519 (92.3)		32
KKB3_RdhA14	cbdbA1588 (98.4)		btf_1454 (98.4)		DET0318 (PceA) (92.5)	8658318VS (94.6)	30
KKB3_RdhA15	cbdbA1595 (99.3)		btf_1460 (97.3)		DET1535 (92.6)	8658324VS (96.4)	34

KKB3_RdhA16	cbdbA1598 (100)		btf_1463 (100)		8658327VS (93.3)	11
KKB3_RdhA17	cbdbA1618 (99.6)		btf_1481 (99.40		8658346VS (95)	10
KKB3_RdhA18	cbdbA1627 (100)	dcmb_1438 (100)	btf_1491 (99.8)		8658355VS (97.4)	13
KKB3_RdhA19	cbdbA1638 (99.8)	dcmb_1444 (99.8)	btf_1497 (99)	DET1545 (94.6)	8658361VS (97.6)	15
KKB3_RdhA20		dcmb_1428 (99.5)				
KKB3_RdhA21		dcmb_1430 (99.8)				
KKB3_RdhA22		dcmb_1434 (100)				51
KKB3_RdhA23		dcmb_1436 (100)				
KKB3_RdhA25				DET1528 (97.8)		

[(a)]Reductive dehalogenase ortholog groups (RD_OG) were assigned based on the naming system proposed by Hug et al. (2013) and were last updated in May 2015.

Figure 4.1. Microbial community structure of enrichment cultures H1-3-2 (A) and KKB3 (B) determined by the abundance of binned genomes in metagenomes of the corresponding cultures.

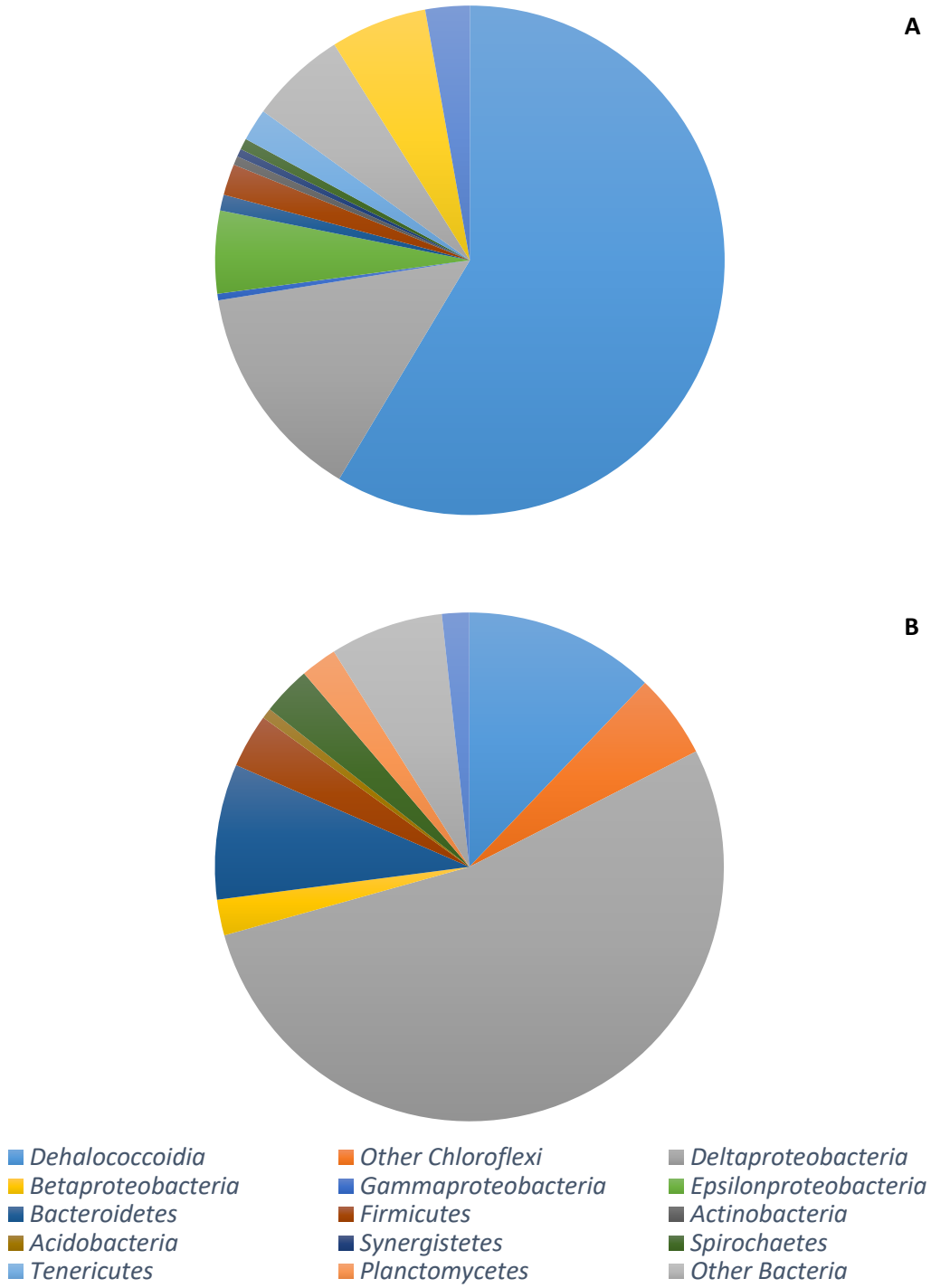


Figure 4.2. Maximum likelihood phylogenetic tree of 16S rRNA genes of 5 *Chloroflexi* representatives retrieved from metagenomes of H1-3-2 and KKB3 enrichment cultures. 16S rRNA gene sequences of representative *Chloroflexi* bacteria were imported from NCBI, their accession numbers are presented in parentheses. The tree is drawn to scale with branch lengths measured in number of substitutions per site. Bootstrap values from 500 iterations are indicated at the nodes.

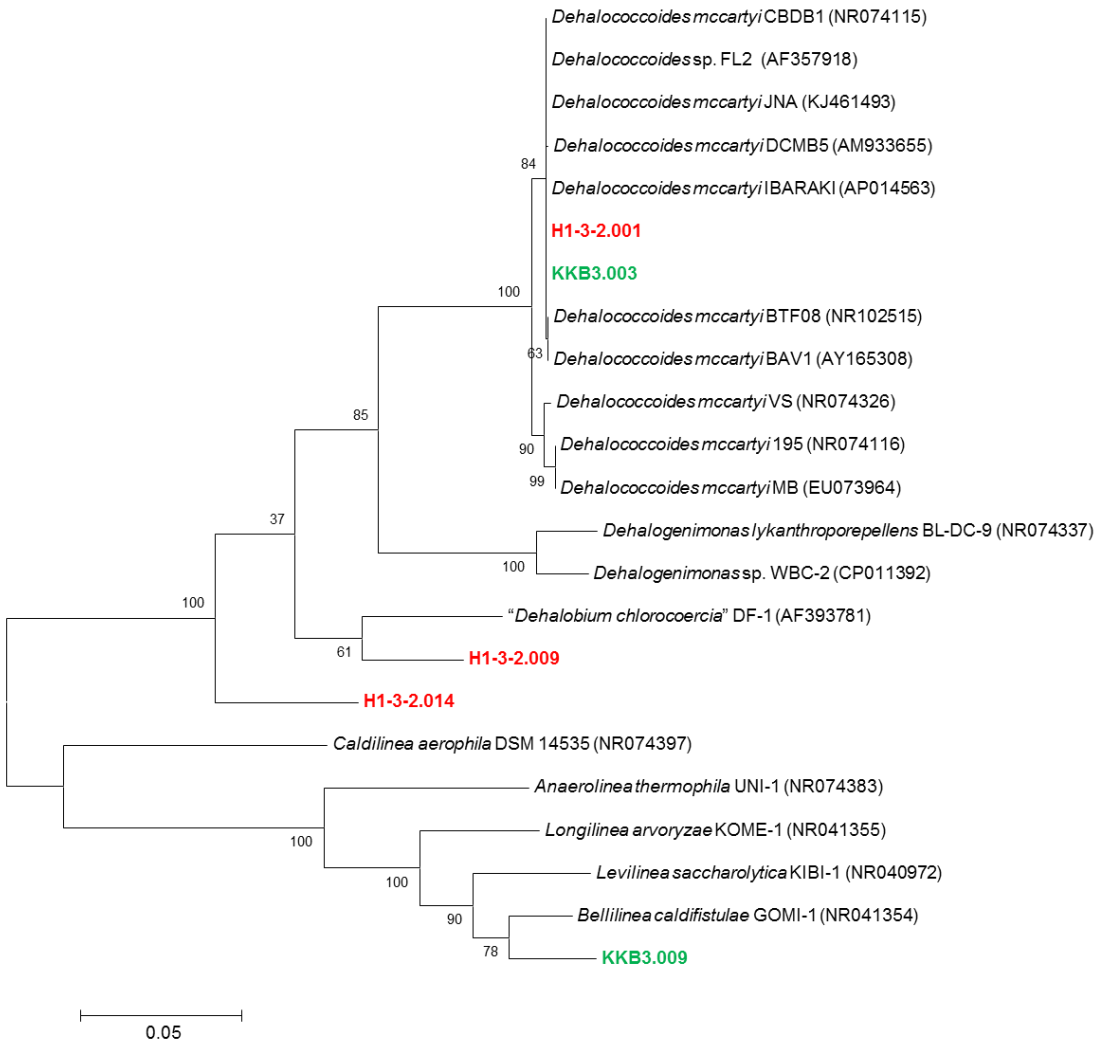
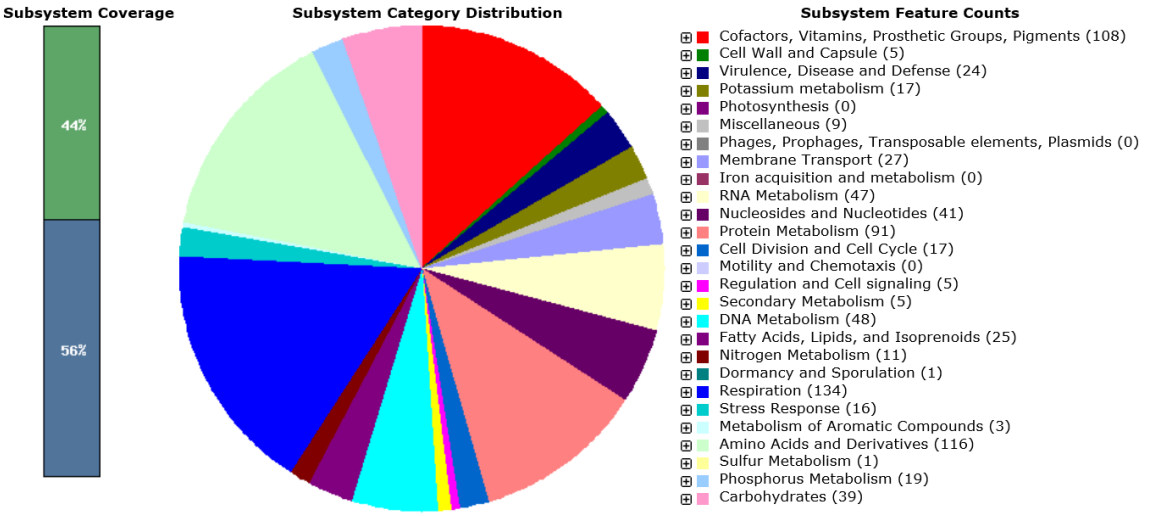


Figure 4.3. Functional categories of *Dehalococcoides* strains H1-3-2.001 (A) and KKB3.003 (B) using the Rapid Annotation using Subsystem Technology (RAST) server.

A



B

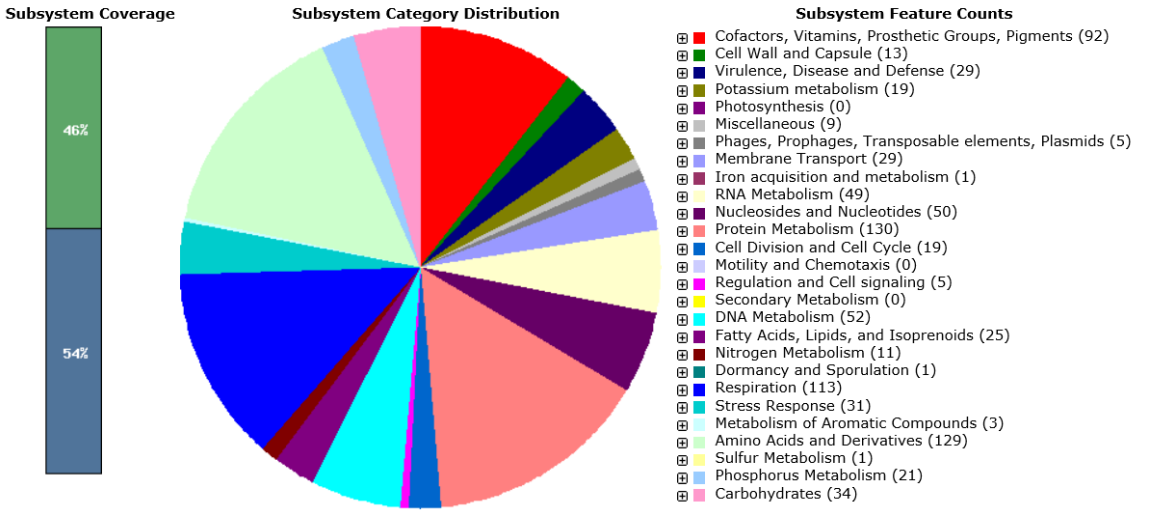


Figure 4.4. Maximum likelihood phylogenetic tree of RdhA protein sequences found in binned genomes of *Dehalococcoides* strains H1-3-2.001 and KKB3.003 and 159 representative reference RdhA sequences in the database. The tree with the highest probability is shown (bootstrap values are not shown) The tree is drawn to scale, with branch lengths measured in number of substitution per site. Only sequences of more than 300 amino acids are included. Names of RdhA's are color coded according to the organisms that harbor them.

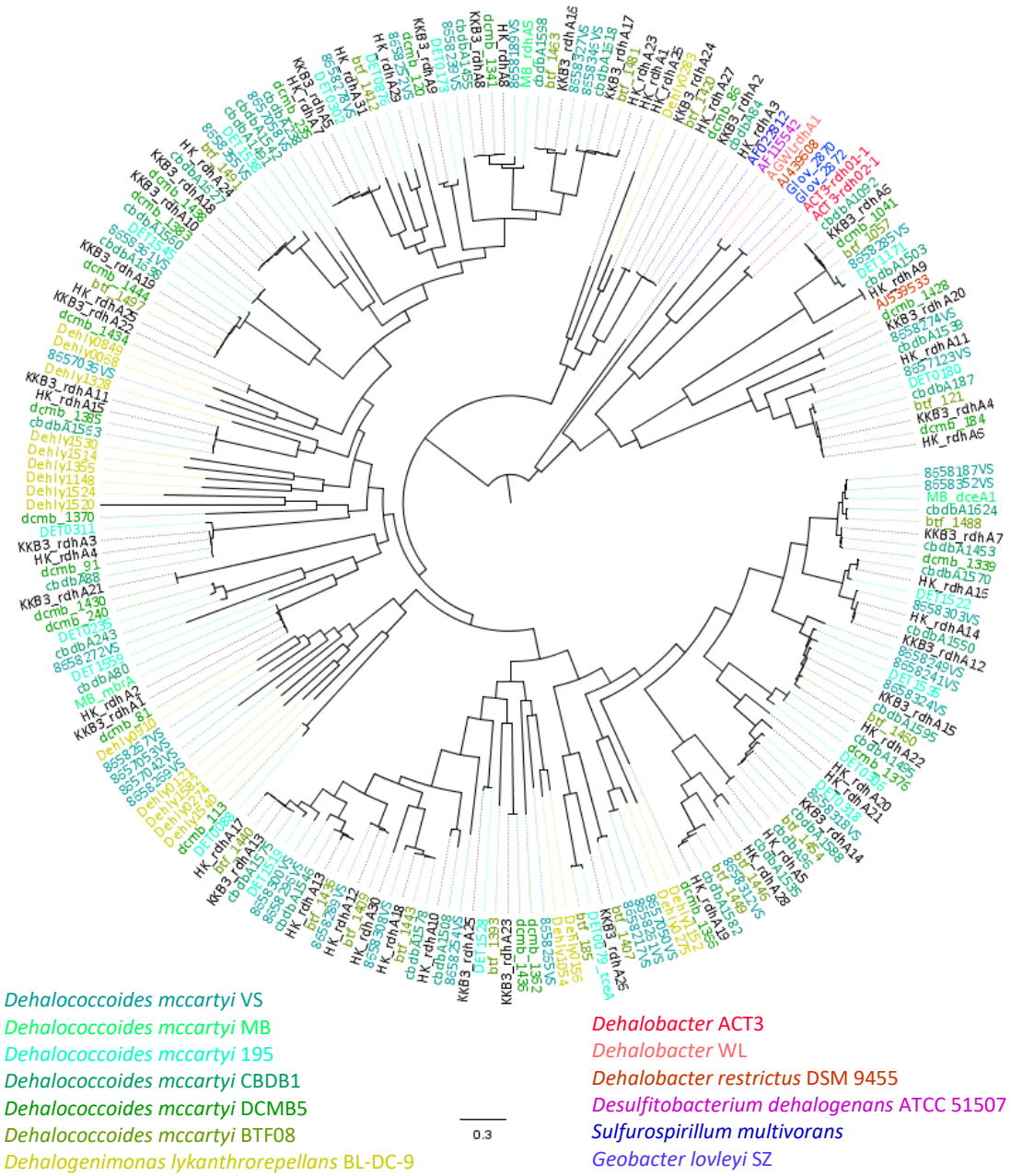
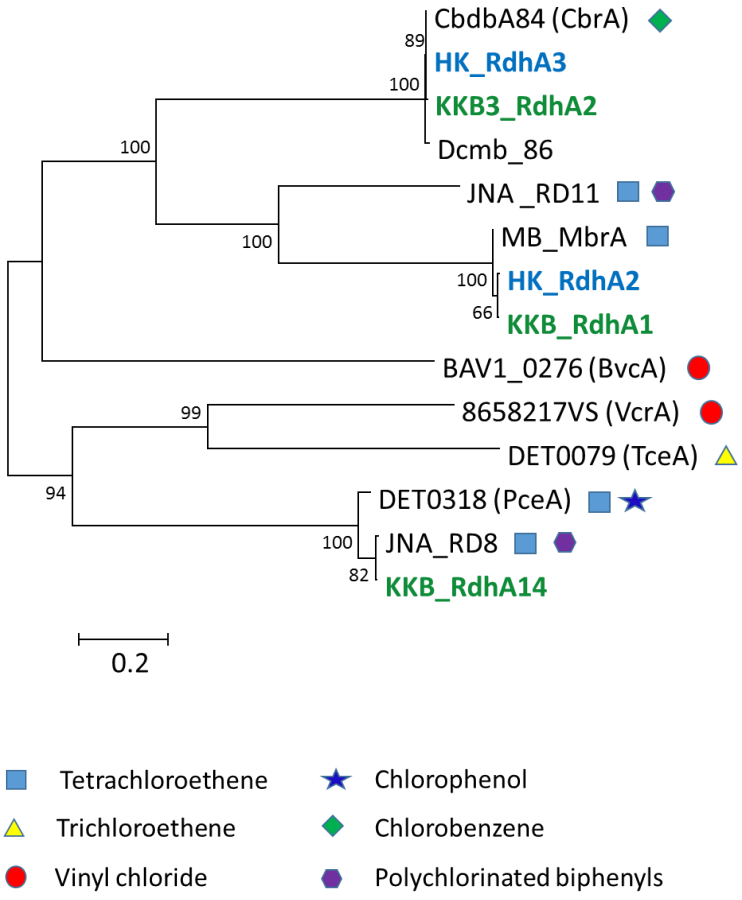


Figure 4.5. Maximum likelihood phylogenetic tree of HK_RdhA3, KKB3_RdhA2 and characterized RdhAs of *Dehalococcoides mccartyi* strains. Scale shows the amount of genetic change. Bootstrap value from 500 itrations are shonw at the nodes. Symbols next to RdhAs represent substrate of the corresponding enzymes.



SUPPLEMENTARY DATA

Table S4.1. Comparison between RdhAs found in binned genomes other than HK1-3-2.001 and KKB3.003 in the metagenomes of Hackensack and Kymijoki River enrichment cultures, and representative RdhAs of *Dehalococcoides* spp. belonging to the Pinellas, Cornell and Victoria subgroups. RdhAs of more than 90% similarity at the amino acid level are shown. Pairwise amino acid identity is presented in parentheses. Assigned ortholog group of the corresponding RdhA of *Dehalococcoides* sp. KKB3.003 is presented in the last column.

RdhAs found in metagenomes	Pinellas			Cornell	Victoria	Assigned ortholog group [(a)]
	D. mccartyi CBDB1	*D. mccartyi* DCMB5	*D. mccartyi* BTF08	*D. mccartyi* 195	*D. mccartyi* VS	
Hackensack River culture						
HK002_k107_430601	cbdbA1495 (100)	dcmb_1376 (100)				36
HK002_contig10074		dcmb_240 (99.1)		DET0235 (94.4)		29
HK002_contig02746	cbdbA243 (99.8)					47
HK002_k107_558148					8658239VS (96.1)	
HK003_k107_355736	cbdbA1535 (100)					37
HK003_k107_598748		dcmb_120 (100)		DET0173 (92.5)		48
HK003_k107_195978				DET1559 (98.3)		35
HK003_k107_692251			btf_1393 (95.4)			

HK004_k107_513369	cbdbA1560 (14) (100)	dcmb_1383 (100)			14
HK004_k107_451949			btf_1446 (99.6)		
HK014_k107_245481		dcmb_1434 (99.2)			51
HK030_k107_589030	cbdbA1491 (100)				
Kymijoki River culture					
KY007_contig02462	cbdbA1495 (100)	dcmb_1376 (100)			36
KY007_k107_383400	cbdbA96 (100)				21
KY007_k107_132383		dcmb_1370 (99.8)			50
KY007_k107_201655		dcmb_1362 (99.8)			
KY007_k107_379181	cbdbA1542 (99.8)				
KY007_k107_283765	cbdbA1503 (99.8)				
KY007_k107_177920	cbdbA1546 (100)		btf_1436 (99.6)		
KY032_k107_431400	cbdbA1539 (99.4)				
KY032_k107_282846				DET1559 (99)	35

[(a)]Reductive dehalogenase ortholog groups (RD_OG) were assigned based on the naming system proposed by Hug et al. (2013) and were last updated in May 2015.

Chapter 5

Reductive Dechlorination of Polychlorinated Dibenzo-*p*-dioxins in Enrichment Cultures from Soils and Sediments from Southern Vietnam and the Kymijoki River

Abstract

The long-term presence of polychlorinated dibenzo-*p*-dioxins (PCDDs) in the environment enriches for organisms that can transform PCDDs either via detoxification mechanisms or to gain energy or carbon. In order to determine if dechlorination of PCDDs is prevalent in anoxic environments, enrichment cultures were established using soils and sediments collected from Vietnam and Finland, and amended with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD), dechlorination was monitored using gas chromatography/mass spectrometry. Reductive dechlorination towards 1,2,3,4-TeCDD was observed at different rates and to different extents in all enrichment cultures regardless of the different contamination background of the inocula. The priming effect of 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) on enhancing dechlorination of 1,2,3,4-TeCDD was inconclusive in Vietnam soil enrichment cultures. Microbial community analysis revealed the presence of *Dehalococcoides* spp. in Kymijoki cultures, but not in Vietnam enrichment cultures.

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are often found together with polychlorinated dibenzofurans (PCDFs) in contaminated environments. These two groups of chlorinated compounds with similar molecular structures are released from various sources via natural or human-related events. The source of PCDD/F emission can be either identifiable (point sources) or unidentifiable (non-point sources). “Hot spots” of PCDD/F contamination are usually associated with heavy industrial activities such as industrial zones found in the Passaic River, New Jersey (USA), Bitterfeld (Germany) or Finland (Bopp *et al*., 1991; Götz *et al*, 1994, 2007; Verta *et al*., 2009). Non-point sources can bring PCDD/F concentrations to above background levels, they affect areas with high anthropogenic activities such as the New York/New Jersey metropolitan area (USA) (Friedman *et al*., 2012).

Each PCDD/F source produces different PCDD/F patterns in which the composition and proportion of each congener differs. Contaminated sites, therefore, have divergent PCDD/F fingerprints. Thermal processes usually produce more octachlorodibenzo-*p*-dioxin, whereas 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is the signature PCDD congener of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)-based herbicide production (Götz *et al*., 2007; Hites, 2011; Dopico & Gómez, 2015). PCDD production was assumed to be produced exclusively from anthropogenic sources. However, they can also be naturally produced from combustion, enzymatic activities of extracellular enzymes on chlorophenols, or sponge associated systems in marine environments (Sheffield, 1985; Öberg & Rappe, 1992).

Excessive production and utilization of PCDD containing chemicals results in deposition of PCDDs in large quantities in the environment and leads to changes in the local microbial community in which some microorganisms adapt to transform PCDDs for detoxification and sometimes to gain energy or carbon (dehalogenating bacteria). The enzymatic machinery that is

adapted to transform newly introduced PCDD congeners as well as other xenobiotic compounds have usually evolved from existing enzymes that catalyze the transformation of similarly structured compounds of natural origin. Horizontal gene transfer is the major means of genetic acquisition to introduce genes encoded for xenobiotic degradation into new microorganisms. Enzymes that are involved in xenobiotic degradation are often found in mobile genetic elements such as plasmids (Springael & Top, 2004). Similarly, the majority of reductive dehalogenase genes, which encode for enzymes involved in dechlorination of organohalides, are embedded in genomic islands or sandwiched by mobile gene elements (Kube *et al*. 2005; Nonaka *et al*., 2006; McMurdie *et al*., 2009). This allows for the adaptation of dehalogenating bacteria to use many kinds of chlorinated compounds.

In this study, we compared enrichment cultures established from soils and sediments collected from various locations, spanning different climate zones, and with different PCDD/F patterns. Soils and sediments collected from Vietnam were presumably contaminated with PCDDs from the 2,4,5-T containing herbicide (Agent Orange) which was sprayed during the Vietnam war (Stellman *et al*., 2003). Sediments collected from the Kymijoki River (Finland) were contaminated with a mixture of PCDD/Fs due to production and utilization of a chlorophenol-based wood preservatives (Verta *et al*., 2009). We aimed to compare the dechlorination activity of enrichment cultures established from these different soils and sediments.

Materials and methods

Culture establishment and maintenance

Vietnam enrichment cultures: Anaerobic enrichment cultures (50 mL) were established in triplicate from soils and sediments collected from Quang Tri province, southern Vietnam in 2008. Table 5.1 lists all four sets of enrichment cultures used in this study and locations from which soils

and sediments were collected. They were established and maintained by Drs. Vien Duong and Joong-Wook Park for over three years. All enrichment cultures were prepared in carbonate buffered mineral medium as described in Monsserate & Häggblom (1997) except that sodium chloride concentration was reduced to 1.17 g/L to adapt to cultivation of microorganisms from terrestrial environments and freshwater sediments. Cultures were kept under strictly anaerobic conditions in 60 mL serum bottles sealed with Teflon™ lined gray chlorobutyl-isoprene septa and crimped with aluminum caps. 1,2,3,4-TeCDD was amended at a nominal concentration of 10 µM, and a mixture of acetate, propionate, and lactate at 1 mM concentration was added as electron donor and carbon source. In August 2009, the cultures which showed dechlorination activity were spiked with 20 µM 1,2,3,4-TeCDD (V Duong & JW Park, personal communication) and incubated at 28 °C until September 2011 when they were adopted for this dissertation project. To determine if 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) can enhance dechlorination activity of 1,2,3,4-TeCDD, a replicate of culture 2T4 was re-spiked with 1,2,3,4-TeCDD at 20 µM, while the other was re-spiked with both 1,2,3,4-TeCDD at 20 µM and 1,2,3,4-TeCB at 50 µM. After 6 months, both cultures were split into 2 cultures and were considered duplicates.

Kymijoki River enrichment cultures: Sediments used to establish Kymijoki enrichment cultures were collected during the sampling trip on the Kymijoki River in June 2013 in Kuusankoski, Finland, and were kept at 4 °C until culture setup. The two samples (designated KKA and KKB) were collected a few meters apart (M Häggblom, personal communication). They had different physical properties. KKA sediment was course and contained large plant debris while KKB sediment contained fine, clay-like particles. Fifty milliliter (50 mL) anaerobic enrichment cultures were established in triplicate using 20% w/v of sediment as inoculum in 60 mL serum bottles which were then sealed with Teflon™ lined gray chlorobutyl-isoprene septa and crimped with aluminum caps, and the headspace was filled with 3%H_2/97%N_2 to maintain anaerobic conditions. To spike

electron acceptor into enrichment cultures, a 250 µL stock solution of 1,2,3,4-TeCDD in toluene (10 mM) was added to cover 0.2 grams of silicon oxide (200 Mesh or finer, Sigma Aldrich Inc., St. Louis, MO, USA). After evaporation of toluene leaving 1,2,3,4-TeCDD coated on silicon oxide particles, 50 mL sediment slurry was added. A mixture of acetate, propionate, and lactate was added as electron donor and carbon source at a concentration of 150 µM each, 100 µg/L cobalamin was added to provide an external source of corrinoid for reductive dechlorination. Killed controls were established from two sediment samples similar to experimental cultures but were autoclaved for 1 hour on 3 consecutive days.

Sampling and analytical methods

Samples were taken periodically from enrichment cultures to monitor dechlorination over time. One milliliter of sediment slurry was withdrawn from a well-shaken culture using a N_2 purged syringe. Chlorinated compounds were extracted into toluene phase using 1.5 mL extraction solvent which consisted of a mixture of toluene and acetone (1:1). Extraction was carried out for 3 hours. Toluene extracts were subjected to gas chromatography/mass spectrometry (GC/MS) analysis as described in Chapter 2. Chlorinated compounds (1,2,3,4-TeCDD and 1,2,3,4-TeCB) as well as their dechlorination products were identified based on their retention times and their most dominant molecular ions in comparison to authentic standards. Quantification was performed using an external standard four-point calibration curve of a mixture of chlorinated dibenzo-*p*-dioxins (1,2,3,4-TeCDD, 1,2,3-TriCDD, 1,3-DiCDD, 1-MoCDD, and DD) at concentrations ranging from 0.2 µM to 20 µM each, a mixture of chlorobenzenes (1,2,3,4-TeCB, 1,2,3-TriCB, and 1,2-DiCB) at concentrations ranging from 0.2 to 50 µM each.

Bacterial community analysis

Bacterial communities in enrichment cultures KKA and KKB 2 years after being established and incubated and duplicates of enrichment culture 2T4 on 1,2,3,4-TeCDD were investigated

using PCR-TRFLP. Genomic DNA from these cultures was isolated using the PowerSoil®DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). Amplification of 16S rRNA genes was carried out using universal bacterial primer pair 27F and 1101R as described in Chapter 3. Restriction digest with *MnlI* and TRFLP analysis were implemented as described by McGuinness *et al*. (2006).

Results

Dechlorination of 1,2,3,4-TeCDD in Vietnam enrichment cultures

All Vietnam cultures showed reductive dechlorination towards 1,2,3,4-TeCDD. After re-spiking with 1,2,3,4-TeCDD and incubating for 2 years, the molar percentage of the residual 1,2,3,4-TeCDD ranged from 0 mol% (in culture 2T4-1) to 55 mol% (in culture 1T4-3) (Figure 5.1). However, the extent of dechlorination differed not only between cultures established from different locations but also between replicates. MoCDD and DiCDD were the most abundant congeners detected at the time of chemical analysis (Figure 5.1). Complete dechlorination of 1,2,3,4-TeCDD, in which dibenzo-*p*-dioxin (DD) accumulated, was observed in several Vietnam enrichment cultures including 1T4-3, 3T4-2, and 3T4-3, at relatively high proportions, and were 45, 72, and 65 mol%, respectively (Figure 5.1).

Bunge *et al*. (2008) revealed that 1,2,3,4-TeCDD dechlorinating bacteria were also capable of reductive dechlorination of chlorobenzenes at a faster rate. 1,2,3-TriCB was used to enhance growth of the 1,2,3,4-TeCDD dechlorinating bacterial community. In this study, we split replicates of enrichment culture 2T4 and amended 1,2,3,4-TeCB along with 1,2,3,4-TeCDD to determine if 1,2,3,4-TeCB had a priming effect in boosting the dechlorination rate. However, after 6 months of incubation, no significant difference in the extent of 1,2,3,4-TeCDD dechlorination was observed between cultures with or without 1,2,3,4-TeCB (Figure 5.2). In the subcultures established by

transfer (50%), 1,2,3,4-TeCB was dechlorinated at a substantially faster rate than 1,2,3,4-TeCDD over the course of 251 days. 100 mol% of 1,2,3,4-TeCB from the first amendment was dechlorinated within 41 days, and dechlorination continued after being re-spiked. 1,2,3,4-TeCDD, however, was not dechlorinated at a faster rate compared to cultures amended with only 1,2,3,4-TeCDD (Figure 5.3).

The bacterial communities in enrichment culture 2T4 amended with only 1,2,3,4-TeCDD were investigated by amplifying bacterial 16S rRNA genes followed by fingerprinting using TRFLP. TRF 111, which corresponds to *Dehalococcoides* spp., was not detected in TRFLP profiles of the enrichment culture 2T4 amended with 1,2,3,4-TeCDD. Therefore, we conclude that *Dehalococcoides* spp. may not contribute to reductive dechlorination of 1,2,3,4-TeCDD in enrichment culture 2T4. TRF 128 was the most dominant TRF found in TRFLP of 2T4 cultures (36 ± 4% in total bacterial community). Other TRFs present at relatively higher abundance in 2T4 cultures included TRFs 208, 212, and 295 which accounted for 10 ± 2%, 6 ± 1%, and 6 ± 1% of the total bacterial community, respectively.

Dechlorination of 1,2,3,4-TeCDD in Kymijoki enrichment cultures

Dehalogenating enrichment cultures (KKA and KKB) were established in triplicate from Kymijoki river sediments. 1,2,3,4-TeCDD was added in carbonate buffered sediment slurry at a nominal concentration of 50 µM which is a higher concentration than in Vietnam cultures and Hackensack River cultures, because the Kymijoki River sediments were contaminated with PCDD/Fs at very high concentration (Verta *et al*., 2009). MoCDD was the most predominant congener detected in both Kymijoki enrichment cultures after 2 years of incubation, similar to what was observed in Liu *et al*. (2014). While the same proportions of MoCDDs and DiCDDs accumulated in culture KKA as compared to KKB (approximately 2 mol% of DiCDD and 55 mol% of MoCDD was detected in the two sets of cultures), the proportion of the residual 1,2,3,4-TeCDD

was different (27 ± 3 mol% in culture KKA, and 43 ± 10 mol% in culture KKB), attributed by the accumulation of TriCDDs in enrichment culture KKA but not in culture KKB (Figure 5.4). Similar to most other cultures, dibenzo-*p*-dioxin was not detected (limit of detection, 0.2 µM) (Figure 5.4).

The bacterial community in Kymijoki River enrichment cultures was also investigated using PCR-TRFLP as in Vietnam enrichment cultures. TRF 111 bp, which corresponds to *Dehalococcoides* spp., increased in relative abundance in both KKA and KKB cultures, from non-detectable at time 0 to 23 ± 13% in KKA and to 10 ± 3% in KKB (Figure 5.5). Comparing the shift of bacterial communities between KKA and KKB cultures after 2 years of incubation, we noticed that TRF 121 in KKA decreased from 12% to 1 ± 0.3%, in contrast, it increased substantially from 0% to 15 ± 4% in KKB culture. TRF 212 increased from 2% at time-point 0 to 18 ± 5% in KKB cultures, while it remained relatively unchanged in KKA culture (6% at time 0 and 7 ± 1% after 2 years).

Discussion

Complete dechlorination of 1,2,3,4-TeCDD to DD was only observed in some of the enrichment cultures established from Vietnam soils and sediments (Figure 5.1). In these cultures, either MoCDD or DiCDDs accumulated. Complete dechlorination to DD was not observed in any of the enrichment cultures established from Kymijoki River sediments (Figure 5.4) and Hackensack River sediments, except for a trace amount of DD detected in Hackensack River enrichment culture H3 (Chapter 2). Long-term incubation (more than 3 years by the time the chemical analysis was performed) may have induced growth of bacteria that can dechlorinate 1,2,3,4-TeCDD and its chlorinated daughter products completely to DD in Vietnam enrichment cultures. *Dehalococcoides* spp. were not detected in these cultures using TRFLP. It is possible that dehalogenating bacteria in 1,2,3,4-TeCDD dechlorinating enrichment cultures established from

Vietnam soils and sediments are distinctly different from cultures established using the Hackensack and Kymijoki River sediments.

Dechlorination of mono-chlorinated compound such as vinyl chloride (VC), monochlorobenzene (MCB) is rare. While a diverse group of dehalogenating bacteria can dechlorinate tetrachloroethene (PCE), trichloroethene (TCE), only a few have been shown to use VC in a respiratory process (He *et al*., 2003; Cupples *et al*., 2003). *Dehalococcoides mccartyi* CBDB1 is able to respire TeCB and trichlorobenzene (TCB) and dechlorinate them to dichlorobenzene (DCB) as the final product, DCB could not be dechlorinated further to MCB and benzene (Adrian *et al*., 2000). Only recently was MCB shown to be terminal electron acceptor for growth of some *Dehalobacter* strains (Fung *et al*., 2009; Nelson *et al*., 2011).

Trichlorodibenzo-*p*-dioxins (TriCDDs) were minor dechlorination end-products in all Vietnam enrichment cultures, except in one replicate of culture 17T4 where 8% TriCDDs accumulated. TriCDDs were also detected at a very low molar percentage in Hackensack River cultures H4 and H5. In other Hackensack River enrichment cultures, they reached the highest proportion after 5 months of incubation, and then were quickly dechlorinated further to DiCDDs and 2-MoCDD (Chapter 2). However, TriCDDs accumulated at a surprisingly high proportion even after a long time incubation in one set of Kymijoki River cultures (KKA). Dechlorination of 1,2,3,4-TeCDD is usually the rate-limited step and TriCDDs are often found as transient dechlorination product and are quickly dechlorinated to daughter products with fewer chlorines. Chemical analysis after a long-term incubation usually results in no detection of TriCDD congeners (Chapter 2; Ballerstedt *et al*., 1997). In the KKA cultures, dechlorination of TriCDD to DiCDD is another rate-limited step. After 2 years of incubation, TriCDDs were detected at 15 mol% of the total dibenzo-*p*-dioxin congeners (Figure 5.4). Compared to dechlorination activities of 1,2,3,4-TeCDF of enrichment cultures established using sediment collected from the same Kymijoki site, TriCDF congeners

accumulated at the greatest proportion among all daughter products of 1,2,3,4-TeCDF dechlorination after 29 months of incubation (Kuokka *et al*., 2014). Different dechlorination patterns observed between the KKA and KKB cultures might be explained by a difference in *Dehalococcoides* species/strains that might be indistinguishable by TRFLP profiles or by contribution by another dehalogenating bacteria. Park *et al*. (2011) also found the same putative *Dehalococcoides* TRF in TRFLP profiles of PCB dechlorinating cultures amended with 1,2,3,4-TeCB and pentachloronitrobenzene (PCNB), however the PCB dechlorination rate was different between the two treatments.

PCDDs are historical contaminants, their concentrations in the environment vary depending on the source and how they were released from point or non-point sources. Dehalogenating bacteria usually represent a very low proportion of the microbial community and may not efficiently dechlorinate contaminants of interest. Alternative chlorinated compounds (haloprimers) at higher concentrations than PCDD and are less toxic than the target contaminants, might be use to stimulate the growth of dehalogenating bacteria to enhance dechlorination rate of the target contaminants. Various halogenated co-substrates, including monobromophenol, 2,3,4,5-tetracholoroanisole and 1,2,3,4-tetrachlorobenzene, have been shown to enhance dechlorination of 1,2,3,4-TeCDD in in anaerobic sediment cultures (Vargas *et al*., 2001; Ahn *et al*., 2007). 2,6-Dibromobiphenyl was also used to enhance growth of dehalogenating bacteria which in turn stimulate reductive dechlorination of PCB (for review, see Bedard, 2008). However, a priming effect of 1,2,3,4-TeCB on dechlorination of 1,2,3,4-TeCDD was inconclusive in this case. In a similar study, 1,2,3,4-TeCB also did not significantly enhance dechlorination of PCBs in Anacostia enrichment cultures (Krumins *et al*., 2009). This might be because different bacterial strains are responsible for reductive dechlorination of 1,2,3,4-TeCB and 1,2,3,4-TeCDD.

Acknowledgements: We would like to thank Drs. Vien Duong and Joong-Wook Park for establishing and maintaining enrichment cultures from Vietnam. We also thank William Bartos for help with processing and establishing enrichment cultures.

Table 5.1. List of soil and sediment samples collected in Vietnam to establish dechlorinating enrichment cultures in 2008 (V Duong, personal communication).

Culture name	Description of samples used to establish cultures
1T4	Sediment, VP creek, Quang Tri province
2T4	Sediment, Mai Loc water reservoir, Cam Nghia, Quang Tri Province
3T4	Sediment, Doi 4 water reservoir, Cam Nghia, Quang Tri province
17T4	Soil, paddy rice field, Cam Nghia, Quang Tri province

Figure 5.1. Relative mole distribution of 1,2,3,4-TeCDD and its dechlorination products in Vietnam enrichment cultures 2 years after 20 µM 1,2,3,4-TeCDD was re-spiked. Data of all cultures in triplicate are presented.

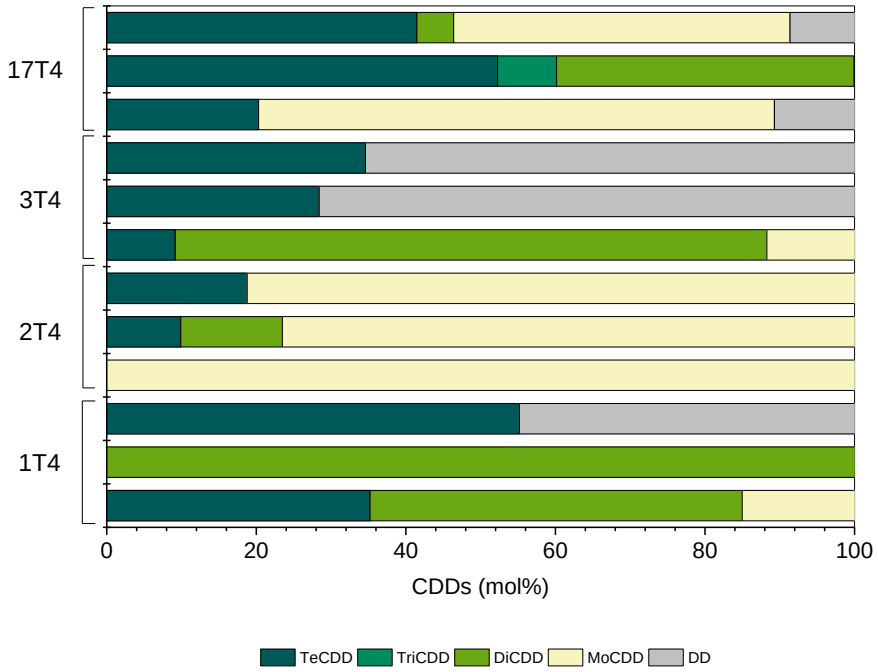


Figure 5.2. Dechlorination of 1,2,3,4-TeCDD in 2T4 enrichment cultures 185 days after the cultures were re-spiked with different combinations of electron acceptors: 1,2,3,4-TeCDD only, and mixture of 1,2,3,4-TeCDD and 1,2,3,4-TeCB. Relative mole distribution of 1,2,3,4-TeCDD and its daughter products at time point 0 and after 185 days are presented.

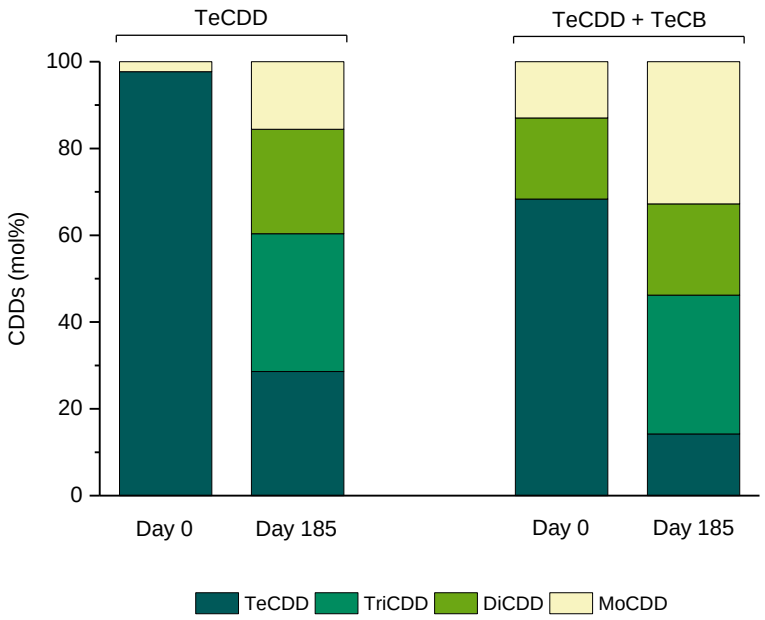
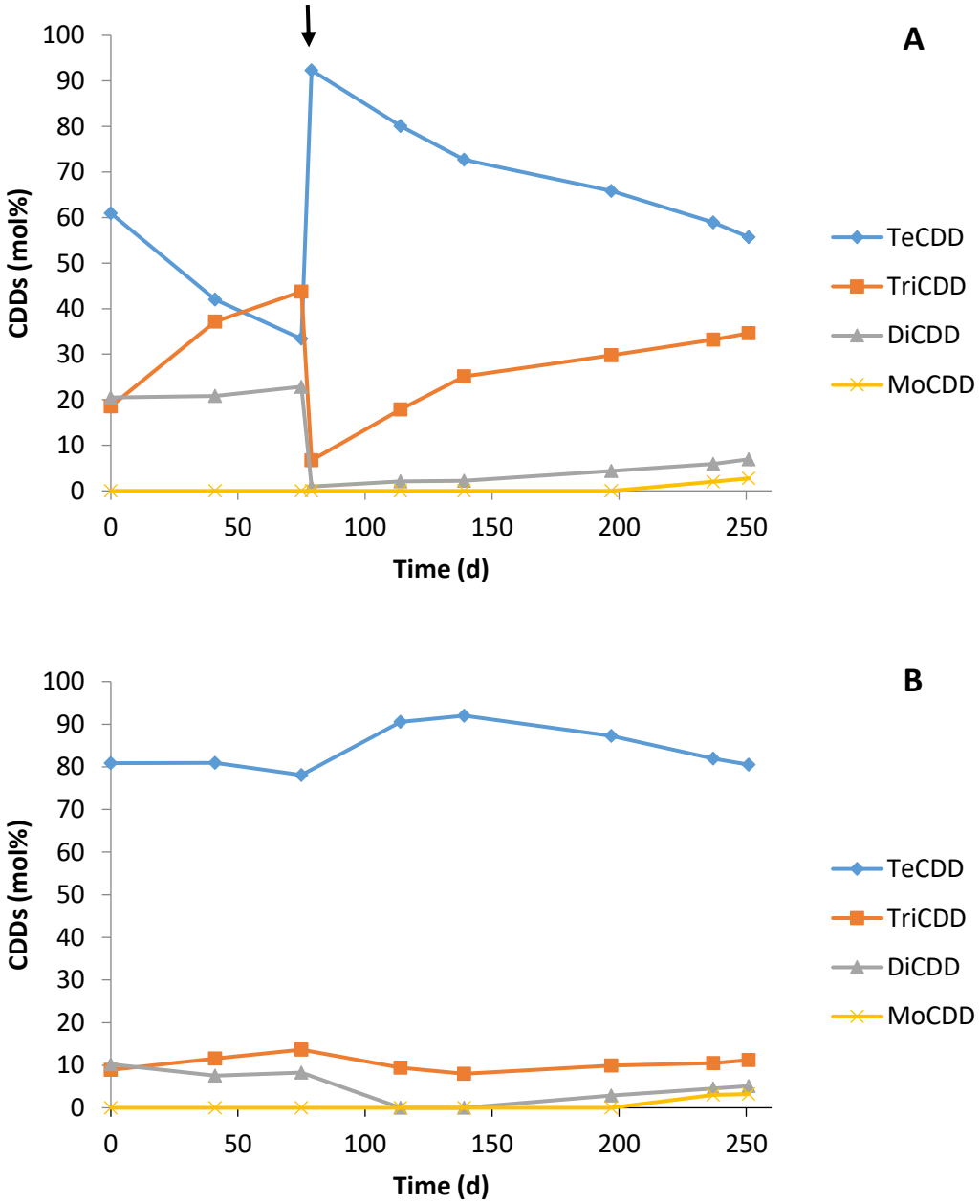
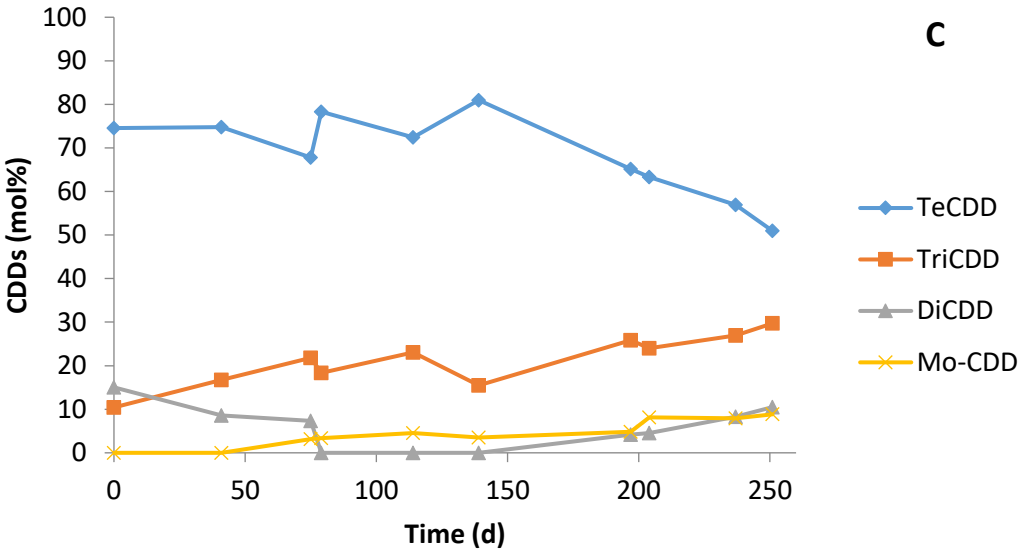
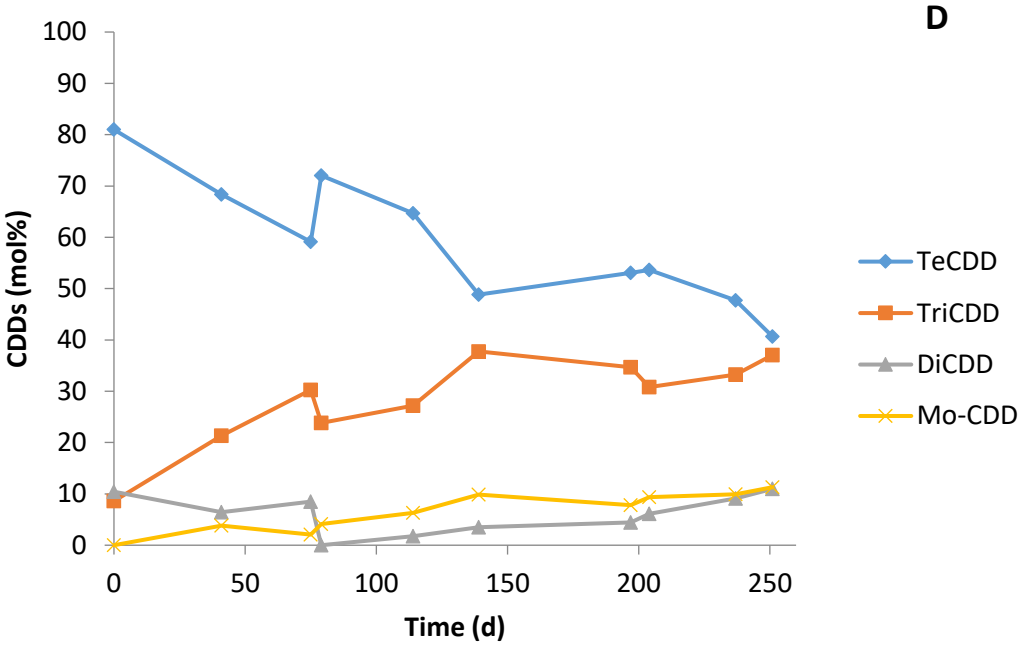
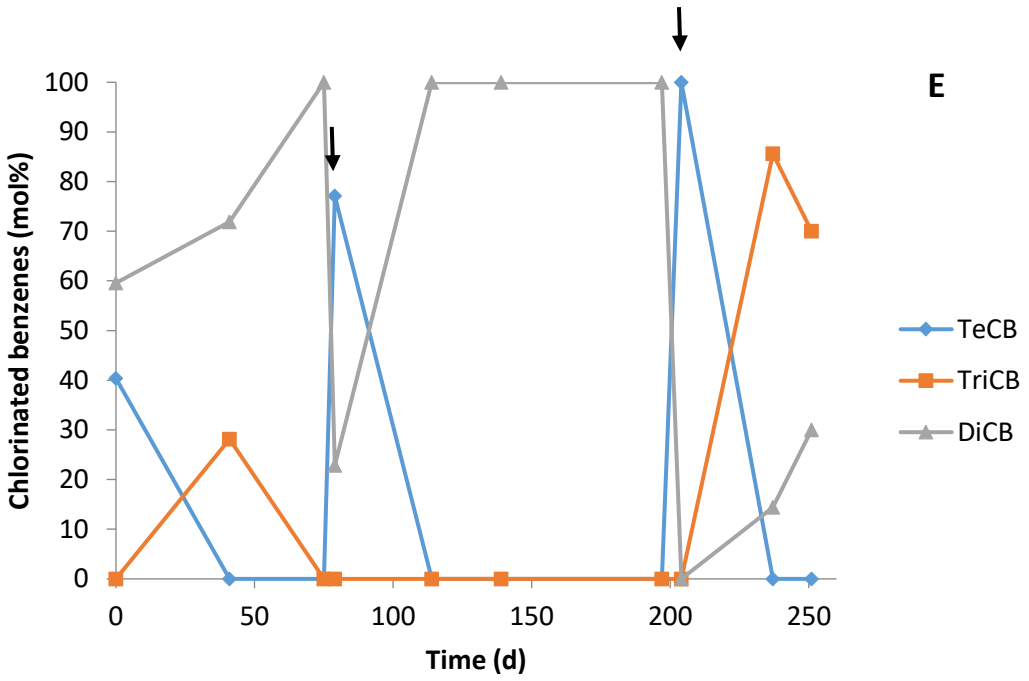


Figure 5.3. Time course of dechlorination in duplicate enrichment cultures 2T4 after transfer (50%) into fresh medium. (A, B) Dechlorination of 1,2,3,4-TeCDD in duplicate 2T4 cultures amended with 1,2,3,4-TeCDD only; (C, D) Dechlorination of 1,2,3,4-TeCDD in cultures amended with 1,2,3,4-TeCDD and 1,2,3,4-TeCB; (E, F) Dechlorination of 1,2,3,4-TeCB in cultures spiked with 1,2,3,4-TeCDD and 1,2,3,4-TeCB. Black arrow indicates when cultures were re-spiked with chlorinated substrates.




C
CDDs (mol%)
Time (d)
TeCDD
TriCDD
DiCDD
Mo-CDD


D
CDDs (mol%)
Time (d)
TeCDD
TriCDD
DiCDD
Mo-CDD


E
Chlorinated benzenes (mol%)
Time (d)
TeCB
TriCB
DiCB

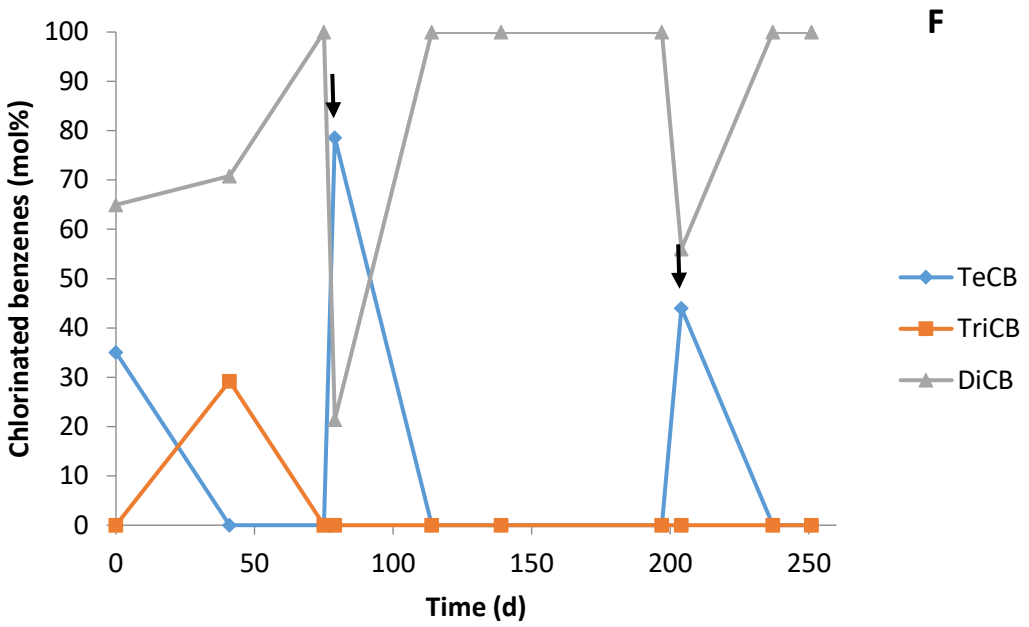

F
Chlorinated benzenes (mol%)
Time (d)
TeCB
TriCB
DiCB

Figure 5.4. Dechlorination of 1,2,3,4-TeCDD in Kymijoki enrichment cultures after 2 years of incubation. Average and standard deviation of relative mole distribution of 1,2,3,4-TeCDD and its daughter products in KKA and KKB cultures are presented.

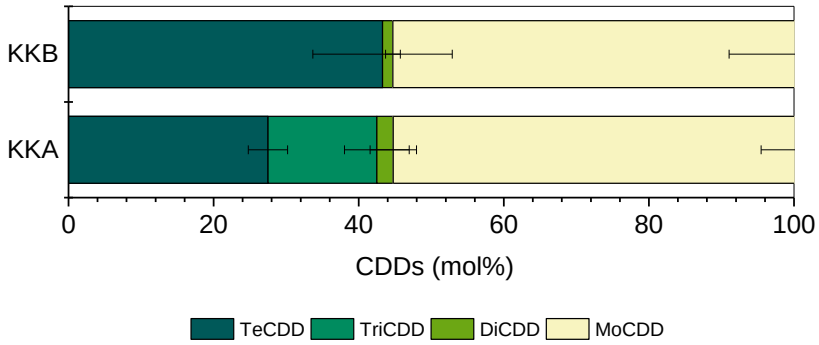
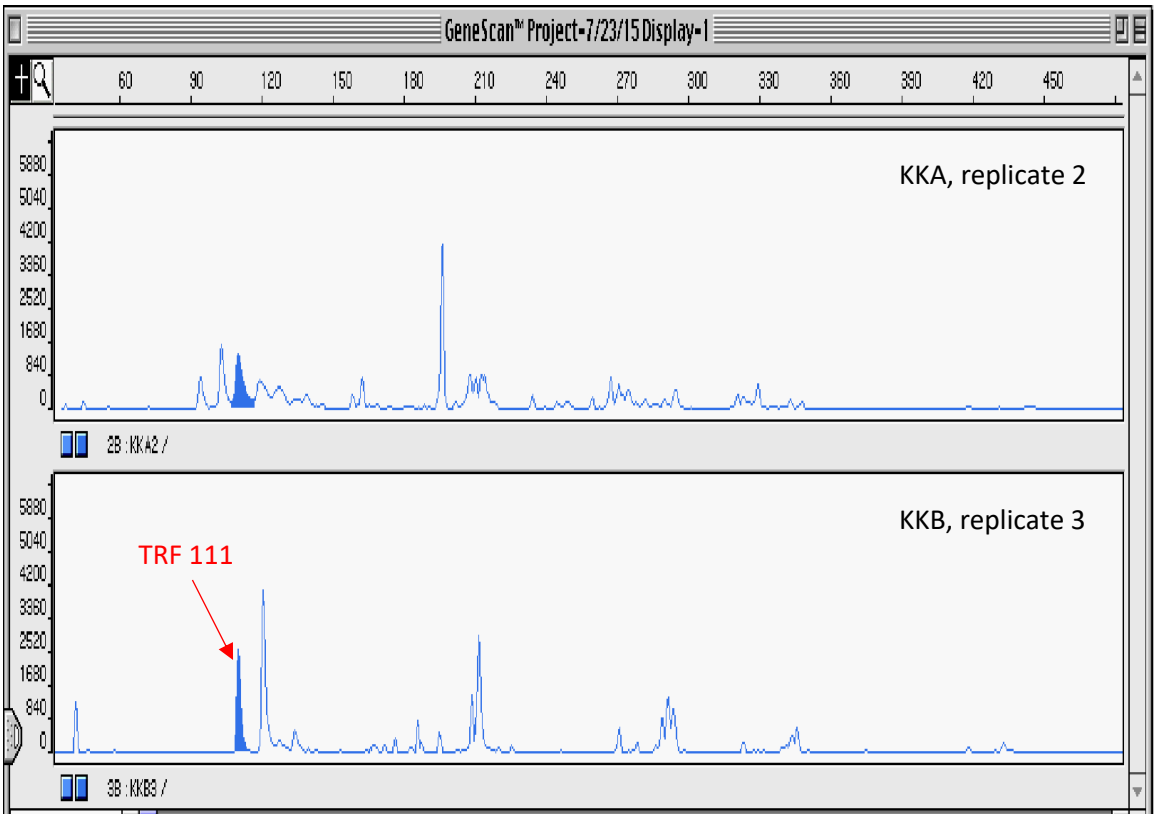


Figure 5.5. TRFLP profile of bacterial community in Kymijoki enrichment cultures revealed the presence of TRF 111 corresponding to *Dehalococcoides* spp.



Chapter 6

Conclusions

Polychlorinated dibenzo-*p*-dioxins (PCDDs) have been part of the carbon and chlorine cycles a long time long before the industrial era began. They have started to cause negative impacts on the environment and biological systems since the beginning of chlorinated biocide production and utilization due to their persistence and toxicity. The main research questions we set out to study were: do PCDDs in the environment sustain the growth of indigenous dehalogenating bacteria and will we be able to find PCDD dehalogenating bacteria in anoxic soils and sediments, regardless of the PCDD contamination background? In this study, we successfully established PCDD dechlorinating enrichment cultures using sediments from the Hackensack and Kymijoki rivers. PCDD dechlorinating cultures established from Vietnam soils and sediments were also adopted to use in this study. A total of 11 sets of anaerobic enrichment cultures were maintained throughout the course of the study. 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) was amended as the terminal electron acceptor at concentrations ranging from 20 to 50 µM in methanogenic medium.

Dechlorination activities were observed in all enrichment cultures although dechlorination rates and extents differed among cultures. There are several lines of evidence indicating that *Dehalococcoides* spp. were actively involved in reductive dechlorination of 1,2,3,4-TeCDD and coupled this with growth. Microbial community analyses revealed the presence of *Dehalococcoides* spp. in four (H1, H2, H3, and H4) out of five sets of Hackensack River sediment enrichment cultures (Figure 2.4) and in both sets of Kymijoki River sediment enrichment cultures (KKA and KKB) (Figure 5.5). Quantification of *Dehalococcoides* spp. in the Hackensack River enrichment cultures revealed the correlation between 16S rRNA gene copy numbers to the extent

of 1,2,3,4-TeCDD dechlorinated (Figure 2.5). Stable isotope probing experiments indicated that *Dehalococcoides* spp. were dominant in the heavy DNA fraction isolated from cultures amended with 1,2,3,4-TeCDD and ^{13}C-labeled acetate, but were detected at a very low proportion in cultures without 1,2,3,4-TeCDD (Figure 3.3), implying that they mediated respiratory dehalogenation of 1,2,3,4-TeCDD. Nonetheless, *Dehalococcoides* spp. were not detected in Vietnam culture 2T4 or in Hackensack River culture H5 which was established from sediments collected at the station closest to Newark Bay (Figure 2.4 and Chapter 5), suggesting the activity of potentially other PCDD dechlorinating species.

In most of our 1,2,3,4-TeCDD dechlorinating enrichment cultures, TriCDDs rarely accumulated after long-term incubation (13 – 24 months). TriCDDs were detected at a very low proportion within 5 months and were not found at concentrations above the detection limit (0.2 µM) at the end of the incubation period (Figures 2.1B and 2.1C). However, some cultures showed accumulation of TriCDDs, even after 2 or more years of incubation. These cultures included the Hackensack River cultures H5, Vietnam culture 17T4-2, and Kymijoki River cultures KKA. Dechlorination of 1,2,3,4-TeCDD may have been contributed by the same *Dehalococcoides s*train as in the other Hackensack River cultures (H1, H2, H3, and H4) although present at lower relative abundance, or different *Dehalococcoides* strains or even other dehalogenating genera/species.

Although dechlorination of MoCDD to DD is thermodynamically favorable (Huang *et al*., 1996), dechlorination of 1,2,3,4-TeCDD only resulted in the accumulation of MoCDD as the major end-product in almost all dechlorinating enrichment cultures and MoCDD was not dechlorinated further to DD (Figure 2.1; Figure 5.4). Complete dechlorination to DD has also not been observed extensively in mixed cultures or pure cultures in the other studies (Ballerstedt *et al*., 1997; Bunge *et al*., 2003; Fennell *et al*., 2004; Pöritz *et al*., 2015). Similar scenarios have been observed in dechlorination of chlorobenzenes and chloroethenes. So far, only a few studies have found

respiratory reductive dechlorination of chlorinated compounds with one chlorine in the molecule (e.g. vinyl chloride, monochlorobenzene) (He *et al*., 2003; Cupples *et al*., 2003; Fung *et al*., 2009; Nelson *et al*., 2011). However, accumulation of DD as the major dechlorination product were observed in 3 out of 12 Vietnam enrichment cultures. The long-term accumulation of MoCDD in Vietnam enrichment cultures may have enriched for groups of bacteria that can dechlorinate MoCDD completely to DD. Interestingly, *Dehalococcoides* spp. were not detected in Vietnam enrichment culture based on TRFLP profile, suggesting that dechlorination of 1,2,3,4-TeCDD in the Vietnam cultures may have been mediated by distinctly different dehalogenating bacteria.

Estuarine and marine sediments become ultimate sinks of PCDDs, because of the migration of PCDDs from upstream river sediments. A high concentration of sulfate in estuarine and marine environments has been known to support the growth of sulfate reducing bacteria which use the same source of electron donors as organohalide-respiring bacteria, and therefore complete with organohalide-respiring bacteria. Here we investigated the impact of salinity and sulfate concentration on dechlorination of PCDDs. The rate and extent of 1,2,3,4-TeCDD dechlorination was found to be impaired by high salinity and sulfate concentrations. Among five sets of enrichment cultures established using sediments collected at 5 locations along the Hackensack River, dechlorination of 1,2,3,4-TeCDD was fastest and most complete in enrichment culture H1 in which sediment was collected from the uppermost section of the river. Dechlorination of 1,2,3,4-TeCDD produced a high relative mole distribution of TriCDD in culture H5 of which inoculum was sediment collected from only 2 km away from the mouth of the river. Time course dechlorination of 1,2,3,4-TeCDD revealed the longer onset of dechlorination in H5 compared to H1 cultures (Figures 2.1B and 2.1C).

Dehalococcoides spp. grow very slowly in pure cultures, and dechlorination activity has been observed to be lower in pure cultures of *Dehalococcoides* spp. than in mixed cultures. Supportive

members of dehalogenating community provide dehalogenating bacteria with essential growth elements such as corrinoids, as well as provide them with a mechanism to cope with oxidative stress (Maymó-Gatell *et al*., 1997; He *et al*., 2007; Hug *et al*., 2012). During the course of this dissertation project, we did not try to isolate PCDD dechlorinating bacteria. However, we managed to enrich for the growth of PCDD dechlorinating bacteria while simplifying microbial community to facilitate genomic study on dehalogenating bacteria (Figure 2.4; Figure 4.1).

High throughput sequencing and bioinformatics analysis allowed for reconstructing the genomes of the two *Dehalococcoides* spp. that dominated in enrichment cultures H1-3-2 and KKB3 from the metagenomes of the corresponding enrichment cultures. Although their 16S rRNA gene sequences were identical and they possessed highly similar core genomes (Chapter 4), they harbored different suites of reductive dehalogenase genes which suggested a distinct capacity to dechlorinate different ranges of chlorinated compounds. Considering that they were enriched from sediments contaminated with not only different PCDDs but other organohalide pollutants, this is not surprising. However, both of these *Dehalococcoides* strains possessed a reductive dehalogenase homologous gene (*rdhA*) (HK_rdhA3 and KKB_rdhA2) which is very similar to the *cbrA* gene found to be responsible for reductive dechlorination of chlorobenzene in *Dehalococcoides mccartyi* CBDB1 (Adrian *et al*., 2007). The protein encoded by the *cbrA* ortholog (dcmb_86) was the most expressed *rdhA* gene in the proteome of *Dehalococcoides* sp. DCMB5 after induction by pentachlorobenzene, trichlorobenzene, and tetrachloroethene (Pöritz *et al*., 2015). So far, only the two *Dehalococcoides* strains CBDB1 and DCMB5 have been shown to respire chlorinated dibenzo-*p*-dioxins and both of them contain *cbrA*. Other *Dehalococcoides* strains that do not dechlorinate chlorinated dibenzo-*p*-dioxin in respiration do not harbor *cbrA* orthologs. Therefore, we postulate that the *cbrA* orthologs in *Dehalococcoides* sp. H1-3-2.001 and KKB3.003 are responsible for reductive dechlorination of 1,2,3,4-TeCDD as a respiratory process.

This thesis provided data to support the hypothesis that even though PCDDs are extremely hydrophobic and persistent, the capacity for microbial reductive dechlorination of PCDDs in anoxic environments is not rare. In fact, PCDD dechlorinating anaerobic bacteria appear ubiquitous, and their activity was observed in almost all enrichment cultures established using soils and sediments collected from sites of different contamination backgrounds. *Dehalococcoides* spp. have been identified as the main bacterial species that actively dechlorinate PCDDs and couple that to growth. Comparative genome analysis suggested that a reductive dehalogenase *cbrA* ortholog is responsible for reductive dechlorination of 1,2,3,4-TeCDD in a respiratory process. Genome analysis provided preliminary evidence for the function of reductive dehalogenases in PCDD dechlorination which have not been studied before due to the complex nutritional requirements and slow growth of *Dehalococcoides* spp.

PCDD dechlorinating bacteria were found in all contaminated environments which were investigated in this thesis. Under anaerobic conditions established in the laboratory, they readily consumed spiked 1,2,3,4-TeCDD as an electron acceptor. However, PCDDs are still present at high concentration in some places all over the world. One explanation can be that PCDDs present in the environment are highly chlorinated (with more than 4 chlorines per molecule), are thus have low water solubility and bioavailability for dehalogenating bacteria. In addition, dehalogenating bacteria in the environment are not limited to using PCDDs as electron acceptors, they can use a wide variety of naturally produced organohalides as well. This thesis did not provide a direct solution for remediation of PCDD contaminated sites, however, it contributed to the understanding of the potential of dehalogenating bacteria in biotransformation of PCDDs. Understanding the organisms and specific genes involved in dechlorination of PCDDs may help design a strategy to evaluate the bioremediation capacity of PCDD contaminated sites and

develop an effective bioremediation approach to reduce negative effects of residual PCDDs in the environment.

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Appendix A

Partial 16S rRNA gene sequences of *Dehalococcoides* strains H1-3-2.001 and KKB3.003 retrieved from metagenomics data

Partial 16S rRNA sequence of *Dehalococcoides* sp. H1-3-2.001

TTGAGCTTGGAGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGGCGTGCCTTATGCATGCAAGTCGAACGGTCTTA
AGCAATTAAGATAGTGGCGAACGGGTGAGTAACGCGTAAGTAACCTACCTCTAAGTGGGGGATAGCTTCGGGAAA
CTGAAGGTAATACCGCATGTGGTGGGCCGACATATGTTGGTTCACTAAAGCCGTAAGGCGCTTGGTGAGGGGCTTG
CGTCCGATTAGCTAGTTGGTGGGGTAATGGCCTACCAAGGCTTCGATCGGTAGCTGGTCTGAGAGGATGATCAGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGCAAGGAATCTTGGGCAATGGGCGAAAGCCT
GACCCAGCAACGCCGCGTGAGGGATGAAGGCTTTCGGGTTGTAAACCTCTTTTCATAGGGAAGAATAATGACGGTA
CCTGTGGAATAAGCTTCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGAAGCAAGCGTTATCCGGATTTAT
TGGGCGTAAAGTGAGCGTAGGTGGTCTTTCAAGTTGGATGTGAAATTTCCCGGCTTAACCGGGACGAGTCATTCAA
TACTGTTGGACTAGAGTACAGCAGGAGAAAACGGAATTCCCGGTGTAGTGGTAAAATGCGTAGATATCGGGAGGA
ACACCAGAGGCGAAGGCGGTTTTCTAGGTTGTCACTGACACTGAGGCTCGAAAGCGTGGGGAGCGAACAGAATTA
GATACTCTGGTAGTCCACGCCTTAAACTATGGACACTAGGTATAGGGAGTATCGACCCTCTCTGTGCCGAAGCTAAC
GCTTTAAGTGTCCCGCCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCA
GCGGAGCGTGTGGTTTAATTCGATGCTACACGAAGAACCTTACCAAGATTTGACATGCATGTAGTAGTGAACTGAAA
GGGGAACGACCTGTTAAGTCAGGAACTTGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTTGG
TTAAGTCCTGCAACGAGCGCAACCCTTGTTGCTAGTTAAATTTTCTAGCGAGACTGCCCCGCGAAACGGGGAGGAA
GGTGGGGATGACGTCAAGTCAGCATGGCCTTTATATCTTGGGCTACACACACGCTACAATGGACAGAACAATAGGT
TGCAACAGTGCGAACTGGAGCTAATCCCCAAAGCTGTCCTCAGTTCGGATTGCAGGCTGAAACCCGCCTGCATGAA
GTTGGAGTTGCTAGTAACCGCATATCAGCATGGTGCGGTGAATACGTTCTCGGGCCTTGTACACACCGCCCGTCACG
TCATGA

Partial 16S rRNA gene sequence of *Dehalococcoides* sp. KKB3.003

AGAAAGGAGGTGATCCAGCCGCAGCTTCCGCTACGGCTACCTTGTTACGACTTCGTCCCAATTACCAGTCCCACCCTC
GGCGACTGCCTCCTTGCGGTTGGCACATCGACTTCAAGTGTTACCGGCTTTCATGACGTGACGGGCGGTGTGTACAA
GGCCCGAGAACGTATTCACCGCACCATGCTGATATGCGGTTACTAGCAACTCCAACTTCATGCAGGCGGGTTTCAGC
CTGCAATCCGAACTGAGGACAGCTTTGGGGATTAGCTCCAGTTCGCACTGTTGCAACCTATTGTTCTGTCCATTGTAG
CGTGTGTGTAGCCCAAGATATAAAGGCCATGCTGACTTGACGTCATCCCCACCTTCCTCCCCGTTTCGCGGGGCAGT
CTCGCTAGAAAATTTAACTAGCAACAAGGGTTGCGCTCGTTGCAGGACTTAACCAAACACCTCACGGCACGAGCTGA
CGACAGCCATGCAGCACCTGTGCAAGTTCCTGACTTAACAGGTCGTTCCCCTTTCAGTTCACTACTACATGCATGTCA
AATCTTGGTAAGGTTCTTCGTGTAGCATCGAATTAAACCACACGCTCCGCTGCTTGTGCGGGCCCCCGTCAATTCCTT
TGAGTTTTAGCCTTGCGACCGTACTCCCCAGGCGGGACACTTAAAGCGTTAGCTTCGGCACAGAGAGGGTCGATACT
CCCTATACCTAGTGTCCATAGTTTAAGGCGTGGACTACCAGAGTATCTAATTCTGTTCGCTCCCCACGCTTTCGAGCC
TCAGTGTCAGTGACAACCTAGAAAACCGCCTTCGCCTCTGGTGTTCCTCCCGATATCTACGCATTTTACCACTACACC
GGGAATTCCGTTTTCTCCTGCTGTACTCTAGTCCAACAGTATTGAATGACTCGTCCCGGTTAAGCCGGGAAATTTCAC
ATCCAACTTGAAAGACCACCTACGCTCACTTTACGCCCAATAAATCCGGATAACGCTTGCTTCCTACGTATTACCGCG

Appendix B

Reductive dehalogenase (RdhA) sequences of *Dehalococcoides* strains H1-3-2.001 and KKB3.003

HK_RdhA1

IANNDPGDTLKDMALYTSTRYVQARVKPPAHGIPELKFNCPSTRVELGIPKHEGTPEDNLRMITAALHFFGARYVRGHEIT
EKTKKTFYKCLGPQYGGLKYTFADVDAPYEDYAHGIALVHPNKYKWAICYEMPQTRLSGLTQLGLGEAGVSSGYQDLAIV
QAKLMSFLRGLGYDSISSGAGLGGGGGGVHVALGILSGIAEQGRVSYAVSPRNGALVRLTDWVMTDLPVTSNKPIDAG
MFRFCHTCKKCAELCPSGAISKTDEPSWEGFSWSRPGVKCWNTDMEKCLPYRGSFDTEGLFAACCSVCQANCVFSKLNE
ASIHGIIKNVISTTGAFNGFFKSMDDVFGYGSPIYLPQNGERDPFATDWWNRDLETDPWRPSPYGGTVMGL

HK_RdhA2

DFMRGIGLASVGLGTLSSVTPRFTDLDEIISDEKANYKQPWWVREQDQGVTEVDWDKKQTFNQAQYDNRVAHLRPSPL
DDEYRTLPGTPFSELYYSNMKTFIQENKPGFSLRDRGIYWGWASPMIKDVYGYRRAGGIGSGSGESFTGYDFLDLVDTPE
SIGVPRWEGTPEENSRMLRSALKLMGASTVGFVEINDNNKKLVFTGSSLVGKPVVWEDTENIYQTNEKQVIPNKCKWAV
VYTIRQPLETTKRGPSWISDGAVGIAYDQCDIVQYRLQAFLKCIGYQAVGGNLFGLGPLPAWGELSGLGEVGRLQNLITPE
FGPLIRESKFNLIDLPVAPTKPVNFGAQRFCHTCLKCADACPGNALQKNREPSWDITPKYDEYVKPELFNSPGHKTWYFD
HFKCRKFWEESASYCGVCQGTCVFSKEKLQSIHEFVKPVIGQTSLFNSFFYNMDRAFGYGLHSEDIENWWDMNLPTFNI
DYSEKPYI

HK_RdhA3

MSNFHSTVSRRDFMKALGLAGVGLGAAGAATPVFHDLDELISSKPEVRESPWWVKEREFEDPTVEIDWSLKTHFDYNLV
HSWVSKETAQEWQEKQAELIREAVANNTPGNTLKDMALANMGLHYAGSDLFNYSQLSRPEYSTVILDTFDVSGQIDNK
YGLTRTQLGIPKWQGTPEENSAMVAAVLHFLGSTRVGYVSINENNKKVWFSPDKANRIISWGDVEEPVNTPGILWPGN
KLGSLVLPNKCNSLISFVIPQSGISKYHHTALSRAATFLGYAESTIISARLQIFLKTLGYDGVGSDASANNVGFGVLAGNGELG
RLNYLVNPWHGALIRKADFMLTDLPLAPTRPIDSGITRFCATCKKCAEMCPGSALSLADGPSWDTLSAQNGLGVKNYTN
DWHKCRPWAWPPSPNTVGSCGVCQAVCVFSKLEESSVHDIIKPVVSQTPLFNRFFKRMDDMFNYNNPENPEEWWSR
DYKNYPYSRAVPGN

HK_RdhA4

MSAFHSTLSRRDFMKAVGLAGAGIGAASAASPVFHDLDEASQIGGVAEKRPWYVRELEYAKPTVEIDWNMIQRQTHYN
NWEDHLDQTEKDNRAHMYYDNTKKMVLENNPGNTMFDVAIRHPAWTAVRRNMDYFFGVEGIMKDTPPGFEMGFD
PESGHCMMSPVWATDLFGIITPGNMGVPRWEGTPEQNAALIRMAARWCGGAEVGYLKADEYTKKLVHKTCGILPILAD
KNGREVVWENVDQPYETDKKLVIPEKCDNIIVVTIREERNPALMAPSYRADATTAKSYALSIAFDIHFRGFLHAIGYTSAGS
GWGPWNNVPFGVLSGIGELGRMRGQITPSCGPLIRKVEVFFTDLPLPPTNPIDFGANRFCRDCGLCAKACPASAIPTFREP
TYDITPADDANSNPTKLHPEYFNLSGKKVVWPNNDFACHNFWVTSGKHGCAACVASCVFSKDIKSSIHEVVKGVVSQTG
MFNGFFANMDHAFGYGIVSDQDMWNNFWFEPDKYWPLEGIDTNL

HK_RdhA5

MSQFHSILSRRDFMKSLGLASAGIGAASLGGVNATAPFFHDVDEITSSPSAITKKPWWVKLQDKPTVPMDLSLLEPGRTK
VWCMPPDDPSGPVTLRAPGYSEEETRQKFTDYFKKEWGTWDPGPTMEGFGDFPERTTEHIGPIRDNALLAGLMPFLFG
KLPDEIIAAEKGQYLSYLLDKPCGWRYAAPLEQRGGTKWQGTPEENLRTVRAAARFYGADDVGAIEVDEDFLRVMWGV
SRFPFIPVPVQFEWGDVDDFVPTPSATRPTKIIIPRRCKYFVHWTMRQPPSRLKHDSGTQQGPSQGWTYSRNPMVNVNI
QEFLWGLGYIALTNWSGYLIPTGYAGVASGAGELSRWSGVLTPKFGNQVRGMYGFLTDLSLAPTKPINFGGYEFCKTCGI
CADACPMGAIQKGEPSWDASEIWQNPGYLGWRLDLTKCSHCPVCQGVCPFNTTDDSFVHNLVKGTIPNVKLFNGFFA
NMERDFGYGRKPYEDWWENAADGKEPVFGIDSTQ

HK_RdhA6

MDEKINRRDFVKGAGLASAATVATLMATASTVSAEELVNPGGKAMDEMGNASIKRDLPESFYKRIENKAGYIGTTRVVA
PTQRLDAREHGFSQIVRRGSTGDWSGEPGDWGPILLAAVQEKKKHAAEISPLEAADYTWSNAFQIAMDRWHITLEPGR
YQQAPIAATKVELSPEEMTARIKKICRWFGCEQVGICEVTEDMKPFFYSVGRTRGTYTTGHANYVDNGREIPWPYPYKYCI
VMADKCDTDTLNALTGPLVEASAKIACSQSDFAPHYLESIIRSLGHDAKANIFSDTDIMDTPFAVKAGLG

HK_RdhA7

MNKFHSSVSRRDFMKGLGIAGAGLGAAAATVPVFHDLDEAVSAPVAGGFRRPWWVKEREYEDPTCEVDWSQIERSDN
SWIMHGVRNGVKGGYLFAGQKYLDWQKEGSDQAFNGVKNNEPGLTLRDMALEGGASPLLMGLNKVVNFVLPEIDQD
QVLAQFGFTAAAWPNASSFWVASAPPDFWGVPKWQGTPEENSRMLRSAMRFFGASEVRFAELNEKTKKLIFTHHVHN
TPIVFEDVDKAYEVAGQKFVLPDKPLYIVSVAVQMSKEMYRQGNAGIRFAANNMRYRLNNVVQVATQSFLKGIGYQGIG
YPSESLFHGMMPSQADAILTGFAEMARNNNYCISPEFGTVAGYYSILTDLPLAPDKPIDAGYFRFCHTCRKCAEACPSQAI
SFDSEPTWDIPPSSVDPAKATLYSTPGKKVFHTDSPACYSRWIGLHGCARCMGTCVFNTNSSAMVHDMVRATIGTTGLF
NGFLWNADKAFGYGLIPPEKWEEWWDKDYPVLGQDSTIGSYYGGY

HK_RdhA8

MPNFHHTISRRNFMKGLGILGTGLGTAAATAPVFHDLDDIISSPKAEWKRPWWVKYREADNPTTEIDWSLMNRWDAR
QTAQAPGIQAKYLGADEIKKRYANVLTNKVKAITHDTPGQTLRDYALSSGAGYFMNLPYVTTFMGPQKVATPQSLSVPV
WQGTPEENSRMLRSAVIFYGGGQVGFGVIDQKIKDKLVFTNHKGAANSIGFVENFPPPPALGKSYLFEDVEQGYEGATTF
VLPSNKQLYEFCFTVPMSKDMFRTANESQIMYSANLSRYRLFGNIQNCIQEFIRSLGYTCYGYASPFSGMMPAIASAQLTG
ITEGNRNNGFCTSPEYGPILGVFSLVTDMPLEPTNPIDAGIWRFCQTCTKCADACPVNAIPKDHEPSWELPTVYGKPDITH
SPGRKQYWTNALDCWLFLTEYNGCGACMATCTFNTNNAPIHELVRTTLATTPLLNSFLWQADKFFGYGLTEDKEAWW
DLSMPVYGFDSAATSSQGGYNK

HK_RdhA9

VSRRDFAKYMGLGSAGLVASAAAAYTWSKQDGSAIASYMGQENRQGVEYFNRKPFEVDKVGTWDWEIIGPNLDGDKS
PNSIHRIDMGTQWNVRFPDRYNPDPTIKRCIPNDSFPSNGLAGLTPYWQAYYTAYPMELDRDKEYMYSWLPQFRERLT
ATDADRQRFNTNEGFIANIRSNAQTAVMGAGVNDPPAISDWQGVATKRAVFESPEAASELIKNLGQDLEAIWVGISKLN
PAWVLYTHSAKAAGMATPKPGDRGFGYDKPIQIPAWWEYQIAVVGNMNWGALSADPNYGDSMTGYNTASQVAQRL
VTAIKQMGYPARWHSPLGGYDVCVPPMAVEVGLGQVGRTSNCIAPDLGGNARPAFVTTSLPMAVDKPIDFKMDEFCS
RCMLCAQVCPVQAISYSPKPDYEIRGLRRFYTNHLKCSDGFNIGAGPAGCRACVAVCPWTKKNTWVHRFVREVLSHDST
GISQNMAVWAEKNLYPKNFQDSLNPPDFKGVYDPPAWMITKDYISGFTETPMGVK

HK_RdhA10

MSNYHSTVSRREFMKGLGIAGAGFGAASAVTPVFRDLDELTSSAAAHPKRAWYVKEREFGDIGIEIDWNILKRRDTRGYS
YWNPMIWKQHYPAYDMEAFNKALDNKTKELWPDYAGPTTRDYSLKNAMYSVGLGCPHYLYNVEQFGVTLPHPAPRPE
AIGMPNWAGTPEENFQMIQAAFSLIGLGPSIGITELDDKSRRFVWEYNNYGQHIIFDDNITETYRTANPPTIHIPSSHRYVI
ATHNMGADEILRRAPSTIGACTESISYARVAYAKSFVEQFIRGLGYNVVYGHSLQAAPAMDFWSGVGEHARMGQVCVT
PENGAMMRTHAIFFTDLPLSPTKPIDAGITKFCETCGICAESCPVGAVPAKGVNRNWDSNCDGQSFDNDIESGGTEVMY
NVPGYKGWRVDGFRCLADCNGCKGSCPFNAIPNGSFIHSLVKATTATTPLFNGFFTQMEKSLHYGKQDKDPESWWHEP
NAWHVYGSNPGLLG

HK_RdhA11

MVDPQKWDLNRRDFIKKTGLASVAATAVVAVNTTGIIADEFVKPGIKAMDPLGNASIKRELPENFYKKIEHRPGYIGTTQI
VSSGERMDAREHGFAQIVRRSSTGDWSGEPGDWGPVLLAAVKEKNKHLAEISDIERLDYTWSSTLQVAMDRWHINLAP
GKFEPAPISATKVEMSPAEATAKIKKMARWLGAEQVGICEVTEDMKPFFYSIGRKHGNYSGPVANYEEDEGREIPWPYPY
KYCIVLADKCDTDTLSALEGPLVEASARVSCSICDFLPHYLESIIRALGYDAKANPFSDTDIIEAPFAVKAGLGELGRSGLLVSP
WGAQLRLLEVFTNMPLVPDMPIDFGLQEFCKVCKKCAENCPSQAISMDDEPSEVITTVKSIRWLQDEKKCLARRLAYGCA
KCQSVCPWSKPDTLMHEIGRMIGQNPALASFLVKVDDFFYNRFPEGHSAADYAPWR

HK_RdhA12

GAASATAPVFHDLDEAASSTKAVLKRGWYVKERDYGDYGIEIDWNIMKRRDQRGFPNWNFGVTMNAYPEGPQAFAAL
KHAGEEATAEKAKEFWPDYKGPTTRDEALGSAFEAMSYGNSGYCGNLVQGGTSTNLPAHRPEEIGMPKWQGTPEENLL
MLRAVFSLVGLGPMVGVAELNEQTKKFVWDYMPFQTSARASTGPNLLPAIPATDGQRRIIFDDNATESYLTEDPPAFHLP
SAQRYVIATHNLSCDEIARRGLGAALGGCTENMSYARVAYAKNIVEQFIRGLGYNVSYGHDMQPALAWDIASGVGEHA
RLGQTIGSPEYGGLMRTHAIFYTDLPLALTKPVDAGFTKFCETCGICAETCPVGAIPERGINRSWDNNCGQSWSDDKMEG
GTKVMFNMPGYKGWRCNLFKCAYTPCGGACKGNCPFNTIADGSFVHSIVKSTVATTPLFNSFFTSMEGILHYGKQDKDP
ESWWNSPDEWHIYGTHPNSLRQ

HK_RdhA13

SCFTSWLVCKRKRLWRPHNKIDWSLMKRRDLRGYSNWDFPTLMFTYPGGPEAFQKHLHDQGTAVTAKAKEIWPEYTG
PSTRTYALSNAFAASAYSNSGYVLNANQFGMKTKASAPRPQDIGMPVWQGTPEENTAMIRAVFSLVGLGPMIGTTMLD
EKSENFVWEYNGKGVTGGDAKYGNKHIIFDPNISEAYSDDTTFRIPTSHKYVIATHNLSCDEFLRTGLSGSGAYGTEEMSY
VRVAYAKSVVEQFIRGLGYNVAYGHDLQAATAWDIWSGVGEHCRMGQITGSPELGGLLRTHAVFYTDLPLELTKPIDAG
FAKFCETCGTCADTCPVGAISPRGVDRNWDSNTGQDWVNDKQAGGTQVMYNMPGFKGWRCNSFACAFSPCGSACK
GACPFNTIADGSFIHSIVKSTVATTPVFNSFFTSMEGILHYGKQDKDPEKWWNNPEAWHIYGTNPNNLRQ

HK_RdhA14

MSKLHSTLSRRDFMKGLGLAGAGLGAVAASAPVFHDVDELTSLGSNVNRYPWYVKEREFKNPTVEIDWNVLSRQNANN
FKSHAKPTPADYDAAGVVGRYMYDLETPAEALILYDYCEKEFPGWDKGWGGSGDVRTTALDNACKFMMMGMWPGD
MYQGGKRINVRNAIIAAGGTGSYSSFLGPQCFSIRPQDVGASRWQGTPEENYKTVRNAFRFLGAQDVGCAEIDSDTVKF
FHKAKGGASGMFAGQGDAGGKQVAFKDIDVPYETGDEYAIPNKCKYIITFTARQSFEGTRRQAGITEGFAVWYSYARYIK
MMCHMQEFIRGLGYDCLNMSGLCFSNPLSAITGLGEHGRMSSPTIHPKNGTTNRANGWAFLTDMPIAPTKPIDFGAYK
FCETCGICADSCPFGIIQKGPSTWENPDAAGNGLAQGQFRGWRTDNVKCPHCPTCQGTCPFNSTSESFIHDMVKATTT
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HK_RdhA15

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PAWNGSPEETTAMIRQAFRFLGTGTISIVELNENNRKLVYGVDWDGKAIVFENVEKAYETDKKRVIPEKCRYAVVFSMP
MSEEMNKRAPTLLGDATTALSYSLSTLFQIRAQRFFRMLGYQGLGSFTYVNNTSINPALAVISGMGEQGRLGQCVFPEYG
TMARLGSVITDLPLVPDKPIDSGVWNFCKTCKLCASHCPSGALNPDDVPSWDVKYSGNHPGKKVYHCDGMNCRGYWY
DLTSLCSICVASCVFAKKNKAGIHDIIKATTAVTPAFNSFFRTMDQAFGYKYSNRDPESWWDINGEPMFGIDSRY

HK_RdhA16

VNISHSTLSRRDFMKGLGLAGAGLGAVAASAPVFHDVDELTSLGSNVNKYPWYVKERDFKNPTVEIDWDTLERPNANNF
KSHPRPSAADFTAAGVVGGNMTDLETPAEALALYDYCEKEFPGWDKGYAGAGDVRSTALDNACKFMMMGQWPAEIY
QGGKRINVRNAILAAGGTATFSSFLGPQAATTIRPQDFGAAKWQGTPEENFKTLRNAFRFLGCQDVGCAEIDSDTVKFFH
KIKGAGSGFNTGEAGGKQIAFKDIDEAYETADEYAIPNKCKYIITFTARQSFEGTRRQAGITESFSVWYCYARYVKMICHM
QEFIRGLGYQCLNMSGLYFSNPLSVITGLGEHGRMSSPAIHPKNGTTNRANGWALLTDLPVAPTNPIDFGAYKFCETCGIC
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FFANMER

HK_RdhA17

MNQFHSTVSRRDFMKGLGLTGVTLGSASALSPQFRDLDELANSAKVVNKRGWWVKERDYGNPTIEIDWNLMKRRDLR
GFSNWDYASLMMAFPGGPPAFKANTPKQAAAVTAKAKEIWPDYAGPTIRDKALSSSFWASAYGHSGYCRSQNQHGM
PTEIPAPRPSAINTPVWEGTPEENAAMLRAVFSLVGLGPVIGTTMLDEKSQNFIWEYSGVSWTGDESVPGNKHIVLDSGI
TESYVDATSFHIPTSQKYVIATHNISCDGFLRRSMAGAGFSSTEEMSYVRVAYAKSIVEQFIRGLGYNVTYGHDLQSAVAW
DMWSGVGEHCRMGQVIGSPEYGGLLRTHAVFYTDLPLPVTNPIDAGFVKFCETCGICAETCPVGAIQERGIDRSWDNNC
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HK_RdhA18

MIKKHSTVSRRDFMKGLGLAGAGIGAAAAVTPVFHDLDELMSSSQATLSRPWYIKNREFGDIGIELDWNLIKRRDLRQYD
NFTMKHLPFQYPGGPPAFMQGMESAAQKNSDRLKELWPDYKASTRDLALANALGSVGTFGTYALNTTQGGWKVDKA
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TQNPNTLHIPSSHKYVISTHNLSLDELTRRTYSPLGTPAESISYSRVAIAKNFVEEFIRGLGYHVVYGHALQPALVWDFLS

HK_RdhA19

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HGAPTTFEFGDVDDIVCTPAIRPTKVVIPRRVKWFLQFSSRQPGEVTRHALGTTQNAGQSYYYTSWVKIVKSIQDFLWGL
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MGAISKDEPTWD

HK_RdhA20

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NSSGNMVDVASLMLAAGGGIMFDSYAGPKMAKTPEELGIPKWQGTPEENLRTLQAGVRFFGGQNVASFELNENYKKL
TFTIDPVGSAIEFGDVEEVVDTPPKKIIPNKCKYVFMWSMTQPYELTRRQSGVYEGIATSTGYERGHIAKVHFQDFVRGLG
YQMVGGAGNDSGPAGAFPIFGGLGELSRASYVNDPVYGLTNRVTWSMFTDMPLPDTRPIDFGGRKFCETCGICAEACP
FGAINPGEPTWEETNTFGNPGYLGWRCDYTKCPHCPICHGTCPFNALSGSFIHDIVKGTVSTTPVFNTFFKNMEKTFKYG
RKDPAEWWELDDYQFGINSSY

HK_RdhA21

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GNMVNVVDAIRGAGGYLGTIDSYAGPKMVQTPEEMGGTKYQGTPEDNLRTLKAGIRYFGGEDVGALELDDNLRKLVFS
TDLYSKNIEFSDVDECIETPTQVTIPNKCKYIFLWTMRQPYELSRRQSGRFEGAATDTSYERAYNIKTHFQDFARGLGYQMI
GAGSSAMTPAGAWATLGGLGELTRASYISHPLYGITVRVTWAFLTDMPLPPSRPIDFGNRKFCETCGICAEACPFGAINP
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HK_RdhA22

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LRQVVRFLGGCDVGAQEMDSDVFKLFHEQSGGKQLVIENVDEAAETPTKLVIPAKAKYILQWTARQPYESTRRQAGEYE
DAAVYYSYQRFPFVGAIIQEFIHALGYTAVSTHMMGYHTNAIATLTGMGEHCRMSSPTLVPKYGTTNRAMWVMMTD
MPLMSTKPIDFGVYDFCKTCGICADACPFGLIEKGDPSWEATQPGSRPGFNGWRTNTTICPHCPVCQSSCPFNTNGDGS
FIHDLVRNTVSTTPIFNSFFANMEDHGIRTQGPARLVEYR

HK_RdhA23

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RPFNGAIPTIATATLTGLGEGARNNGAFISPEFGPCVGLFSLVTDLPLEPTPPIDAGMWRFCQTCTKCADECPAQCISFEHE
PTWDVPKIYGKEDTTHIPGRKQFWTDGIACWSYKATIGGCGACMGTCTFNTDIANIHTIVRATLSTTPVFNSFLW

HK_RdhA24

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VYPALEGAAPIPELVGQTYTHESFGVPRWESTPEENFALLKSAMRFFGAGQIASIELDANVKSMFYPVDASRMFFNGPP
MAYGFEDCDNGYVTDTHFIIPNKARWVVTYTTPMPKEMYRTAPSGVCYAANMSRYRLNQETMACVQKFLLGLGYQGL
QSAPWPNGICPSPAVATLSGLGEMDRINQCVIPEEGAVVGIYKFITDLPLPVSKPIDFGAFRFCHSCRKCADTCPAKAISFEE

EPTWEPAGPWSTGGKKAYYKNEPECKLYQHSTGATCQICTGVCVFNVNTKAMIHEIVKSTLSTTGIFNSFLWKADVAFGY
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HK_RdhA25

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AADAGYGK

HK_RdhA26

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DHSFDESLGVPPPGPQINNADPTPDAWPDWGRPGEPKFKKVPRWSGTPEEASKMITAAAHYFGAYKVGFLEVDDKVKK
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FCETCGVCADLCPNEAISKEKEPTWELPPTKLGRPEYGIPAGTPNTWSRPGVKNWHVDYVRCRGCSYCQGYCVFSQQNF
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HK_RdhA27

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HK_RdhA28

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FPMEVIMATGGKYMALTDRPEGWREVPYITQRGGTKWQGSPEEALKVVRAAARFYGFDDVQAIPVDEKFLKVMWGTK
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HK_RdhA29

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HK_RdhA30

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HK_RdhA31

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VIPSKPMYVISVVIQMSKELFRHGTGILRTCGNGSRYYIWAGVQARTQAFLNGLGYQGLGYPIRHWGLMSSMADAVLTG
LAETSRNSNVCISPEYGTVAGYFSLLTDLPLAPTPPADAGMFRFCHTCRKCADACPVQAISHDSEPSWDIPRSALAPNTEP
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KKB_RdhA1

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IGVPRWEGTPEENSRMLRSALKLMGASTVGFVEINDNNKKLVFTGSSLVGKPVVWEDTENIYQTNEKQVIPNKCKWAV
VYTIRQPLETTKRGPSWISDGAVGIAYDQCDIVQYRLQAFLKCIGYQAVGGNLFGLGPLPAWGELSGLGEVGRLQNLITPE
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DYSEKPYI

KKB_RdhA2

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GLTRTQLGIPKWQGTPEENSAMVAAVLHFLGSTRVGYVSINENNKKVWFSPDKANRIISWGDVEEPVNTPGILWPGNKL
GSLVLPNKCNSLISFVIPQSGISKYHHTALSRAATFLGYAESTIISARLQIFLKTLGYDGVGSDASANNVGFGVLAGNGELGRL
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YKNYPYSRAVPGN

KKB_RdhA3

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GWGPWNNVPFGVLSGIGELGRMRGQITPSCGPLIRKVEVFFTDLPLPPTNPIDFGANRFCRDCGLCAKACPASAIPTFREP
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KKB_RdhA4

MDEKINRRDFVKGAGLASAATVATLMATASTVSAEELVNPGGKAMDEMGNASIKRDLPESFYKRIENKAGYIGTTRVVA
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GAQMRIMEVFTNLPLVPDKPIDFGLQEFCKVCKKCADNCPASAISMDDEPSEVDTVVKSIRWFQDGKKCLAQRLAYGCS
KCQGVCPWSKPDTLIHEVGRMVGQNPAFAPFLVKLDDFFYNRYPEGHATGEWAPWR

KKB_RdhA5

MNKFHSSVSRRDFMKGLGIAGAGLGAAAAAVPVFHDLDEAVSAPVAGGFRRPWWVKEREYEDPTCEVDWSQIERSDN
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YPSESLFHGMMPSQADAILTGFAEMARNNNYCISPEFGTVAGYYSILTDLPLAPDKPIDAGYFRFCHTCRKCAEACPSQAI
SFDSEPTWDIPPSSVDPAKATLYSTPGKKVFHTDSPACYSRWIGLHGCARCMGTCVFNTNSSAMVHDMVRATIGTTGLF
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KKB_RdhA6

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EPPKWITTNEYVSSFVNTPMGVK

KKB_RdhA7

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KKB_RdhA8

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VLPSNKQLYEFCFTVPMSKDMFRTANESQIMYSANLSRYRLFGNIQNCIQEFIRSLGYTCYGYASPFSGMMPAIASAQLTG
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KKB_RdhA9

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KKB_RdhA10

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PSWDVPDVDGKPRVFCNPGHKGFWPDMAGCNYYSKGGGTSGCWVCYANCTFSEDKAAMMHNIIRGTVSTTSLFNGF
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KKB_RdhA11

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TMARLGSVITDLPLVPDKPIDSGVWNFCKTCKLCASHCPSGALNPDDVPSWDVKYSGNHPGKKVYHCDGMNCRGYWY
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KKB_RdhA12

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FWDIEQPTYGFDTTA

KKB_RdhA13

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PTEIPAPRPSAINTPAWEGTPEENAAMLRAVFSLVGLGPVIGTTMLDEKSQNFIWEYSGVSWTGDESVPGNKHIVLDSGI
TESYVDATSFHIPTSQKYVIATHNISCDGFLRRSMAGAGFSSTEEMSYVRVAYAKSIVEQFIRGLGYNVTYGHDLQSAVAW
DMWSGVGEHCRMGQVIGSPEYGGLLRTHAVFYTDLPLPVTNPIDAGFVKFCETCGICADTCPVGAIQERGIDRSWDNN
CGQSWADDKQAGGSKVMYNIPGYKGWRCNLFSCAFTPCASACKSNCPFNAIGDGSFVHSIVKSTVATSPIFNSFFTSME
GVLHYGKQDKDPASWWNSPDEWFIYGTHPNLLRQ

KKB_RdhA14

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ARMDPSLPVYDNLKAIGAPVTRWLDWADKKAEDEILFAKAREEFPGFEPGIDGFGDLRTTALTHASEMFAFGQFPQKM
NLGGNMVDLVPAIRAAGGYLGSTDSYAGPKIVHTPEEMGGTKYQGTPEDNLRTLKAGIRYFGGEDVGALELDDNLKKLIF
TVDQYGKTLEFGDVEECVETPRQVIIPNKCKYIFLWTMRQPYEXXRXQSGRFEGAATXTSYERAXNXKAHFQDFARGLGY
QMISAGNNSLSPAGAWAVLGGLGELSRASYVNHPLYGITVRVTWGFLTDMPLPPSRPIDFGARKFCETCGICAEACPFGA
INPGEPTWKDDNAFGNAGFLGWRCDYTKCPHCPICQGTCPFNSHPGSFIHDIVKGTVSTTPVFNSFFKNMEKSFKYGRK
NPATWWDEVDDYPYGVDTSY

KKB_RdhA15

VKTFHSTLSRRDFMKALGLAGAGVGAVSAAAPVFHDVDELTASSGGVQKLPWWVKEREFKDPSVPIDWQNLPKMEGT
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GEINMGGNRVNVMQAILKAGGTATFPSFMGLRSSETLRPQDFGVPRWEGTPEENLLTLRQVVRFLGGCDVGAQEMDS
DVFKLFHEQSGGKQLVIENVDEAAETPTKLVIPAKAKYILQWTARQPYESTRRQAGEYEDAAVYYSYQRFPFVGAVIQEFI
HALGYTAVSTHMMGYHTNAIATLTGMGEHCRMSSPTLVPKYGTTNRAMWVMMTDMPLMSTKPIDFGVYDFCKTCGI
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KKB_RdhA16

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RMVGQVRSVQIKYLGEEEVNRRNAVGAKFTADGLKNDTPGLKVRDQALAAGVMSMLPMAMGMIPSISFMGPATATP
EARGVAKYQGTPAENSRMLRSALIFYGAAQVGYGEVTQRYKDKLFRTFDKGNAATAYQGAWPPPLTQCKQYFFEDVPV
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TIASA

KKB_RdhA17

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GTPEENSKMIRAAMIHFGAAQVGMAEITDRVKTKLVREYDKDTTHKKYIFEDVPKGYEGADKLVFPDKVPLYDFAFTHPL
NKEMFRSSPSSDIGSAGNSLRYSQFSIIQPRIQMFMQVLGYTCYGYTRPFNGAIPTIATATLTGLGEGARNNGAFISPEFGP
CVGLFSLVTDLPLEPTPPIDAGMWRFCQTCTKCADECPAQCISFEHEPTWDVPKIYGKEDTTHIPGRKQFWTDGIACWSY
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DY

KKB_RdhA18

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VGQTYTHESFGVPRWESTPEENFALLKSAMRFFGAGQIASIELDANVKSMFYPVDASRMFFNGPPMAYGFEDCDNGYV
TDTHFIIPNKARWVVTYTTPMPKEMYRTAPSGVCYAANMSRYRLNQETMACVQKFLLGLGYQGLQSAPWPNGICPSPA

VATLSGLGEMDRINQCVIPEEGAVVGIYKFITDLPLPVSKPIDFGAFRFCHSCRKCADTCPAKAISFEEEPTWEPAGPWSTG
GKKAYYKNEPECKLYQHSTGATCQICTGVCVFNVNTKAMIHEIVKSTLSTTGIFNSFLWKADVAFGYGHHDAAEWWDLD
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KKB_RdhA19

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PKWTGTPEEASRMLRAAMRAYGASLVGYTELTQEHRDHVIFSYEKGDSNNEKYIGTTIPVTAARPIVFENVPKAYETTEKL
VIPNVPLWEIALSTQGSNELWRSAGTLLGGMANGNTFYNCANLHASTYNFLRYLGYQLIGTIGNDARYVGSEGGAAIMA
GLGEASRQKLYTLTPEYGAPGRLYGVLTDLPLEPTHPIDAGIYRFCHSCQKCADSCPPQCISKEKEPSWDLPLTEGKETIYSV
KGTKAFYNNLPLCRQYSNETSHGCRICWGECTFTVNRGALVHQIIKGTIANIPLFNTYFYKLGDAFGYGTDPEKAEAWWD
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KKB_RdhA20

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KYCIVCGNFEDLQTAKANTGGLNNISVATECSDNDIYPIYLETVIRQLGWDAKAQPFANKDIMEGPFAVAAGLGEQGRSG
LVVSPWGSHVRFYEVLTNMPLVPDKPIDFGLRDFCRVCKKCADNCPSGAISKETEPAEVMTTTKRMGWEWNAYKCMA
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KKB_RdhA21

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KPYVFEDVPQAYETGATSVERKPQEGKRVIPERCRWLIHFSLNEVYEWPYVFSGDSWLRYAEARQIQYRLQAFIKGLGYQ
AIGPCNFTNNMTENIALAVLGGEAELGRNNMAISPLLGTSCGQYSSIITDLPLAPSKPVDAGIHHFCHTCQQCAEACPGG
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SCVFWKGIKGTLLAGIQ

KKB_RdhA22

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LRTALRFFGAADIGILELDENVRKLVYTYPRVAPYKRYEFEEVDKGYEDDEKWVIPSKKKLYVVTIISQSSIDGYTTTPSWIQR
AASDNTVRLNAGILAATQEFLRTLGYQAMAGHERNAIGPAPAFAALSGLGELCRNTQVLTPDYGLMIRAVRLITDLPLAPT
KPIDAGIWRFCHDCAKCSEACVSQAIPHDKEPSWDVPGGWNNPGKKVWYVNAVKCQSTREMLTRDCGLCMTACVYT
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KKB_RdhA23

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AQTVLGRRWPSLQSNAAVMGVYNFLSWAEGNLQEFLHGIGYEANIVDNITNSEPCGLMAGVGENVRMGYTVVSPETG
AMARCVLRVITDLPLATTKPVDAGLNRFCNTCKICAEACPYNAIVLDDAPSWDNAAIPGSPPGFNGWRLKAMSCAHCGS
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KKB_RdhA24

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QLENIKAKWTTPNTGMTAKDYAFFAGSASDAIGASVPLTKGDATLMYTFGGTTQPGGYNYKQLGLPRWSATPEENLKM
VTAVLRFWGAQDVGAHEINEKTQKVFYSADTGGRPYTFADVDNASSDSSHACLIPNKAKTVLTWVVPMSRVGQYSAPD
GFNILNKVSMGIGYSMGDIIQNRILSFLGALGYLSISRNCGGMNVAHGNLAGLGEHGRLDYLVNMDYGANVRYTDFVVT
DLPIASTKPIDAGIWKFCQTCKKCAEMCPSGAIPIEGDPSYTIEHPESNGYGLKSYHVNYDNCHPYRGNPGSVVNGGCGIC
QNVCVFSKMEGGNIHEVIKATVGTTDVFNSFFRQMDDFFGYGEFKQADEFWDTMPNTYGTFVPWGRGNSMPGK

KKB_RdhA25

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FRSFGVSSVTVAPVDEKTRKLFYSYVGKQKVTFEDIDLFESTADRFAIPNNCQWVIHWTNLQDTELTSRMPSHVGRAGFP
MAYTHVKRIDLLIEQFLRGLGYQSINAGNYCPSAAFGIMTGVGEHTRMGTTLNSPEYGSHLRGQYRVVTDLPLAITKPIDA
GMERFCETCGVCGTQCPFGAIAMGDKSWDNACGQDWASDQSVGGDTCMWNIPGYNGWRLDYRKCMGNCCSCM
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KKB_RdhA26

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QNRFKRSTSPGLDPASNVFYPMQIFIGGRIQNFFKGLGYQALGGSINLWGPGGAWGNMSGLGEQGRHAAMISVKYGS
GTKGDNRNVVDLPLAPTRPVDAGIHRFCETCGICINTCVTDAIQKGPPTWDSGRWNNVQGYKGYRIDWSNCSACTNCQ
VYCPFFRMSDASWIHSIVRSTVATTPVFNGFFRNMEEVFDYGSKGNLDEWWN