MICROBIAL DECHLORINATION OF POLYCHLORINATED DIBENZO-*P*-DIOXINS AND DIBENZOFURANS: PATHWAYS, KINETICS AND ENVIRONMENTAL IMPLICATIONS

by

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ABSTRACT OF THE DISSERTATION

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Dissertation Director:

Donna E. Fennell

This dissertation describes research on the dechlorination of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) by a *Dehalococcoides* containing mixed culture and in freshwater sediments, including elucidation of pathways, assessment of kinetics and extrapolation of the results to address potential environmental implications. First, the dechlorination pathway of a selected PCDF congener was elaborated. A toxic 2,3,7,8-substituted congener-1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) was detoxified to non 2,3,7,8-substituted congener upon reductive dechlorination. The amendment of halogenated compounds, including tetrachloroethene (PCE) and 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB), in addition to the selected PCDD/F congeners, resulted in differences in the pattern and extent of PCDD/F dechlorination. The addition of 1,2,3,4-TeCB enhanced the extent of dechlorination of 1,2,3,4,7,8-HxCDF by approximately three fold, while the addition of PCE did not affect 1,2,3,4,7,8-HxCDF dechlorination. Second, the dechlorination kinetics were examined using 1,2,3,4-

tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) as a model compound. The dechlorination rate increased with an increase in the nominal concentration of the compound and exhibited pseudo-first order kinetics. Kinetic parameters were also estimated based on a Monod-type biokinetic model. Third, the carbon isotope fractionation during reductive dechlorination of 1,2,3,4-TeCDD using compound specific isotope analysis (CSIA) was investigated. A unique pattern was observed in that the dechlorination intermediate, 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) was enriched in ^{13}C relative to the educt, 1,2,3,4-TeCDD. The final product 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) was depleted in ^{13}C compared to both the educt and the intermediate. The enrichment of ^{13}C in the intermediate could be a result of the further dechlorination of 1,2,4-TrCDD to 1,3-DCDD. Finally, the dissertation addressed approaches to enhance the dechlorination of PCDD/F in sediments. The approaches examined included addition of electron donors, amendment with additional halogenated compounds, bioaugmentation with *Dehalococcoides*, and combinations of these approaches. The addition of alternate halogenated compounds enhanced the dechlorination of 1,2,3,4-TeCDD in two sediments, those from Gulf Island Pond, ME and Lake Roosevelt, WA. In heavily PCDD/F contaminated River Kymijoki sediment, all active treatments exhibited similar extensive dechlorination of 1,2,3,4-TeCDD, however, the amendment of halogenated compounds and bioaugmentation expedited the dechlorination at the onset of the experiment. This study addressed several theoretical and practical aspects of reductive dechlorination of PCDD/Fs pertinent to the better understanding of this subject. The outcomes could be used to direct the practice of the remedial operations for PCDD/F contaminated sediments.

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Chapter 1

Introduction

1. Rationale

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDD/Fs) are two groups of compounds with similar tricyclic planar structures. They are produced inadvertently and released to the environment from a wide range of sources including chemical manufacturing, paper and pulp production, and combustion and incineration. PCDD/Fs are hydrophobic, have low solubility and volatility, accumulate in soils, sediments and biota, and are ubiquitous in the environment (U.S.EPA 2006). Contamination of soil and sediment with PCDD/Fs is a serious environmental problem. PCDD/Fs are generally present in the environment at very low concentration, however, they are of great public concern because of their toxicity and potential to bioaccumulate. Seventeen 2,3,7,8-substituted PCDD/F congeners are the focus of regulatory effort and scientific investigation owing to their high toxicity to humans and wildlife (Van den Berg, et al. 2006).

Biotransformation of PCDD/Fs may occur under both aerobic and anaerobic conditions. Lightly chlorinated PCDD/Fs may be biotransformed or mineralized under aerobic conditions (Wittich 1998), while highly chlorinated PCDD/Fs undergo reductive

dechlorination under anaerobic conditions (Adriaens et al. 1994, 1995; Beurskens et al. 1995; Barkovskii et al. 1996; Ballerstedt et al. 1997; Albrecht et al. 1999; Bunge et al. 2001; Vargas et al. 2001). During anaerobic dechlorination, many different lightly chlorinated daughter products could be formed from various highly chlorinated congeners that exist in environmental mixtures.

Recently two bacterial strains have been identified which dechlorinate PCDD/Fs under anaerobic conditions — *Dehalococcoides* sp. strain CBDB1 (Bunge et al. 2003) and *Dehalococcoides ethenogenes* strain 195 (Fennell et al. 2004). The focus of this study, *D. ethenogenes* strain 195, dechlorinates tetrachloroethene (PCE) to vinyl chloride (VC) and ethene. It grows on tetrachloroethene (PCE), trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-1,2-DCE), 1,1-DCE, selected chlorinated benzenes, and chlorophenols (Maymó-Gatell et al. 1997, 1999; Fennell et al. 2004; Adrian et al. 2007). *Dehalococcoides* is an important bacterial genus which has been widely utilized in field scale remediation of groundwater contaminated with chlorinated ethenes. Recently, in microcosms developed from contaminated sediments, native *Dehalococcoides* spp. have been detected and implicated in dechlorination of PCDD/Fs (Ballerstedt et al. 2004; Yoshida et al. 2005; Ahn et al. 2007). It is therefore important to continue the investigation of PCDD/F dechlorination by these organisms to expand the knowledge base regarding their potential for PCDD/F dechlorination and ultimately sediment bioremediation. *D. ethenogenes* strain 195 dechlorinates selected polychlorinated biphenyls (PCBs), chlorinated naphthalenes, and PCDD/Fs *(13)* and certain polybrominated diphenyl ethers (He et al. 2006). In this study, the dechlorination of PCDD/Fs was investigated with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD)

and 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) as model compounds, using a mixed culture containing *Dehalococcoides ethenogenes* strain 195.

Investigation of microbial biotransformation of PCDD/Fs provides information needed for attempts to enhance bioremediation in contaminated environmental matrices. Although we know transformation occurs, more information on the theoretical and practical aspects is needed to answer fundamental questions pertinent to bioremediation feasibility studies and operational strategies. For example, integration of information such as identification of functioning indigenous and exogenous microorganisms, understanding the effectiveness of amendments that enhance biotransformation activities, development of methods to monitor the effectiveness of amendment implementation and succeeding microbial activities, and establishing model frameworks to predict the fate and transport of PCDD/Fs *in situ* are needed for successful employment of bioremediation. In this study, pathways of dechlorination were investigated to determine if strain 195 could detoxify PCDD/Fs by first removing a lateral (2,3,7, or 8) chlorine substituent, thus greatly reducing the toxicity of the parent compound. Rates of dechlorination were also ascertained under a variety of conditions to lay the groundwork for more comprehensive predictive models for PCDD/F fate. The possibility of using compound specific isotope analysis to monitor the dechlorination of PCDD/Fs was explored in an effort to address the great complexity of monitoring *in situ* bioremediation. Finally, amendments to enhance the dechlorination of PCDD/Fs by native microorganisms in sediments were investigated. Results revealed useful information that will contribute to the goal of application of bioremediation to treat PCDD/F-contaminated sediments.

2. Goals and Objectives

The overall goal of this study was to investigate the dechlorination of PCDD/Fs by *Dehalococcoides ethenogenes* strain 195 and by native dechlorinating bacteria in sediments. Specific objectives were:

1) To examine the spectrum of PCDD/F dechlorination by *Dehalococcoides ethenogenes* strain 195, delineate dechlorination pathways and explore the environmental relevance
2) To elucidate the kinetics of PCDD/F dechlorination by *Dehalococcoides ethenogenes* strain 195 and estimate the kinetic parameters
3) To explore the feasibility of using compound specific isotope analysis to monitor *in situ* PCDD/F dechlorination
4) To test strategies for enhancing PCDD/F dechlorination in sediments

3. Dissertation Overview

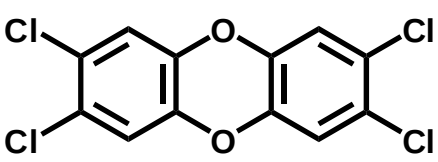
This dissertation is composed of seven chapters. Chapter 1 is the introduction. Chapter 2 is the review of literature in the different research fields. Chapters 3 to 6 address the research related to the four objectives in the order in which they were listed. The final

chapter, Chapter 7, summarizes the dissertation and elaborates the environmental implications of the research results from this study.

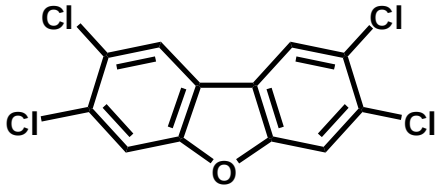
Chapter 2

Literature Review

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are two groups of compounds with similar tricyclic planar structures (Figure 2-1). There are 75 congeners in the PCDD group and 135 congeners in the PCDF group, differing in the number and position of chlorine substituents in the carbon backbone (Table 2-1).



2,3,7,8-tetrachlorodibenzo-*p*-dioxin



2,3,7,8-tetrachlorodibenzofuran

Figure 2-1. Molecular structures of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 2,3,7,8-tetrachlorodibenzofuran.

PCDD/Fs are generally present in the environment at very low concentrations; however, they are of great public concern because of their toxicity (Van den Berg et al. 2006) and their potential to bioaccumulate (Cai et al. 1994). Seventeen 2,3,7,8-substituted PCDD/F congeners are the focus of regulatory effort and scientific investigation owing to their high toxicity to humans and wildlife. Toxic equivalency factors (TEFs) such as those published by the World Health Organization (WHO) indicate an order of magnitude

estimate of the toxicity of the 2,3,7,8-substituted PCDD/F congeners and other dioxin-like compounds including certain co-planar polychlorinated biphenyls (PCBs), relative to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TeCDD), the PCDD/F congener considered the most toxic of these compounds (Table 2-2) (Van den Berg et al. 2006). The WHO TEFs were established through comparison of physiological responses mediated by binding of dioxin-like compounds to the aryl hydrocarbon receptor and were tabulated for mammals, fish, and birds. A toxic equivalent (TEQ) expressed as equivalents of 2,3,7,8-TeCDD can be computed for mixtures of 2,3,7,8-substituted PCDD/F congeners by summing the products of individual congener's concentrations multiplied by their respective TEFs:

$$TEQ = \Sigma_{i\text{-}n}\, TEF_i \times [Congener_i] + TEF_j \times [Congener_j] + \ldots TEF_n \times [Congener_n] \quad (1)$$

where:

TEQ = toxic equivalent concentration;

$[Congener_n]$ = concentration of a specific congener; and

TEF_n = toxic equivalency factor for a specific congener.

Table 2-1. The abbreviations for dibenzo-p-dioxin, dibenzofuran and polychlorinated dibenzo-*p*-dioxins and dibenzofuran congeners (PCDD/Fs).

Abbreviation	**Full name**
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
CDD	chlorinated dibenzo-*p*-dioxin
CDF	chlorinated dibenzofuran
DD	Dibenzo-*p*-dioxin
MCDD/mono-CDD	monochlorodibenzo-*p*-dioxin
DCDD/di-CDD	dichlorodibenzo-*p*-dioxin
TrCDD/tri-CDD	trichlorodibenzo-*p*-dioxin
TeCDD/tetra-CDD	tetrachlorodibenzo-*p*-dioxin
PeCDD/penta-CDD	pentachlorodibenzo-*p*-dioxin
HxCDD/hexa-CDD	hexachlorodibenzo-*p*-dioxin
HpCDD/hepta-CDD	chlorodibenzo-*p*-dioxin
OCDD	octachlorodibenzo-*p*-dioxin
DF	dibenzofuran
MCDF/mono-CDF	monochlorodibenzofuran
DCDF/di-CDF	dichlorodibenzofuran
TrCDF/tri-CDF	trichlorodibenzofuran
TeCDF/tetra-CDF	tetrachlorodibenzofuran
PeCDF/penta-CDF	pentachlorodibenzofuran
HxCDF/hexa-CDF	hexachlorodibenzofuran
HpCDF/hepta-CDF	heptachlorodibenzofuran
OCDF	octachlorodibenzofuran

Table 2-2. Toxic equivalency factors (TEFs) of 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxin and dibenzofurans (PCDD/Fs) for human risk assessment and mammals (Van den berg et al. 2006).

PCDDs		PCDFs	
Congener	TEF	Congener	TEF
2,3,7,8-TCDD	1	2,3,7,8-TCDF	0.1
1,2,3,7,8-PeCDD	1	1,2,3,7,8-PeCDF	0.03
1,2,3,4,7,8-HxCDD	0.1	2,3,4,7,8-PeCDF	0.3
1,2,3,6,7,8-HxCDD	0.1	1,2,3,4,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDD	0.1	1,2,3,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-HpCDD	0.01	1,2,3,7,8,9-HxCDF	0.1
1,2,3,4,6,7,8,9-OCDD	0.0003	2,3,4,6,7,8-HxCDF	0.1
		1,2,3,4,6,7,8-HpCDF	0.01
		1,2,3,4,7,8,9-HpCDF	0.01
		1,2,3,4,6,7,8,9-OCDF	0.0003

TEFs are intended to predict the relative toxicity of congeners that are in animal tissues or their diets. TEFs are also widely used to quantify TEQs for dioxin-like compounds in abiotic environmental media. The process of summing converted TEQ concentrations of individual congeners to a single total TEQ value is useful for comparing different environmental matrices (e.g., sediments) and prioritizing remedial efforts.

1. Sources of PCDD/Fs

PCDD/Fs are produced inadvertently and released to the environment from a wide range of sources. These sources have been grouped into six categories: combustion sources, metals smelting, refining and process sources, chemical manufacturing sources, natural sources, and environmental reservoirs by the United States Environmental Protection Agency (US EPA) (2006). US EPA reported that in the year 1987, 1995, and 2000, the releases of dioxin-like compounds were 13,965 g, 3,444 g, and 1,422 g TEQ, respectively. The same report also summarized advances in the control of PCDD/F release in the USA. A decrease in the mass of dioxin-like compounds released to the environment was reported to be about 90% between 1987 and 2000. In 1987, the release from municipal waste combustion was 8,905 g TEQ, which was the largest release source for dioxin-like compounds. However, this source was reduced more than 99%, to 83.8 g TEQ, by 2000. Similarly, another major source in 1987, medical waste incineration was reduced up to 85% from 2,570 g TEQ to 378 g TEQ in 2000. The reduction from these two major sources, which both were release sources to the air, accounted for 79% of the reduction from 1987 to 2000. This report illustrated the effectiveness of the control of the emissions from incineration facilities. The leading release source in 2000 was the backyard burning of refuse in barrels, which amounted to 498.5 g TEQ. The environmental release of dioxin-like compounds were from a wide range of sources, however, the dominant sources were combustion sources to the air. The percents of release to the air took up 96.5%, 90.0%, and 92.4% of the total release to the air, water and land, in year 1987, 1995, and 2000, respectively. Although the total amount of release decreased greatly from 1987 to 2000,

the release of dioxin-like compounds to the atmosphere remained the largest input source to the environment. The successful reduction in release of dioxin-like compounds were a result of application of combined approaches including strict emission regulations, the improvement of emission control techniques, the decreased number of facilities as emission sources, and voluntary efforts by industry.

2. Physical-Chemical Properties

The PCDD/F congeners have similar physical-chemical properties. They have stable chemical structures, with high melting points, low aqueous solubilities, and low vapor pressures. The melting points of PCDDs range from 80-90°C for monochlorodibenzo-*p*-dioxins (MCDDs) to 330°C for octachlorodibenzo-*p*-dioxin (OCDD) (Pohland & Yang 1972; Kende et al. 1974) (Table 2-3). The melting point of PCDD congeners generally increases with an increasing number of chlorines in the structure. However, 2,3,7,8-TeCDD has a very high melting point of 305-307°C relative to other TeCDDs in the same homolog group. This reflects the stability of the structure of 2,3,7,8-TeCDD with chlorines on all the lateral positions. The non-chlorinated dibenzo-*p*-dioxin (DD) has a melting point of 123 °C *(6)*. The aqueous solubilities of PCDDs range from $3.2 \pm 0.2 \times 10^{-7}$ g/L for 1,3,6,8-TeCDD to $0.4 \pm 0.1 \times 10^{-9}$ g/L for OCDD at 20°C determined by Friesen et al. (1985). Shiu et al. (1988) summarized and determined the aqueous solubility of some PCDD congeners (Table 2-4). Aqueous solubility decreased with the increasing number of chlorines in general, from DD with a solubility of 8.42×10^{-4} g/L to

OCDD with a solubility of 7.4×10^{-11} g/L at 25°C. The solubility of 2,3,7,8-TeCDD was measured using a variety of methods (Shiu et al. 1988;Crummett & Stehl 1973; Webster et al. 1983; Marple et al. 1986; Adams & Blaine 1986), and ranged from 7.91×10^{-9} g/L to 3.17×10^{-7} g/L. The aqueous solubilities of most PCDD congeners follow a trend that they increase by about an order of magnitude with an increase in temperature from 5°C to 45°C (Table 2-5). The vapor pressure of 10 PCDDs in the solid form has been determined by Rordorf (1985, 1989) to be from 5.5×10^{-2} Pa for DD to 1.1×10^{-10} Pa for OCDD at 25 °C. Four PCDF congeners have vapor pressures ranging from 3.5×10^{-1} Pa for dibenzofuran (DF) to 5.0×10^{-10} Pa for octachlorodibenzofuran (OCDF) (Table 2-6). Vapor pressure predictions have been made for other PCDD/F congeners with known melting points (Rordorf 1989). Vapor pressure decreases with an increase in the number of chlorines and with decreasing temperature, as determined by thermodynamic calculations. If the vapor pressure of the solid and the solubility of the solid form of PCDD/Fs are known, the Henry's law constant may be calculated as the ratio of the former to the latter. Shiu et al. (1988) calculated the Henry's law constant for one representative isomer from each PCDD/F homolog group. The values were from 12.29 $Pa \cdot m^3/mol$ for DD to 0.683 $Pa \cdot m^3/mol$ for OCDD (Table 2-4). The partitioning of PCDD/Fs to the air and aqueous phases thus can be estimated based on their Henry's law constants. Another very important parameter that governs the environmental behavior of PCDD/Fs is the octanol-water partitioning coefficient (K_{ow}), which describes the partitioning of PCDD/Fs between an organic solvent phase and the aqueous phase. The log K_{OW} of some PCDD congeners with representatives from each homolog group of MCDDs to OCDD plus DD were measured at 25 °C (Shiu et al. 1988; Sarna et al. 1984).

The log K_{OW} ranged from 4.30 for DD to 8.20 for OCDD, showing the hydrophobicity of this group of compounds (Table 2-7).

Table 2-3. Melting point for dibenzo-*p*-dioxin and some polychlorinated dibenzo-*p*-dioxins (PCDDs) (Pohland et al. 1972; Kende et al. 1974; Shiu et al. 1988).

PCDD Congener	Melting point **(°C)**
Dibenzo-*p*-dioxin	123
1-Chloro-	80-90
2-Chloro-	88-89
1,3-Dichloro-	113.5-114.5
2,3-Dichloro-	163-164
2,7-Dichloro-	209-210
2,8-Dichloro-	150.5-151
1,2,4-Trichloro-	128-129
2,3,7-Trichloro-	157-158
2,3,7,8-Tetrachloro-	305-306
1,2,3,4-Tetrachloro-	188-190
1,3,7,8-Tetrachloro-	193.5-195
1,3,6,8-Tetrachloro-	219-219.5
1,2,3,4,7-Pentachloro-	195-196
1,2,3,4,7,8-Hexachloro-	275
1,2,4,6,7,9-Hexachloro-	238-240
Octachloro-	330

Table 2-4. Aqueous solubility (at 25°C) and Henry's law constant for dibenzo-*p*-dioxin and some polychlorinated dibenzo-*p*-dioxins (PCDDs) (Shiu et al. 1988; Friesen et al. 1985; Crummett & Stehl 1973; Webster et al. 1983; Marple et al. 1986; Adams & Blaine 1986).

PCDD Congener	Solubility (µg/L)	Henry's law constants (Pa•m3/mol)
Dibenzo-*p*-dioxin	842	12.29
	900	
1-Chloro-	417	8.38
2-Chloro-	278	14.82
	318	
2,3-Dichloro-	14.9	6.61
2,7-Dichloro-	3.75	8.11
2,8-Dichloro-	16.7	2.13
1,2,4-Tricholor-	8.41	3.84
1,2,3,4-Tetrachloro-	0.63	3.77
	0.47	
1,2,3,7-Tetrachloro-	0.42 (20°C)	0.77
1,3,6,8-Tetrachloro-	0.32 (20°C)	0.71
2,3,7,8-Tetrachloro-	0.2	1.63
	0.317	
	0.0193	
	0.00791	
1,2,3,4,7-Pentachloro-	0.118 (20°C)	0.264
1,2,3,4,7,8-Hexachloro-	0.0044 (20°C)	4.52
1,2,3,4,6,7,8-Heptachloro-	0.0024 (20°C)	0.133
Octachloro-	0.0004 (20°C)	0.683
	0.000074	

Table 2-5. Temperature dependence of aqueous solubility of dibenzo-*p*-dioxin and some polychlorinated dibenzo-*p*-dioxins (PCDDs) (revised from Shiu et al. 1988).

Solubilities (μg/L)	5°C	15°C	25°C	35°C	45°C
Dibenzo-*p*-dioxin	205 ± 14	460 ± 16	842 ± 14	1762 ± 35	3262 ± 26
l-Chloro-	136 ± 3.6	233 ± 3.3	417 ± 16	725 ± 24	1240 ± 23
2-Chloro-	63.5 ± 13	137 ± 10	278 ± 10.5	653 ± 34	1109 ± 104
2,3-Dichloro-	36.8 ± 0.1	7.16 ± 0.8	14.9 ± 0.38	30.4 ± 0.8	58.6 ± 3.3
2,7-Dichloro-	1.09 ± 0.05	2.01 ± 0.14	3.75 ± 0.097	7.27 ± 0.35	13.4 ± 0.15
2,8-Dichloro-	4.42 ± 0.14	8.59 ± 0.55	16.74 ± 0.5	27.53 ± 0.9	51.5 ± 2.9
1,2,4-Trichloro-	2.19 ± 0.087	4.77 ± 0.087	8.41 ± 0.16	16.68 ± 1.92	28.22 ± 0.32
1,2,3,4-Tetrachloro-	0.348 ± 0.028	0.45 ± 0.025	0.63 ± 0.025	1.14 ± 0.064	2.085 ± 0.022

Table 2-6. Vapor pressure of dibenzo-*p*-dioxin (DD) and some polychlorinated dibenzo-*p*-dioxins (PCDDs) at different temperatures (Rordorf 1989).

PCDDs	Vapor pressures for t (°C)				
	25	50	75	100	125
DD	5.50E-02	9.80E-01	1.20E+01	9.70E+01	6.30E+02
1-MCDD	1.20E-02	2.60E-01	3.60E+00	3.60E+01	2.60E+02
2-MCDD	1.70E-02	3.60E-01	4.80E+00	4.50E+01	3.20E+02
2,3-DCDD	3.90E-04	1.10E-02	1.80E-01	2.20E+00	1.90E+01
2,7-DCDD	1.20E-04	3.30E-03	5.60E-02	6.40E-01	5.40E+00
2,8-DCDD	1.40E-04	4.20E-03	7.80E-02	9.70E-01	8.80E+00
1,3,7-TrCDD	3.60E-05	1.40E-03	3.10E-02	4.50E-01	4.70E+00
1,2,4-TrCD	1.00E-04	4.20E-03	1.00E-01	1.60E+00	1.80E+01
1,2,3,4-TeCDD	6.40E-06	2.60E-04	6.20E-03	9.60E-02	1.10E+00
2,3,7,8-TeCDD	2.00E-07	9.50E-06	2.60E-04	4.60E-03	5.70E-02
OCDD	1.10E-10	1.30E-08	7.20E-07	2.40E-05	5.10E-04
DF	3.50E-01	5.10E+00	5.00E+01	3.60E+02	2.10E-03
3,6-DCDF	2.00E-04	6.30E-03	I.2E-01	1.60E+00	1.50E+01
2,4,8-TrCDF	9.10E-05	3.00E-03	6.10E-02	8.20E-01	8.00E+00
OCDF	5.0E-I0	5.30E-08	2.90E-06	9.10E-05	1.90E-03

Table 2-7. Octanol-water partition coefficient of dibenzo-*p*-dioxin and some polychlorinated dibenzo-*p*-dioxin (PCDDs) (Shiu et al. 1988; Sarna et al. 1985; Burkhard & Kuehl 1986; Kaiser 1983).

PCDDs	Log K_{OW}
Dibenzo-*p*-dioxin	4.3
1-Chloro-	4.75
2-Chloro-	5.00
2,3-Dichloro-	5.60
2,7-Dichloro-	5.75
2,8-Dichloro-	5.60
1,2,4-Trichloro-	6.35
1,2,3,4-Tetrachloro-	6.60
1,2,3,7-Tetrachloro-	6.90
1,3,6,8-Tetrachloro-	7.10
1,3,7,9-Tetrachloro	7.10
2,3,7,8- Tetrachloro	6.80
1,2,3,4,7-Pentachloro-	7.40
1,2,3,4,7,8-Hexachloro-	7.80
1,2,3,4,6,7,8-Heptachloro-	8.00
Octachloro-	8.20

3. Fate and Transport of PCDD/Fs in the Environment

3.1. Atmosphere

The physical-chemical properties of PCDD/Fs determine their distribution in the environment. Although the major release route of dioxin-like compounds to the

environment is via the atmosphere, dioxin-like compounds are detected in all environmental compartments, and their fate and transport should be evaluated across all compartments. PCDD/Fs are transported in the air after release from combustion sources. Two mechanisms affect the lifetime of PCDD/Fs in the atmosphere: chemical transformation and deposition. PCDD/Fs exist in the air either in the vapor phase or particle phase depending on their physical-chemical properties and the ambient environment. Congeners with six to eight chlorines are mainly present in the particle phase due to their low vapor pressure. PCDD/Fs with four to five chorines exist in both phases (Cohen et al. 1997). Eitzer and Hites (1989) analyzed the atmospheric PCDD/F congeners in the ambient air of Bloomington, Indiana, and found that there was no seasonal effect on the total concentrations of PCDD/Fs but on the proportion between the vapor and particle phases. They concluded that the controlling parameters on the partitioning of PCDD/Fs between vapor phase and particle phase were the ambient temperature and their vapor pressures. The PCDD/Fs could be eliminated from the atmosphere via dry deposition of particles, thus removing particle phase PCDD/Fs, at a time scale of about one week (Cohen et al. 1997). PCDD/F congeners could be removed by wet deposition from atmosphere to the surface of soil, water, and vegetation in dissolved phase or in particles. PCDD/F congeners in the particle phase mainly exist in the fine particles. Kaupp et al. (1994) measured the particle size distribution of PCDD/Fs in the particle phase in the air at a rural location in Germany. They discovered that approximately 90% of the PCDD/F congeners existed in particles with diameter < 1.35 μm. This result is consistent with the theoretical considerations that particle surface area per cubic meter of air is an important parameter in the vapor/particle partitioning (Junge

1977). The fine particles with which most PCDD/Fs are associated could be removed by dry or wet deposition with an average lifetime of around 1 week (Cohen et al. 1997). In summary, the highly chlorinated congeners are usually associated with the particle phase while lightly chlorinated congeners are mainly in the vapor phase. The congeners with intermediate chlorination will exist in both phases, varying according to the ambient environment. However, their lifetime in the air is expected to be on the order of one week when chemical transformation is neglected. PCDD/Fs also undergo photolysis or react with hydroxyl radicals in the air. If chemical transformation happens, their lifetime in the atmosphere will be much shortened.

3.2 Soils

PCDD/Fs deposited from air to the soil surface are subject to photodegradation and volatilization. However, these processes are limited to the soil surface (Freeman & Schroy 1989). Due to the low solubility and high hydrophobicity, they are prone to bind to soil particles with little upward or downward mobility, especially in soils with high organic matter contents. Yanders et al. (1989) studied the concentration profile of 2,3,7,8-TeCDD in homogenized soil bins using soil taken from Times Beach, Missouri, the site of a significant 2,3,7,8-TeCDD contamination event, over a four year period. They detected no appreciable vertical movement or loss of the congener from a sampling depth of 5 mm to 50 mm and concluded that 2,3,7,8-TeCDD that penetrated below the soil surface during the contamination events remained unchanged over time. Some studies showed that the addition of surfactant or a carrier such as waste oil could mobilize the

bound PCDD/Fs in soil. Puri et al. (1989) examined the effects of waste oil and surfactant on the distribution of 2,3,7,8-TeCDD in soil and concluded that their presence might enhance the translocation of PCDD/Fs in soil. This might explain the observed contamination of groundwater by PCDD/Fs in wood treatment facilities due to the coexistence of pentachlorophenol, petroleum oil, or creosote (Pereira et al. 1985). The PCDD/Fs in soil showed persistent presence after even long period of contamination. The primary transport mechanism for PCDD/Fs from soil to water bodies occurs through soil erosion and transport of the particle-bound pollutants.

3.3 Aquatic Systems

PCDD/Fs enter water bodies through soil erosion, atmospheric deposition, wastewater discharge, and surface runoff. In the water column, they are subject to sedimentation processes and ultimately accumulate in the sediments. Sediment resuspension and remobilization occur under physical forces, such as waves and currents, and via biological activities of benthic organisms. Volatilization from the water to the atmosphere could happen, especially for lower chlorinated congeners. Broman et al. (1992) investigated the presence of PCDD/Fs in the aquatic environment in the Baltic Sea. They determined that the average concentration of PCDD/Fs associated with particles with a diameter > 0.45 mm was 230 pg/m^3; while the apparently dissolved PCDD/F concentration averaged 120 pg/m^3. Further calculation using the partitioning coefficient between the dissolved organic carbon and aqueous phase predicted that the “truly” dissolved PCDD/F concentration was 70 pg/m^3. The comparison of concentrations in

suspended solids and settled solids showed concentrations of PCDD/Fs in the settled solids was an order of magnitude higher (840 pg/m^3) than in the suspended solids, which was attributed to the scavenging of the dissolved fraction during their settling through the water column. They also calculated the fugacity of PCDD/Fs based on the truly dissolved concentration of PCDD/Fs and their concentrations in gas-phase air samples and obtained a net transport from air to water. A study conducted on the partitioning of several PCDD/F congeners in limnocorrals using lake water and natural sediment (Muir et al. 1992) illustrated that 70-80% of PCDD/Fs were associated with particles and <1% for OCDD to 10% for 1,3,6,8-TeCDD were "freely" dissolved. The remaining PCDD/Fs were associated with the dissolved organic carbon. The particles were grouped by filtering through 20 μm, 10 μm, 1 μm, and 0.22 μm filters and the highest concentration of the congener 2,3,7,8-TeCDF in the particle phase either based on water volume or carbon content was observed in the portion with the smallest particle size (0.22 μm-1 μm).

PCDD/Fs in the environment may be taken up by organisms in terrestrial or aquatic systems and thus accumulate along the increasing trophic level of the food chain. In the aquatic environment, benthic organisms capture contaminated particles or water thus allowing entry of these compounds to the food chain. Benthic organisms are consumed by crayfish or other predators in the upper trophic levels. In the terrestrial system, deposition on vegetation and subsequent ingestion by animals is the main route of entry of PCDD/Fs and subsequent accumulation in the food chain.

Overall, PCDD/F congeners are associated with particulate matter in soil, sediment and the water column. The same trend is observed with the highly chlorinated congeners

in the atmosphere while the congeners with fewer chlorine substitutes are mainly present in the vapor phase. The cycling of PCDD/Fs in the environment is a complex process with multiple sources, flows, reservoirs and sinks. The reservoirs refer to environmental compartments such as soil and sediment and anthropogenic materials which contain PCDD/Fs that act as temporary storage of these compounds. They may release PCDD/Fs for circulation in the wider environment later. Sinks include undisturbed soil and sediment which facilitate long term storage and isolation of PCDD/Fs (U.S. EPA). The ultimate destinations of PCDD/Fs in the environment are soils and sediments. PCDD/F contamination of soils and sediment has been reported worldwide and is a serious environmental problem (Bopp et al. 1991; Kjeller & Rappe 1995; Juttner et al. 1997; Isosaari et al. 2002; Verta et al. 2007).

4. Biotransformation of PCDD/Fs

4.1 Aerobic Biotransformation

Biotransformation of PCDDs may occur under both aerobic and anaerobic conditions. Under aerobic conditions, PCDD/Fs undergo oxidation by oxidative enzymes produced by microorganisms. Many studies have focused on the biodegradation of 2,3,7,8-TeCDD since its toxicity a major public health concern. Kearney and coworkers (1972) investigated the degradation of 2,3,7,8-TeCDD and 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD) in two soils. They detected $^{14}CO_2$ from soil incubated with ^{14}C labeled 2,7-DCDD while little $^{14}CO_2$ was detected from ^{14}C labeled 2,3,7,8-TeCDD spiked soil.

Further analysis using thin layer chromatography detected a polar metabolite from 2,7-DiCDD. Hütter and Philippi (1982) investigated the metabolism of 2,3,7,8-TeCDD by *Trichoderma harzianum*, a fungal species isolated from soil from zone A3 of the area affected by the Seveso disaster in Italy. They found that the microbial transformation of this congener was very slow in liquid media or soil, and only about 1% was transformed to an assumed hydroxylated derivative of 2,3,7,8-TeCDD with a molecular weight of 338, within several months of incubation. Camoni and coworkers (1982) studied the microbial degradation of 2,3,7,8-TeCDD in the laboratory using a contaminated soil and a spiked clean soil. They observed that 2,3,7,8-TeCDD decreased over 480 days of incubation at a statistically significant level, however, no metabolites were reported. They concluded this decrease might from a combination of processes including volatilization, photodegradation, binding or irreversible adsorption to the soil in addition to a slow biotic degradation. Matsumura and Benezet (1973) performed a screening test using about 100 microbial strains which were shown to be able to degrade persistent pesticides on their ability to transform 2,3,7,8-TeCDD. They discovered that 5 strains out of 100 showed some activity, although no details were given. Later, Quensen and Matsumura (1983) manipulated the conditions to enhance the degradation of 5 ppb 2,3,7,8-TeCDD by two pure cultures *Nocardiopsis* spp. and *Bacillus megaterium*, which had previously shown activity on 2,3,7,8-TeCDD, and by organisms in three types of soils. The effect of the carrier solvent used to dissolve the congener and subsequently added to the cultures was shown to play an important role. The best degradation was achieved by *Bacillus megaterium* with ethyl acetate as the carrier solvent . The authors speculated that the solvent improved the microbial uptake of the compound. The native microorganisms in

soils showed little activity, if it even occurred. Bumpus et al. (1985) observed the biodegradation of persistent compounds including 2,3,7,8-TeCDD with a white rot fungus *Phanerochaete chrysosporium*, by detecting the production of $^{14}CO_2$ from ^{14}C labeled compounds. During the 30 day incubation period, 27.9 pmol of the originally present 1.25 nmol 2,3,7,8-TeCDD was transformed to $^{14}CO_2$. The extracellular lignin-degrading enzyme system of the fungus which is "highly nonspecific and nonstereoselective", was thought accountable for degradation. In another study, Takada et al. (1996) investigated the degradation ability of another white rot fungus, *Phanerochaete sordida* YK-624, on a mixture of ten 2,3,7,8-substituted TeCDD/F to OCDD/F congeners. They reported biodegradation extents of 40-76% for PCDDs and 45-70% for PCDFs, based on substrate disappearance. A stronger proof for the existence of biodegradation was that 4,5-dichlorocatechol and tetrachlorocatechol were detected as the metabolites of 2,3,7,8-TeCDD and OCDD although they were quantified as less than 1% of the original substrates. Schreiner and coworkers (1997) performed a comprehensive screening test on the degradation of a spiked solution containing all 210 PCDD/F congeners using five pure aerobic bacterial strains. Three strains out of five degraded lower chlorinated congeners. *Pseudomonas* sp. DSM-No.6708 and *Sphingomonas* sp. DSM-No.7135 degraded almost all MCDD/Fs and DCDD/Fs, and some of the trichlorodibenzo-*p*-dioxins and dibenzofurans (TrCDD/Fs), TeCDD/Fs, and pentachlorodibenzo-*p*-dioxins and dibenzofurans (PeCDD/Fs). *Alcaligenes eutrophus* DSM-No.5536 also degraded some MCDD/F and DCDD/F isomers.

More studies on the metabolic pathways elucidated the characteristics of the degradation of dibenzo-*p*-dioxin (DD), dibenzofuran (DF) and PCDD/Fs. Klečka and

Gibson showed that a *Pseudomonas* sp. N.C.I.B. 9816 strain 11 oxidized DD to two compounds, 2-hydroxydibenzo-*p*-dioxin and *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin (1979). The latter was further transformed by the cell extract of the strain in the presence of NAD^+ to 1,2-dihydroxydibenzo-*p*-dioxin.They found that the strain only oxidized DD when sodium salicylate was present as a growth substrate to induce the enzymes. They also performed a degradation test using a *Beijerinckia* mutant strain B8/36 (later assigned to *Sphingomonas*) on DD and other PCDDs. The strain metabolized DD to cis-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin and further to 2-hydroxydibenzo-*p*-dioxin after a non-enzymatic dehydration. These two metabolites were detected in the degradation study by *Pseudomonas* sp. N.C.I.B. 9816 as well. This strain also oxidized two MCDDs to *cis*-dihydrodiols but failed to metabolize certain DCDDS. The *Beijerinckia* sp. wild type metabolized DD to 1,2-dihydroxydibenzo-*p*-dioxin. All the activities were co-metabolic. It has been observed that bacteria initiate the oxidation of aromatic hydrocarbons by first transforming them into dihydrodiols, which was consistent with the studies of DD by *Beijerinckia* sp. and *Pseudomonas* sp. as discussed above (Klečka & Gibson 1979, 1980). Subsequent transformation from these metabolites to catechols would lead to ring fission by dioxygenases and further to complete degradation. The metabolite of *Beijerinckia* sp. wild type, 1,2-dihydroxydibenzo-*p*-dioxin was found to inhibit the activity of two ring fission oxygenases in the organism, which explained the lack of further transformation. A similar pathway was observed in the oxidation of DF by *Beijerinckia* mutant strain B8/36 and a fungus *Cunninghamella elegans* (Cerniglia et al. 1979). DF was oxidized to 2,3-dihydroxy-2,3-

dihydrodibenzofuran. A pathway scheme for both the fungal and bacterial degradation of DF was proposed in which both pathways might lead to the formation of catechols.

Wittich (1998) summarized the pathway of DD and DF from previous studies, which features an oxidative attack for the angular position on the two carbon atoms adjacent to the ether bridge (Figure 2-2). Subsequently, an unstable hemiacetal was formed and spontaneously transformed to 2,2',3-trihydroxydiphenyl ether in the case of DD and 2,2',3-trihydroxybiphenyl in the case of DF. The dihydroxylated aromatic rings of these intermediates were then meta-cleaved. After hydrolysis, salicylate and catechol were formed, respectively for the DD and DF pathway. The complete degradation of these compounds was subsequently carried out via the Krebs cycle.

Some pioneering work on the characterization of enzymatic systems responsible for each step in the oxidative pathway has been done. The dioxin dioxygenase system of the DD/DF degrading strain *Sphingomonas* sp. RW1 has been purified and characterized (Bünz & Cook 1993). The meta-cleaving dioxygenase responsible for the next step with its encoding gene in strain RW1 were characterized as well (Happe et al. 1993). Bunz et al. (1993) also isolated two hydrolases catalyzing the cleavage of the side chain to form salicylate. Further degradation was by known enzymes.

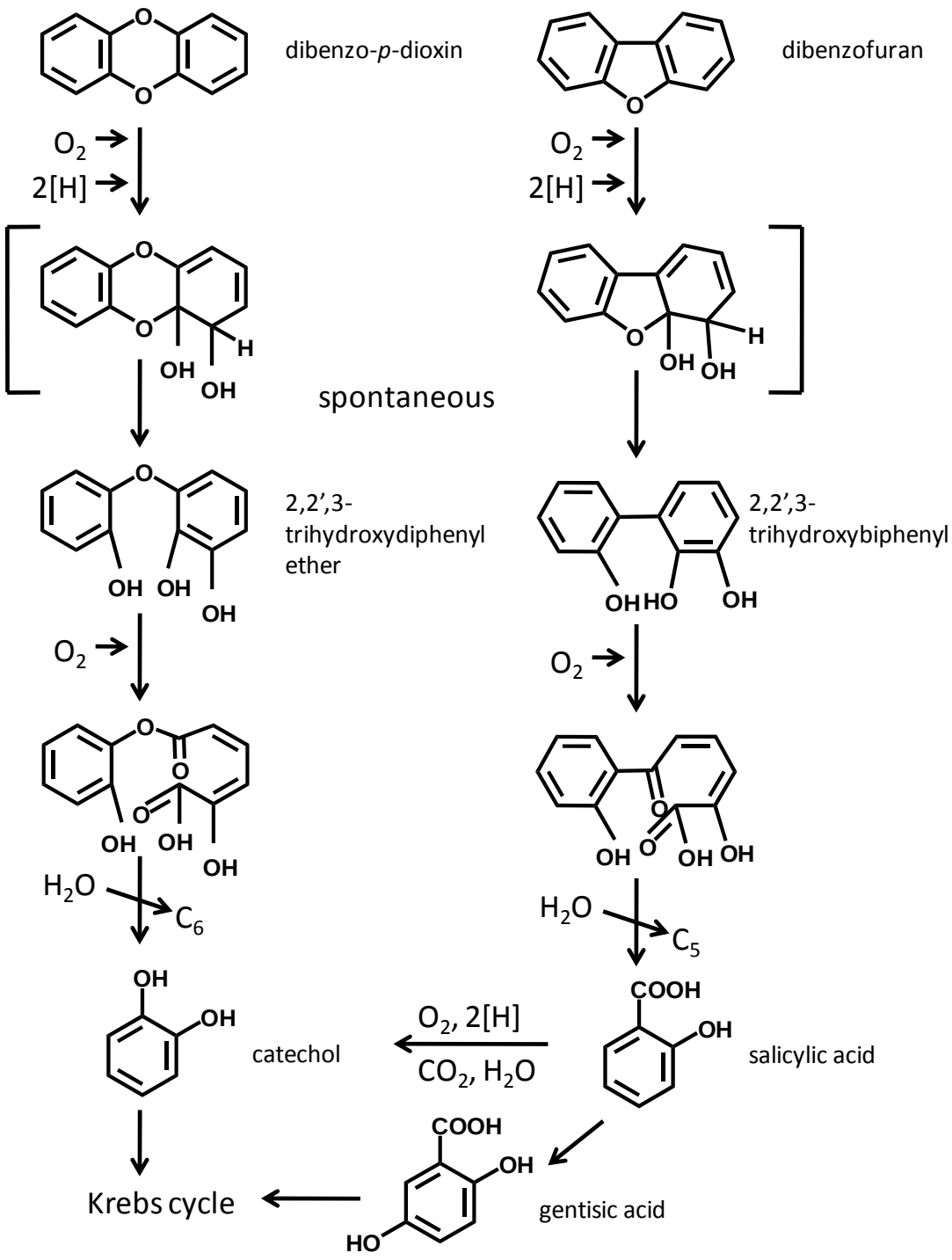


Figure 2-2. The degradation pathway of dibenzo-*p*-dioxin and dibenzofuran under aerobic condition (revised from Wittich 1998).

4.2 Anaerobic Biotransformation

Compared with the aerobic degradation process, anaerobic biotransformation is more relevant for the fate of PCDD/Fs in anoxic sediments. Under anaerobic conditions, a different mechanism, reductive dechlorination, in which chlorine substituents are replaced by hydrogen atoms to yield lower chlorinated congeners, is involved in the biotransformation of PCDD/Fs. Originally, the possibility of anaerobic transformation of PCDD/Fs was revealed by investigation of the fate of PCDD/F congeners in the environment. Beurskens et al. (1993) analyzed sediment cores from Lake Ketelmeer, The Netherlands. They detected the disappearance of PCDD/F congeners in the sediment and attributed the losses to microbial dechlorination reactions in the anaerobic lake sediment. Barabás et al. (2004[a],2004[b]) used geostatistical analysis of historical contamination and detected a reductive dechlorination fingerprint showing formation of 2,3,7,8-TeCDD at the expense of 1,2,3,7,8-PeCDD in Passaic River, New Jersey, USA sediments. The analysis of sediment cores gave an indication of the possibility for the reductive dechlorination and the occurrence of reductive dechlorination was further validated in several laboratory studies. Adriaens and coworkers (1994, 1995) investigated the microbial dechlorination of PeCDD/Fs to heptachlorodibenzo-*p*-dioxins and dibenzofurans (HpCDD/Fs) in sediments. The disappearance of the PCDD/F congeners was observed in both active and autoclaved control microcosms, however, the loss in the active ones were more pronounced, ranging from 11-35% for HpCDDs, and hexachlorodibenzo-*p*-dioxins (HxCDDs) and 25-31% for the PeCDF and HpCDF congeners. Peri-dechlorination was identified as the preferential pathway in the

dechlorination of 1,2,3,4,6,7,8-HpCDD with the formation of 1,2,3,4,7,8-HxCDD and 1,2,3,6,7,8-HxCDD. The dechlorination of 1,2,4,6,8-PeCDF exhibited the same pattern with the peri-removal of chlorine substituents as the dominating route. Dechlorination of HpCDD, HxCDD and PeCDF congeners stopped with the formation of TeCDD/Fs. Beurskens et al. (1995) detected the dechlorination of a model compound 1,2,3,4-TeCDD to less chlorinated congeners with an anaerobic culture enriched from Lake Ketelmeer sediment amended with chlorobenzenes. Two trichlorinated congeners, 1,2,3-TrCDD, and 1,2,4-TrCDD, and two dichlorinated congeners, 1,3-DCDD and 2,3-DCDD were detected in the active microcosms. The ratio of the concentrations of 1,3-DCDD to 2,3-DCDD was about 5:2. The final product, 2-MCDD, was detected at a very low concentration. A pathway was proposed based on the detected metabolites (Figure 2-3). The chlorine substituents were removed from both lateral and peri position at each dechlorination step to form isomers with the same degree of chlorination. This suggested that different microorganisms in the culture might mediate the dechlorination.

More research with 1,2,3,4-TeCDD as a model compound was carried out afterwards. Ballerstedt et al. (1997) set up microcosms with microorganisms eluted from soil, sewage and river sediment and amended them with 1,2,3,4-TeCDD. Dechlorination activity was detected in the microcosms with river sediment only and 1,2,3,4-TeCDD was dechlorinated to 1,2,3-TrCDD and 1,2,4-TrCDD, and further to 1,3-DCDD. Subsequent incubation with the two TrCDDs revealed different pathways. The authors concluded that the dechlorination of 1,2,3,4-TeCDD mainly proceeded through 1,2,4-TrCDD by a lateral chlorine removal, to 1,3-DCDD by a second peri chlorine removal. The formation of 1,2,3-TrCDD was a minor side reaction. However, when incubated alone, 1,2,3-TrCDD

revealed a new pattern, forming 1,3-DCDD and 2,3-DCDD at similar levels. The 2-MCDD was detected as a metabolite from the dechlorination of both TrCDDs. The pathway observed during this study showed a preferential dechlorination route through a first step lateral chlorine removal, which was different from the result obtained by Beurskens et al. (1995). Ballerstedt et al. (1997*)* also noted that river sediment may be a suitable source for PCDD/F dechlorinating bacteria. Another study with 1,2,3,4-TeCDD as model compound focused on the various dechlorination activities of microbial populations from different depths within a sediment (Bunge et al. 2001). Two river sediment cores were separated into 10-20 cm thick layers and incubated with 1,2,3,4-TeCDD. Different dechlorination activities and pathway patterns were observed from these separate incubations. One pathway was characterized with the combined formation of 1,3-DCDD and 2,3-DCDD while another led to exclusive formation of 1,3-DCDD from 1,2,4-TrCDD. Both pathways had been observed in previous studies (Beurskens et al. 1995; Ballerstedt et al. 1997). This finding demonstrated that dechlorinating microbial populations were heterogeneously distributed in this sediment.

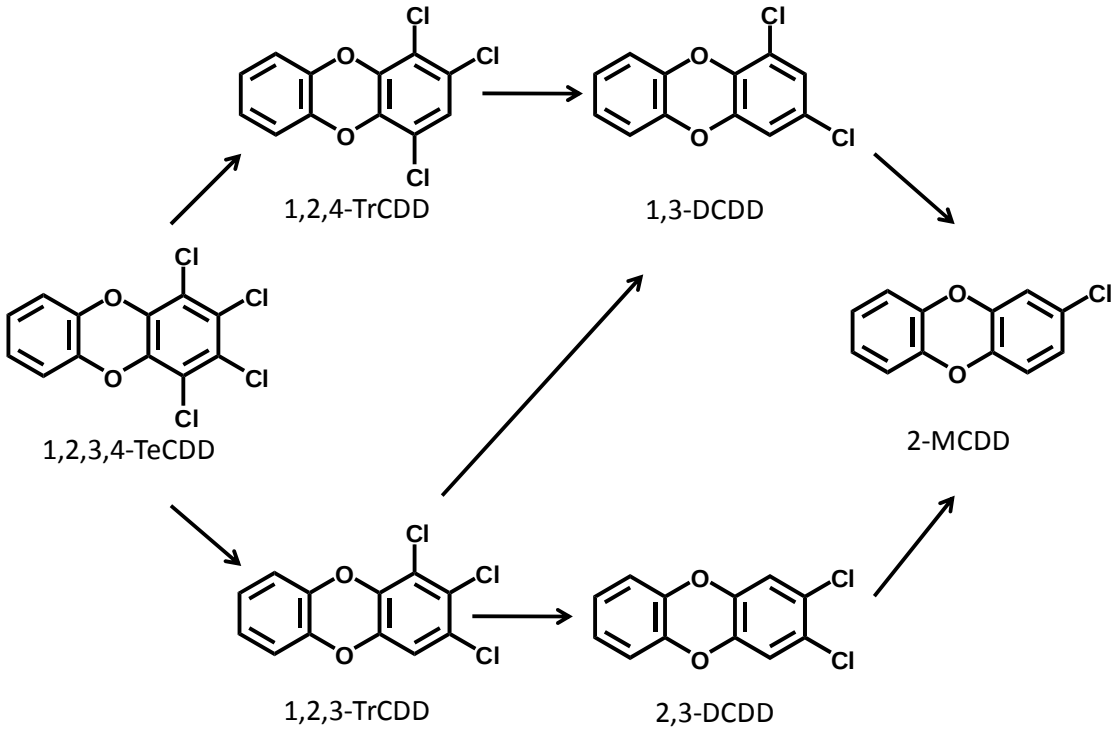


Figure 2-3. Dechlorination pathway of 1,2,3,4-TeCDD in Lake Ketelmeer sediments and river sediments (Beurskens et al. 1995; Ballerstedt et al. 1997; Bunge et al. 2001).

Research focusing on the dechlorination of the historical PCDD/Fs in sediment has been addressed as well. Microorganisms eluted from the historically contaminated Passaic River, New Jersey, USA sediment was added as inoculum for dechlorination of aged and freshly spiked PCDDs (Barkovskii & Adriaens 1996). The 2,3,7,8-TeCDD was detected as the major congener in the historical PCDD contaminants with higher chlorinated PCDDs existing at non-quantifiable trace levels, while no less chlorinated PCDDs were observed. During the seven month incubation, two TrCDDs and some DCDDs were detected, with 2-MCDD as the major product. The freshly spiked mixture

of PCDDs contained OCDD as the vast majority along with a very low level of HpCDDs (1,2,3,4,6,7,8-HpCDD and 1,2,3,4,6,7,9-HpCDD), and HxCDDs (1,2,4,6,7,9-HxCDD and 1,2,3,4,6,8-HxCDD). In the microcosms with freshly spiked PCDDs, the HpCDDs appeared to first increase, then decrease, which was a typical time progress for intermediates in sequential dechlorination. Five HxCDDs including two 2,3,7,8-substituted congeners were detected as intermediates followed by subsequent dechlorination to four TeCDDs (including 2,3,7,8-TeCDD), five TrCDDs, trace DCDDs and possibly two MCDDs. Finally, 26% and 8% of OCDD loss were observed in the active and pasteurized microcosms, respectively. The comparison of intermediates between the active and pasteurized (at 75°C for 30min) treatments revealed that 2,3,7,8-substituted congeners were formed only in the active treatments through a peri dechlorination pathway by non-spore-forming microorganisms (i.e., those that could not survive the pasteurization treatment) in the sediment. The observed peri-lateral dechlorination with the formation of non 2,3,7,8-substituted congeners was a combination of microbial and abiotic activities. In another study with the sediment from Passaic River, the sediment slurry was incubated with one or more amendments of organic acid, 2-monobromodibenzo-*p*-dioxin (2-MBDD), and hydrogen (Albrecht et al. 1999). Variations in the PCDD concentrations in the sediment were observed. The baseline profile contained mainly OCDD, 2:1 ratio of 1,2,3,4,6,7,9-HpCDD to 1,2,3,4,6,7,8-HpCDD, and 2,3,7,8-TeCDD. The amendment with organic acid and hydrogen showed the most significant extent of dechlorination, forming 2-MCDD without significant accumulation of 1,2,3,4,6,7,8-HpCDD or 2,3,7,8-TeCDD, which is a characteristic of a lateral dechlorination pathway. The amendment with organic acid and

2-MBDD resulted in a significant increase of 2,3,7,8-TeCDD, a product from a peri-dechlorination. Results from this study revealed the different potential effects of various amendments on the dechlorination pathway and extent and demonstrated the possibility of enhancing bioremediation of PCDD/F contaminated sediments.

Many factors may influence the dechlorination of PCDD/Fs by microorganisms, for example, the redox condition, the presence of other compounds that could serve as possible electron acceptors or donors, and the contamination history. The addition of alternate halogenated compounds or "haloprimers" was shown by Bedard et al. (1998) and DeWeerd and Bedard (1999) to enhance dechlorination of polychlorinated biphenyls (PCBs). As observed previously, addition of alternate halogenated compounds has been shown to enhance dechlorination of PCDDs. Beurskens et al. (1995) demonstrated dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) in a hexachlorobenzene-enriched culture from Rhine River sediment. Albrecht et al. (1999) demonstrated that OCDD was dechlorinated to 2-MCDD after amendment with 2-MBDD and hydrogen. Effect of terminal electron accepting processes, including methanogenic conditions, sulfidogenic conditions, and iron-reducing conditions, and addition of "haloprimers" on dechlorination were evaluated in sediments from the Arthur Kill, New Jersey, USA spiked with 1,2,3,4-TeCDD (Vargas et al. 2001). After 31 months, limited dechlorination occurred under methanogenic conditions, with formation of 1,2,4-TrCDD and 1,3-DCDD. Very limited dechlorination was detected in the sulfidogenic, iron-reducing, and killed (autoclaved) treatments. A microbial consortium enriched from the same sediment using a mixture of 2-, 3-, and 4-bromophenol was also able to dechlorinate 1,2,3,4-TeCDD to a more extensive extent in a shorter period (17 months)

following the same pathway to 1,3-DCDD. This result showed that relatively harmless brominated compounds could act as "haloprimers" to prime the dechlorination of PCDD/Fs by a microbial community. A study went further in this direction with the investigation on the priming effect of a variety of halogenated compounds (Ahn et al. 2005). The haloprimers examined included bromophenols (BPs), chlorophenols (CPs) and halogenated compounds with greater similarity to PCDD/Fs, such as 1,2,3,4-tetrachlorobenzene (TeCB), 2,3,4,5-tetrachloroanisole (TeCA), 2,3,4,5-tetrachlorophenol (TeCP), 2,3,5,6-tetrachlorobenzoate (TeCBA), and 2',3',4'-trichloroacetophenone (TrCAP). The haloprimers with more analogous structure to PCDD/Fs, such as TeCB and TeCA, enhanced the dechlorination of 1,2,3,4-TeCDD to the most extensive extent over the amendments with BPs and CPs. The primary dechlorination pathway was delineated as 1,2,3,4-TeCDD to 1,2,4-TrCDD, then to 1,3-DCDD, finally to 2-MCDD. This study also compared the dechlorination of 1,2,3,4-TeCDD in different sediments and the most extensive dechlorination was observed in the more contaminated sediments . A recent investigation of the microbial community eluted from Passaic River, New Jersey sediment also revealed that the addition of the priming compound affected both the dechlorination extent and pattern (Fu et al. 2005). The 1,2,3,4,6,7,8-HpCDD was spiked under methanogenic and sulfidogenic conditions with or without the amendment of the priming compound, 2-MBDD. It was observed that the presence of 2-MBDD increased the dechlorination up to 10% and decreased the formation of 2,3,7,8-substituted congeners, showing a preferential lateral dechlorination over peri dechlorination. Dechlorination under methanogenic conditions proceeded from HpCDD to MCDDs, while dechlorination ceased at DCDDs under sulfidogenic conditions. However it seems

that the percent of formation of 2,3,7,8-substituted isomer(s) in the same homolog group was less under sulfidogenic conditions than under methanogenic conditions. Denaturing gradient gel electrophoresis (DGGE) analysis showed that both the terminal electron accepting processes and the priming compound caused shifts in microbial community composition.

Recently as molecular techniques are incorporated more into microbiological research, it is possible to gain knowledge about the microbial community composition and identify the microorganisms responsible for dechlorination in a much shorter time compared with the traditional microbiological approaches. A mixed culture enriched with 1,2,4-TrCDD has been studied for microbial community composition (Ballerstedt et al. 2004). Two isolates belonging to *Sulfurospirillum* and *Trichococcus* were purified from the mixed culture, however they did not exhibit an ability to dechlorinate 1,2,4-TrCDD. Further 16S rRNA gene-targeted methods revealed the presence of *Acetobacterium, Desulfitobacterium, Desulfuromonas* and *Dehalococcoides*. This evidence showed that *Dehalococcoides* species might be involved in the dechlorination of 1,2,4-TrCDD. This was in agreement with results from other studies which also exhibited the dechorination of PCDD/Fs by *Dehalococcoides* species. Two bacterial strains have been identified to dechlorinate PCDDs under anaerobic conditions — *Dehalococcoides* sp. strain CBDB1 (Bunge et al. 2003) and *Dehalococcoides ethenogenes* strain 195 (Fennell et al. 2004). *Dehalococcoides* strain CBDB1 was originally isolated from a mixed culture enriched with 1,2,3-trichlorobenzene (Adrian et al. 2000). Strain CBDB1 is able to dechlorinate several chlorobenzenes and later, it was shown to dechlorinate five PCDD congeners, 1,2,4-TrCDD, 1,2,3-TrCDD, 2,3-DCDD, 1,2,3,4-TeCDD, and 1,2,3,7,8-PeCDD, a

2,3,7,8-substituted congener (Bunge et al. 2003). The successful successive transfer on TrCDD congeners suggested that strain CBDB1 used them as electron acceptors for growth. A pathway study indicated that strain CBDB1 preferentially removed the first chlorine substituents from a peri position. Bunge et al. (2003) also detected *Dehalococcoides* species from four other PCDD-dechlorinating enrichment cultures, using PCR targeting the 16S ribosomal RNA gene of *Dehalococcoides*. The obtained PCR products were identical with the sequence of strain CBDB1.

The other strain able to dechlorinate PCDD/Fs is *Dehalococcoides ethenogenes* strain 195, isolated from an anaerobic sludge digester (Maymó-Gatell et al. 1997, 1999*)*. Strain 195 was isolated with tetrachloroethene as an electron acceptor and hydrogen as an electron donor. Its ability to dechlorinate other chlorinated compounds was revealed by subsequent studies. *D. ethenogenes* strain 195 dechlorinates tetrachloroethene (PCE) to vinyl chloride (VC) and ethene. It grows on tetrachloroethene (PCE), trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-1,2-DCE), 1,1-DCE (Maymó-Gatell et al. 1997, 1999), selected chlorinated benzenes (Fennell et al. 2004), and chlorophenols (Adrian et al. 2007). *D. ethenogenes* strain 195 also dechlorinates certain polybrominated diphenyl ethers (PBDEs) (He et al. 2006), polychlorinated biphenyls (PCBs), chlorinated naphthalenes, and PCDD/Fs (Fennell et al. 2004). Specifically, strain 195 dechlorinates 1,2,3,4-TeCDD to 1,2,4-TrCDD, and subsequently to 1,3-DCDD (Fennell et al. 2004). More *Dehalococcoides* isolates and enrichments (He et al. 2003; Bedard et al. 2007) are able to dechlorinate a wide range of chlorinated compounds, which makes *Dehalococcoides* species important players in the transformation of chlorinated

compounds in the environment and worthy of continued study for bioremediation purposes.

Dechlorination of PCDD/Fs by abiotic reactions with electron shuttling matters may also be an important fate process (Adriaens et al. 1996; Fu et al. 1999). These processes might work alone or together with microorganisms in the anaerobic environment to enhance dechlorination reactions.

5. Practical Considerations for Applying Bioremediation for PCDD/Fs Contaminated Sediments

5.1 Biotransformation Pathways of PCDD/Fs

The discovery of microbial biotransformation of PCDD/Fs suggests that it may be feasible to bioremediate contaminated environmental matrices. The contamination of sediments with PCDD/Fs is reported worldwide (Bopp et al. 1991; Kjeller & Rappe 1995; Juttner et al. 1997; Isosaari et al. 2002; Verta et al. 2007). Reductive dechlorination could be an important process where highly chlorinated PCDD/Fs are transformed to lightly chlorinated PCDD/Fs, rendering them available for further aerobic biotransformation. Indeed in sediments, this process may occur naturally. Yoshida et al. (2005) constructed a PCDD/F-dechlorinating microcosm that contained anaerobic and aerobic zones in which PCDD/Fs were dechlorinated and total concentrations decreased without the accumulation of TrCDD/Fs, DCDD/Fs or MCDD/Fs. Catechol and salicylate, the oxidative products of DD and DF, accumulated in the microcosm, although their

production was not directly linked to PCDD/F oxidation by specific aerobic bacteria. This study suggests that bioremediation could have a profound impact on PCDD/Fs in aquatic sediments and has the potential to detoxify sediments. However, additional information on both the theoretical and practical aspects of these processes is needed to answer fundamental questions pertinent to bioremediation feasibility studies, and to ultimately enable design and operational strategies for bioremediation. For example we need to screen and identify functioning indigenous and exogenous PCDD/F-transforming microorganisms and determine how applying amendments may enhance the biotransformation activities of these microbes. Furthermore we need to develop better methods to monitor the effectiveness of amendment implementation and the microbial activities. Finally, establishing model frameworks to simulate the fate and transport of these contaminants *in situ*, including quantification and prediction of the rates of biotransformation still require work. The successful employment of bioremediation requires the integration of knowledge from all these areas.

While dechlorination may be useful as a remedial process, the possible formation of toxic products is of great concern to regulators, site owners and managers. To fulfill the goal of bioremediation of contaminated environmental media such as sediments, not only is dechlorination desired, but more importantly, detoxification with respect to the PCDD/Fs should be achieved. Thus microorganisms with the ability to transform the 2,3,7,8-substituted congeners to non 2,3,7,8-substituted congeners, i.e. remove the lateral chlorine substitutes, are desirable. The application or enhancement of such microorganisms to contaminated sediments could ensure “safe” bioremediation with less possibility of producing toxic intermediates.

5.2 Rates of Biotransformation

One limitation to a bioremediation approach is the lack of information on the dechlorination kinetics of PCDD/Fs. Although studies on the microbial reductive dechlorination of PCDD/Fs have been reported, because of the difficulty and expense of sample preparation and analyses, dechlorination rates have rarely been estimated. Published dechlorination rates range over several orders of magnitude. The transformation rate of 1,2,3,4-TeCDD by a mixed culture enriched from Saale River sediment was 60 nM/d (Ballerstedt et al. 1997), while dechlorination rates of OCDD in spiked and historical Passaic River matrices were 0.007-0.075 nM/d (Albrecht et al. 1999). Since the concentrations of the responsible microorganisms were not known in those studies, the rates were not linked with biomass concentrations. The dechlorination rates of 1,2,3,4,6,7,9-HpCDD were 0.01-0.17 nM/d and 0.004-0.014 nM/d by Aldrich humic acid and polymaleic acid, respectively (Fu et al. 1999). The discrepancy in rates reported from different studies may result from different experimental conditions, such as the physical-chemical properties of the PCDD/F congeners investigated, the properties of the matrices in which the PCDD/Fs were studied, the affinity of the microorganisms or enzymes that mediate the dechlorination reactions for the PCDD/Fs, effects of the amendments applied to enhance dechlorination, biotransformation mechanisms, or other factors. For example, Ahn et al. (2005) reported half-lives for 1,2,3,4-TeCDD/F of 3 to 4 months when sediments were amended with aromatic chlorinated co-substrates, but in excess of 12 months under unenhanced conditions. The approximate half-life of

PCDD/Fs in the anaerobic-aerobic microcosm of Yoshida et al. (2005) was 14 months. Since factors affecting one specific dechlorination process may vary significantly, it is critical to obtained kinetic parameters for each specific system in order to simulate and predict the fate of PCDD/Fs within that system.

Dechlorination under different experimental conditions would provide essential information on the factors controlling the dechlorination process. Furthermore, kinetic parameters obtained from controlled studies could be used in predictive modeling to help predict bioremediation outcomes and timeframes.

5.3 Monitoring *In Situ* Bioremediation

Another challenge in the bioremediation field is the lack of precise monitoring approaches. In making the case for the application of bioremedial technologies, it is important that the desired biological activity be conclusively documented. Documentation of the existence and effectiveness of *in situ* microbial biotransformation of pollutants is a time consuming and expensive process that may require installation of many monitoring points and many rounds of sampling and analysis to record pollutant concentration changes over time. For the PCDD/Fs consisting of 210 separate congeners, this is especially true.

One promising tool for assessment of biological transformation of environmental contaminants is Compound Specific Isotope Analysis (CSIA). Using CSIA the ratio of the relative abundance of the heavy and light isotopes of a given element is determined in a specific compound. As the compound undergoes transformation, fractionation of the

isotopes in the parent compound, may occur. Stable isotope fractionation during microbial biodegradation is a kinetic isotope effect caused by different reaction rates for a compound containing the heavy isotope (e.g., ^{13}C) versus the light isotope (e.g., ^{12}C) at the location of the reaction. The zero point energy of a molecule made up of heavy isotopes of an element is lower than that of the one consisting of light isotopes (Broecker & Oversby 1971; Hoefs 2004). Thus, more energy is needed to break the bonds in the molecules containing heavy isotopes. Therefore, the reaction is slower than for the molecule with light isotopes of the same element. This typically results in an enrichment of heavy isotopes in the residual substrate. The application of CSIA could identify the characteristic isotope fractionation associated with the biological transformation of compounds as a proof of the occurrence of *in situ* biotransformation of contaminants. This analytical tool is especially helpful when the disappearance of the contaminants could be a result of several factors, including physical-chemical processes and biological processes. Biologically mediated stable isotope fractionation of various contaminants has been examined under different conditions in laboratory and field studies, including benzene, toluene, ethylbenzene and xylenes (BTEX) (Meckenstock et al. 1999; Morasch et al. 2001; Hunkeler et al. 2001[a]; Mancini et al. 2003); methyl-tertiary-butyl ether (MTBE) (Hunkeler et al. 2001[b]; Gray et al. 2002; Kolhatkar et al. 2002; Zwank et al. 2005; Somsamak et al. 2005); polynuclear aromatic hydrocarbons (PAHs) (Mazeas et al. 2002; Richnow et al. 2003; Yanik et al. 2003); phenol and benzoate (Hall et al. 1999); polychlorinated biphenyls (Drenzek et al. 2001); and chlorinated ethenes (Hunkeler et al. 1999; Bloom et al. 2000; Sherwood Lollar et al. 2001; Slater et al. 2001; Barth et al. 2002;

Nijenhuis et al. 2005). An extensive review of this literature was recently provided by Schmitt et al. (2004).

Carbon stable isotope fractionation of chlorinated ethenes has been monitored in order to verify and estimate the extent of *in situ* biodegradation in field studies and can be used to validate a bioremediation effort. CSIA may be used as both a qualitative and a quantitative measurement to monitor the *in situ* biodegradation processes (Sherwood Lollar et al. 2001; Nijenhuis et al. 2005, 2007). CSIA could be of particular use to examine transformation of more complex environmental pollutants whose pathways of biotransformation are difficult to assess in the environment, such as PCDD/Fs. If stable isotope fractionation occurs during the dechlorination of PCDDs, CSIA could be used as a helpful tool to document their environmental transformation.

Chapter 3

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

(This chapter is in press in Environmental Science and Technology)

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are two groups of compounds with similar tricyclic planar structures. They are produced inadvertently and enter the environment from many sources including chemical manufacturing, pulp and paper production, and combustion processes (U.S. EPA 2006). PCDD/Fs are hydrophobic, have low solubility and volatility, accumulate in soils, sediments and biota, and are ubiquitous in the environment (Czuczwa et al. 1984; Brzuzy & Hites 1996; Wagrowski & Hites 2000, Mai et al. 2007; Rappolder et al. 2007). Contamination of sediment with PCDD/Fs is a serious environmental problem (Bopp et al. 1991; Kjeller & Rappe 1995; Juttner et al. 1997; Isosaari et al. 2002; Verta et al. 2007). PCDD/Fs are generally present at very low concentrations; however, they are of great concern because of their toxicity (Van den Berg et al. 2006) and potential to bioaccumulate (Cai et al. 1994). Seventeen 2,3,7,8-substituted PCDD/F congeners are the focus of regulatory effort and scientific investigation owing to their high toxicity to

humans and wildlife. Toxicity equivalency factors (TEFs) indicate an order of magnitude estimate of the toxicity of the 2,3,7,8-substituted PCDD/F congeners and other dioxin-like compounds including certain co-planar polychlorinated biphenyls (PCBs) (Van den Berg et al. 2006). TEF values are assigned relative to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TeCDD), the PCDD/F congener considered the most toxic of these compounds. A toxic equivalent (TEQ) expressed as equivalents of 2,3,7,8-TeCDD can be computed for mixtures of 2,3,7,8-substituted PCDD/F congeners by summing the products of individual concentrations multiplied by their respective TEFs. TEFs are intended to predict the relative toxicity of congeners in animal tissues or their diets. TEFs are also widely used to quantify TEQs for dioxin-like compounds in environmental media, e.g. sediment. Summing converted TEQs of individual congeners to a single total TEQ value is useful for comparing contaminated sediments and prioritizing remedial efforts.

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

D. ethenogenes strain 195 dechlorinates tetrachloroethene (PCE) to vinyl chloride (VC) and ethene. It grows on PCE, trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-

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

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

D. ethenogenes strain 195 was observed to proceed through a lateral, followed by a peri dechlorination step. During the dechlorination of 1,2,3,4,7,8-HxCDF or OCDD, removal of chlorine from one of the 2-, 3-, 7-, or 8-positions would greatly decrease the TEQ of the total PCDD/Fs. This study examined both the pathway and extent of dechlorination of 1,2,3,4,7,8-HxCDF and OCDD by a mixed culture containing *D. ethenogenes* strain 195 under different conditions.

2. Materials and Methods

2.1 Chemicals

1,2,3,4,7,8-HxCDF (98+%), 1,2,3,4-TeCDF, and 1,3,7,8-tetrachlorodibenzofuran (1,3,7,8-TeCDF) were purchased from Ultra Scientific (North Kingstown, RI, USA). The 2,2',5-trichlorobiphenyl (2,2',5-TrCB) and OCDD and standard solutions of 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF), 1,2,3,7,8-pentachlorodibenzofuran (1,2,3,7,8-PeCDF), and 2,3,7,8-tetrachlorodibenzofuran (2,3,7,8-TeCDF) were purchased from AccuStandard, Inc. (New Haven, CT, USA). A 1,2,3,4,8-pentachlorodibenzofuran (1,2,3,4,8-PeCDF) standard solution was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Standard solutions of 1,2,4,7,8-pentachlorodibenzofuran (1,2,4,7,8-PeCDF), 1,3,4,7,8-pentachlorodibenzofuran (1,3,4,7,8-PeCDF), 2,3,6,8-tetrachlorodibenzofuran (2,3,6,8-TeCDF), 1,4,7,8-tetrachlorodibenzofuran (1,4,7,8-TeCDF), 1,3,4,8-tetrachlorodibenzofuran (1,3,4,8-TeCDF), 1,2,4,7-tetrachlorodibenzofuran (1,2,4,7-TeCDF) and 1,2,4,8-

tetrachlorodibenzofuran (1,2,4,8-TeCDF) were purchased from Wellington Laboratories, Inc. (Guelph, Ontario, Canada). The 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA), PCE (99.9+%), TCE (99.5+%), and butyric acid (99+%) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). The *cis*-1,2-DCE was purchased from Supelco, Inc. (Bellefonte, PA, USA). VC (99.5+%) was obtained from Fluka Chemie GmbH (Germany). Ethene (99%) was purchased from Matheson Tri-Gas, Inc. (Montgomeryville, PA, USA).

2.2 Culture Preparation

A mixed culture containing *D. ethenogenes* strain 195 was grown at 25°C on PCE and butyric acid using methods described previously (Fennell et al. 2004; Fennell & Gossett 1997; Fennell 1998). Dechlorination experiments were carried out in 60-mL serum bottles. Dry sterile sediment (0.375 g) prepared as previously described (Fennell et al. 2004) was added to each bottle. The sediment was completely wetted by 0.35 mL of a 535 μM (200 mg/L) 1,2,3,4,7,8-HxCDF-toluene stock solution. The toluene was allowed to volatilize under sterile N_2, leaving behind a coating of 1,2,3,4,7,8-HxCDF on the sediment carrier. Culture (37.5 mL) was transferred to each bottle under anoxic and sterile conditions resulting in a final nominal 1,2,3,4,7,8-HxCDF concentration of 5 μM (1.87 mg/L). Each bottle also received 100 μM butyric acid as an electron donor and hydrogen source and 15 μL of a 50 g/L fermented yeast extract solution (Fennell 1998; Fennell & Gossett 1997) as a nutrient source on days 6, 25, 51, 74, 111, 144, and 165. A vitamin stock solution (Fennell 1998; Fennell & Gossett 1997) was added at set up. Four

sets of triplicate treatments were established. One set of cultures received 1,2,3,4,7,8-HxCDF as the sole halogenated substrate. Because we do not know if PCDD/Fs are growth substrates for *D. ethenogenes* strain 195, in addition to the 1,2,3,4,7,8-HxCDF, one set of triplicate cultures was amended with PCE, a known growth compound, as an additional substrate on the same days when butyric acid and fermented yeast extract were amended. One set of triplicate bottles was spiked with 1,2,3,4-TeCB, which is also a growth supporting substrate for *D. ethenogenes* strain 195, but only on days 0 and 76. The nominal concentrations of PCE and 1,2,3,4-TeCB added to the culture bottles were 25 μM. The fourth set of triplicate bottles was autoclaved for one hour on each of three consecutive days to serve as killed controls. Parallel treatments were prepared with OCDD as the PCDD/F substrate at 5 μM, using the same experimental protocol as described for 1,2,3,4,7,8-HxCDF. The cultures were shaken in the dark at 120 rpm at 28°C and sampled periodically over 195 days.

To ascertain the dechlorination intermediates of 1,2,3,4,7,8-HxCDF, two separate experiments were performed using 1,2,4,7,8-PeCDF or 1,3,4,7,8-PeCDF as the halogenated substrate. In the first experiment, 28 mL tubes were spiked with toluene stock solutions of either 1,2,4,7,8-PeCDF or 1,3,4,7,8-PeCDF, and toluene was allowed to volatilize. Three mL of culture was added and the final nominal concentration of PeCDF was 2 μM. Tubes were sacrificed at set up and after 1 and 2 months. Two active cultures and one autoclaved control culture were prepared for each time point for both of the PeCDF congeners. In the second experiment, dry sediment (0.15 g) was added to 60 mL serum bottles followed by spiking 0.25 mL of a 147 μM (50 mg/L) stock solution of 1,2,4,7,8-PeCDF or 1,3,4,7,8-PeCDF (in toluene), respectively. After volatilization of

toluene, 15 mL culture was transferred to each bottle under sterile and anaerobic conditions to achieve a final nominal concentration of 1,2,4,7,8-PeCDF or 1,3,4,7,8-PeCDF of 2.45 μM. At set up, cultures in both experiments received 12 μM 1,2,3,4-TeCB and butyric acid, fermented yeast extract and vitamin stock solution at the same concentrations as the experiment with 1,2,3,4,7,8-HxCDF. The cultures were shaken in the dark at 120 rpm at 28°C.

2.3 Analytical Methods

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

Samples of 1 or 2 mL of culture/sediment mixture were removed for PCDD/F extraction using a sterile anoxic syringe with an 18 gauge needle. [The entire 3 mL culture in the tube experiment examining 1,2,4,7,8 and 1,3,4,7,8-PeCDF dechlorination was extracted.] Samples were extracted and prepared for analysis as described previously (Vargas et al. 2001). Briefly, after sample centrifugation, the aqueous phase was removed to a separate vial, 2,2',5-TrCB was added as a surrogate standard to the sediment residue, and then the sediment phase was rinsed with 1 mL of acetone to remove water. The solid phase was extracted overnight with 3 mL of 2:1 volume:volume (vol:vol) toluene:acetone

solution, then for 4 h with 1 mL of 2:1 vol:vol toluene:acetone solution, and was then rinsed with 1 mL toluene. After each step, the solvent phase was combined with the aqueous phase. Finally, the pooled solvent was back extracted by adding NaCl. Interfering organic compounds were removed by passing the solvent phase through a 2 mL glass pipette filled with Florisil (Sigma-Aldrich, St. Louis, MO) and eluting with three volumes of toluene. The extract was then concentrated to about 2 mL. PCDD/Fs and chlorobenzenes were analyzed using an Agilent 6890 gas chromatograph equipped with a 5973N mass selective detector (GC-MS) (Agilent Technologies, Inc. Santa Clara, CA) and a HP-5MS (Agilent Technologies, Inc. Santa Clara, CA, USA) column (60 m × 0.25 mm I.D.). The temperature program was: initial temperature 70°C; increased by 10°C/min to 170°C; increased by 2°C/min to 200°C; increased by 5°C/min to 220°C and held for 16 min; increased by 5°C/min to 235°C and held for 7 min; and finally increased by 5°C/min to 280°C. The post run temperature was 300°C, for 5 min. PCDD/Fs were detected and identified based on the retention times of standards and their molecular ions (*m/z*: 460, OCDD; 374, 1,2,3,4,7,8-HxCDF; 340, PeCDFs; 306, TeCDFs; and 186, 2,2',5-TrCB). Qualifying ions were monitored to assure the correct identification (458, OCDD; 376, 1,2,3,4,7,8-HxCDF; 342, PeCDFs; 304, TeCDFs; and 256, 2,2',5-TrCB). The response factors of each PCDD/F congener compared to the surrogate, 2,2',5-TrCB, were calculated over five concentration levels as a linear calibration curve. The approximate detection limit for the PCDD/Fs was 1 ppb.

The PCDD/Fs were quantified based on the calibration curve and presented as averages of duplicate or triplicate data points plus or minus one standard deviation. The 1,3,4,7,8-PeCDF and 1,2,4,7,8-PeCDF co-eluted and it was not possible to quantify them

separately. We therefore used the calibration curve of 1,3,4,7,8-PeCDF to quantify the PeCDF peak detected in samples. The 1,2,4,7,8-PeCDF and 1,3,4,7,8-PeCDF had similar response factors. Results are presented as a molar fraction of an individual congener of the total moles of all PCDFs present. Presentation of PCDFs as mole percent assumed no anaerobic degradation of the dibenzofuran structure and that the PCDFs underwent no significant reactions other than dechlorination.

3. Results

3.1 Dechlorination Pathway

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

substituted PCDFs, thus, dechlorination of 1,2,3,4,7,8-HxCDF by the mixed culture containing *D. ethenogenes* strain 195 was a detoxification process. The 1,2,3,4,8-PeCDF was also excluded as a metabolite based on the retention time in comparison to the standard.

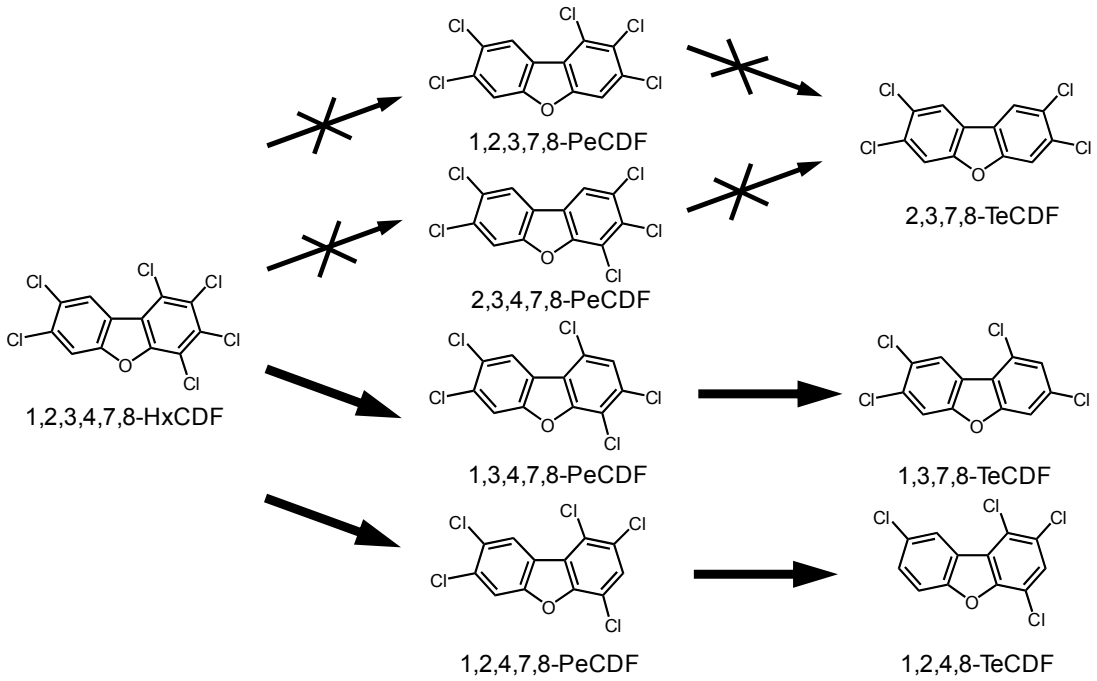


Figure 3-1. Pathways of the dechlorination of 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) by a mixed culture containing *Dehalococcoides ethenogenes* strain 195.

Table 3-1. Retention times of PCDF standards and metabolites on an Agilent 6890 gas chromatograph equipped with a 5973N mass selective detector (GC-MS) (Agilent Technologies, Inc. Santa Clara, CA) and a HP-5MS (Agilent Technologies, Inc. Santa Clara, CA, USA) column (60 m × 0.25 mm I.D.). [The temperature program was: initial temperature 70°C; increased by 10°C/min to 170°C; increased by 2°C/min to 200°C; increased by 5°C/min to 220°C and held for 16 min; increased by 5°C/min to 235°C and held for 7 min; and finally increased by 5°C/min to 280°C.]

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

Because standards of 1,2,4,7,8-PeCDF and 1,3,4,7,8-PeCDF had the same retention time as the PeCDF metabolite in the samples (Table 3-1), we identified the PeCDF metabolite(s) as 1,2,4,7,8- and/or 1,3,4,7,8-PeCDF. The 1,2,4,7,8- and 1,3,4,7,8-PeCDF could not be resolved using our GC method. The formation of 1,2,4,7,8-PeCDF and/or 1,3,4,7,8-PeCDF resulted from a lateral chlorine removal from the fully chlorinated ring of 1,2,3,4,7,8-HxCDF. We determined that 1,3,7,8-TeCDF was the first eluted TeCDF metabolite, which also indirectly confirmed the formation of 1,3,4,7,8-PeCDF from the dechlorination of 1,2,3,4,7,8-HxCDF. The 1,3,7,8-TeCDF was produced through removal of a chlorine from a flanked peri position of 1,3,4,7,8-PeCDF. This dechlorination pattern, first removal from a lateral position, followed by a removal from a peri position, was consistent with the dechlorination pattern of 1,2,3,4-TeCDD reported previously for *D. ethenogenes* strain 195 (Fennell et al. 2004).

We further narrowed the number of TeCDFs to be examined using the retention indices of PCDFs developed by Hale et al. (1985). The order of elution of the TeCDF congeners tested in our study and that of Hale et al. (1985) were almost identical. Through a process of elimination using results from the PeCDF-spiked cultures, expected elution order, theoretically possible dechlorination products, and finally, comparison of the metabolite retention time to that of known standards, the second eluted TeCDF metabolite was identified as 1,2,4,8-TeCDF (Table 3-1), a metabolite from the dechlorination of 1,2,4,7,8-PeCDF. The formation of 1,2,4,7,8-PeCDF was a result of a chlorine removal from a lateral position of 1,2,3,4,7,8-HxCDF, which followed the expected pattern. However, the second dechlorination, 1,2,4,8-TeCDF formation by

removal of a chlorine from a lateral position of 1,2,4,7,8-PeCDF, instead of a peri position, was not expected.

In cultures spiked with 1,3,4,7,8-PeCDF, 1,3,7,8-TeCDF was formed as a dechlorination product in both sets of experiments. The cultures spiked with 1,2,4,7,8-PeCDF formed a trace of 1,2,4,8-TeCDF in the second experiment only, also confirming the second dechlorination route of 1,2,3,4,7,8-HxCDF to 1,2,4,7,8-PeCDF to 1,2,4,8-TeCF. The 1,2,4,7-TeCDF, which had a retention time identical to 1,3,7,8-TeCDF was not detected as a metabolite from 1,2,4,7,8-PeCDF, excluding it as a TeCDF daughter product in the 1,2,3,4,7,8-HxCDF spiked cultures.

We detected no dechlorination products from OCDD in any active treatments or in the killed controls over 195 days. In all experiments where they were added, PCE was dechlorinated to primarily ethene and 1,2,3,4-TeCB was dechlorinated to a mixture of tri- and dichlorobenzene (data not shown).

3.2 Dechlorination and Effects of Additional Substrates

Dechlorination of 1,2,3,4,7,8-HxCDF occurred in all live treatments, regardless of amendments of additional substrates (Figure 3-2). No dechlorination daughter products were detected in killed controls (data not shown). However, the extent of dechlorination varied in different treatments. At day 38, a PeCDF peak was observed in all live treatments spiked with 1,2,3,4,7,8-HxCDF. At day 70, in 1,2,3,4-TeCB-amended cultures, the two tetrachlorodibenzofuran (TeCDF) peaks were observed. At the end of incubation,

the treatment amended with 1,2,3,4-TeCB contained (Figure 3-2C): 57.6 ± 1.5 mol % parent compound 1,2,3,4,7,8-HxCDF; 32.2 ± 0.7 mol % PeCDF intermediates; and 5.8 ± 0.4 mol % 1,3,7,8-TeCDF and 4.4 ± 0.4 mol % 1,2,4,8-TeCDF, the final products. While dechlorination occurred in the other two active treatments, their dechlorination proceeded less extensively than the treatment with 1,2,3,4-TeCB addition. In the bottles spiked only with 1,2,3,4,7,8-HxCDF (Figure 3-2A), the mole % of PCDF congeners at day 195 were 87.2 ± 0.7%, 12.1 ± 0.4%, and 0.3 ± 0.2% and 0.3 ± 0.1%, for 1,2,3,4,7,8-HxCDF, the PeCDF congeners and the two TeCDF congeners, respectively. Similarly, the final mole % of PCDFs in the treatment amended with PCE (Figure 3-2B) were 84.1 ± 4.4%, 15.5 ± 4.2%, 0.3 ± 0.1%, and 0.2 ± 0.0% for the parent compound, intermediates and final products.

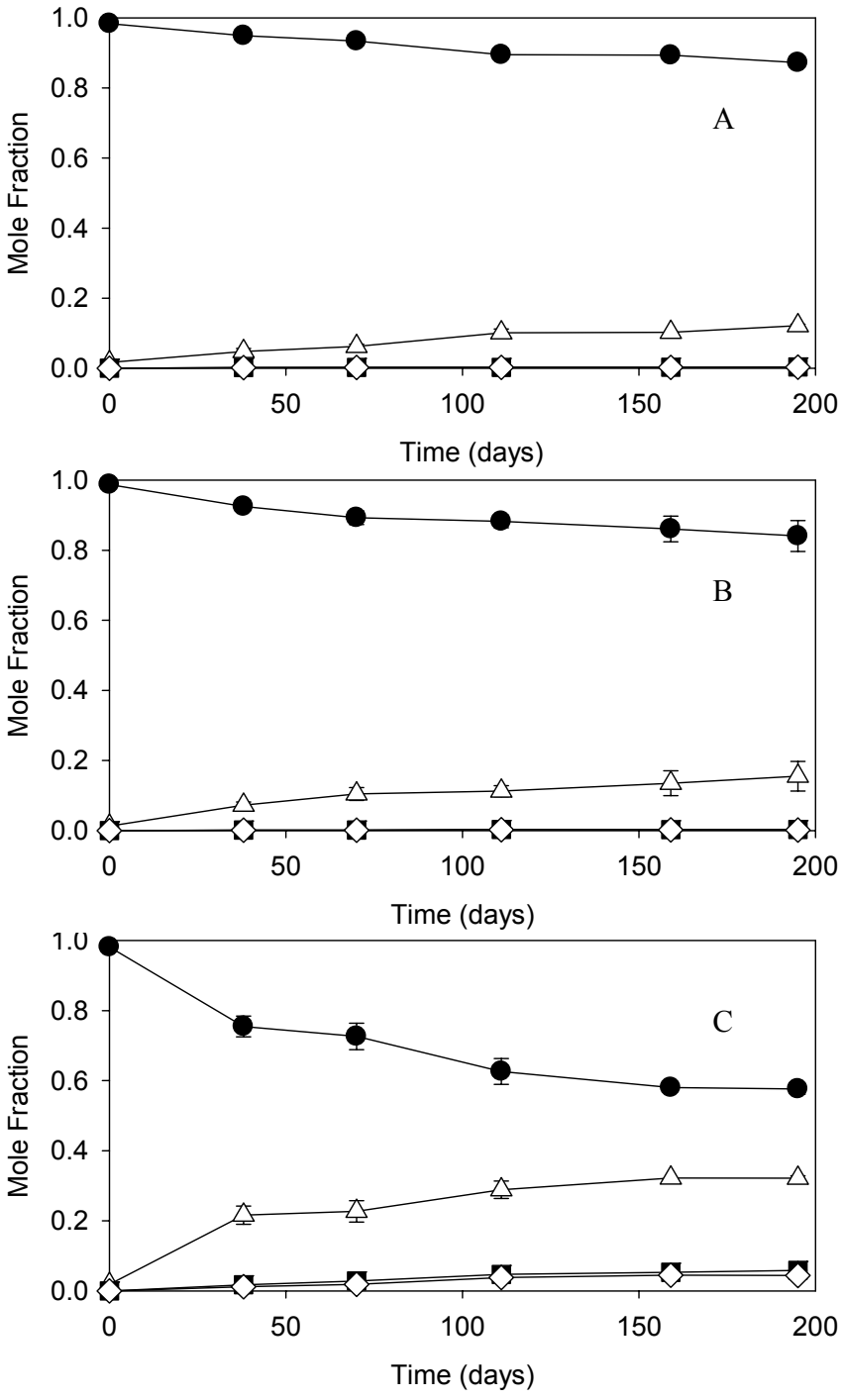

1.0
0.8
0.6
0.4
0.2
0.0
Mole Fraction
A
0
50
100
150
200
Time (days)
1.0
0.8
0.6
0.4
0.2
0.0
Mole Fraction
B
0
50
100
150
200
Time (days)
1.0
0.8
0.6
0.4
0.2
0.0
Mole Fraction
C
0
50
100
150
200
Time (days)

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

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

4. Discussion

4.1 Possible Mechanisms for the Effects of Additional Halogenated Substrates

The 1,2,3,4-TeCB stimulated the most extensive dechlorination of 1,2,3,4,7,8-HxCDF to 1,2,4,7,8-/1,3,4,7,8-PeCDF with formation of TeCDF products. In the other two

treatments, dechlorination of 1,2,3,4,7,8-HxCDF to PeCDF(s) was less extensive and the formation of TeCDFs was negligible. Although both PCE and 1,2,3,4-TeCB are growth substrates for *D. ethenogenes* strain 195 (Fennell et al. 2004; Maymó-Gatell et al. 1997), 1,2,3,4-TeCB stimulated more dechlorination of 1,2,3,4,7,8-HxCDF than PCE, even though it was amended less often than PCE and presumably supported less overall growth. This could mean that the 1,2,3,4-TeCB may have a greater role than just as a growth substrate. For example, it may induce expression of a dehalogenase with a higher affinity for PCDFs. The complete genome sequence of *D. ethenogenes* strain 195 has 17 coding sequences that encode putative reductive dehalogenases (Seshadri et al. 2005), implying a diverse dehalogenation ability. A broad spectrum of dechlorination processes for strain 195 has been confirmed experimentally (Maymó-Gatell et al. 1997, 1999; Fennell et al. 2004; He et al. 2006; Adrian et al. 2007).

The 1,2,3,4-TeCB has been shown to enhance dechlorination of PCDD/Fs in sediments (Ahn et al. 2005) and could perhaps induce a reductive dehalogenase which is able to dechlorinate both 1,2,3,4-TeCB and 1,2,3,4,7,8-HxCDF. In contrast, while the dechlorination of PCE supplies energy for the growth of strain 195, it may not induce the enzyme instrumental in the dechlorination of 1,2,3,4,7,8-HxCDF. It is also possible that PCE suppressed the enzyme needed for dechlorination of 1,2,3,4,7,8-HxCDF, or that PCE competed as a substrate for enzyme or reducing equivalents since it was added more frequently than 1,2,3,4-TeCB, albeit with an electron donor. Two membrane-bound reductive dehalogenases (RDs) were responsible for the dechlorination of PCE to ethene (Magnuson et al. 1998), PCE–RD (PceA) and TCE–RD (TceA). Rahm et al. (2006) found that genes encoding four reductive dehalogenases, *tceA*, DET0162, DET0318

(*pceA*), and DET1559, in strain 195 had high expression in the mixed culture containing strain 195 (the original source of the culture we used) fed with PCE and butyrate. Fung et al. observed that when strain 195 was grown with 2,3-dichlorophenol, the genes *pceA* and DET0162 had high transcript levels and proteomic analysis detected PceA with high peptide coverage (Fung et al. 2007). Since we do not know which enzyme(s) mediates PCDD/F dechlorination it is not possible to determine the importance of additional halogenated substrates on regulation of 1,2,3,4,7,8-HxCDF dechlorination. However, the amendment with 1,2,3,4-TeCB enhanced the extent of dechlorination of 1,2,3,4,7,8-HxCDF approximately three-fold, compared to when 1,2,3,4,7,8-HxCDF was added alone, which may further support the importance of alternate halogenated electron acceptors in the biotransformation of PCDD/Fs (Ahn et al. 2005).

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

4.2 Environmental Relevance

The dechlorination of 1,2,3,4,7,8-HxCDF, an environmentally relevant PCDF congener was demonstrated by a mixed culture containing *D. ethenogenes* strain 195. We confirmed that one route of dechlorination of 1,2,3,4,7,8-HxCDF was through 1,3,4,7,8-PeCDF to 1,3,7,8-TeCDF and the other route was through 1,2,4,7,8-PeCDF to 1,2,4,8-

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

Dechlorination does not always achieve the goal of detoxification. In early studies on microbial dechlorination of PCE and TCE, the production of VC, a potent human carcinogen was recognized as a potential limitation for remediation. In a critical study, Freedman and Gossett (1989) demonstrated dechlorination completely to ethene, a benign product. Dehalogenation resulting in increased system toxicity was also observed for PBDEs when an octa-BDE mixture was dechlorinated to more toxic hexa-, penta- and tetra-BDEs by *Dehalococcoides*-containing cultures (He et al. 2006). Similarly, Adriaens and Grbić-Galić (1994) found that 1,2,3,4,6,7,8-HeptaCDF was dechlorinated to 1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF by a peri chlorine removal in aquifer microcosms. This resulted in increased toxicity because the produced HxCDFs both have TEFs of 0.1, ten-fold higher than that of the parent compound. *Dehalococcoides* sp. strain CBDB1 dechlorinated 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD) with a TEF of 1, slowly (2.8 mol % within 104 days) to mainly 2,3,7,8-TeCDD, also with a TEF of 1, and small amounts of 1,3,7,8-TeCDD (Bunge et al. 2003). Strain CBDB1 further

detoxified the 2,3,7,8-TeCDD intermediate, forming 2,3,7-TrCDD, 2,7-DCDD and/or 2,8-DCDD. This pattern of dechlorination was similar to what had been demonstrated by strain CBDB1 with 1,2,3,4-TeCDD. A pure culture of *D. ethenogenes* strain 195 did not dechlorinate 2,3,7,8-TeCDD after 249 days (Fennell et al. 2004).

Considering the generally slow rates of dechlorination of PCDD/Fs by dechlorinating bacteria (Adriaens et al. 1994, 1995; Beurskens et al. 1995; Barkovskii & Adriaens 1996; Ballerstedt et al. 1997; Bunge et al. 2003; Fennell et al. 2004; this study), transient intermediates might exist in the environment for years and have great environmental impact. The formation of equally or more toxic intermediates will not reduce the threat to the environment and biota. The TEF values of the seventeen 2,3,7,8-substituted PCDD/Fs normally increase with decreasing number of chlorines on the carbon backbone. Dechlorination from a peri position mostly increases the toxicity in the system. On the contrary, dechlorination from a lateral position greatly reduces the toxicity of the 2,3,7,8-substituted congeners. The TEF of 1,2,3,4,7,8-HxCDF is 0.1, while the TEFs of potential 2,3,7,8-substituted products 2,3,4,7,8-PeCDF, 1,2,3,7,8-PeCDF and 2,3,7,8-TeCDF are 0.3, 0.03 and 0.1, respectively. Dechlorination of 1,2,3,4,7,8-HxCDF to 2,3,4,7,8-PeCDF would be a detrimental rather than favorable process and the system TEQ would increase. If 1,2,3,7,8-PeCDF or 2,3,7,8-TeCDF, were formed from dechlorination, the TEQ would decrease or remain the same. We have demonstrated detoxification during the dechlorination of an environmentally relevant 2,3,7,8-substituted PCDF congener by a mixed culture containing *D. ethenogenes* strain 195, and an additional halogenated substrate, 1,2,3,4-TeCB, enhanced this process. Here, TEQ in 1,2,3,4,7,8-HxCDF spiked systems decreased from 187 μg/L at time 0 to 108 μg/L, 157 μg/L, and 163 μg/L in the

treatments amended with 1,2,3,4-TeCB, PCE, and no additional halogenated substrate, respectively. *Dehalococcoides* spp. and closely related *Chloroflexi* have been implicated in PCDD/F dechlorination in environmental samples (Yoshida et al. 2005; Ahn et al. 2007). Obtaining more information about the bacteria and dehalogenases involved in PCDD/F dechlorination could enable advances in using this process for bioremediation.

Chapter 4

Kinetics of Reductive Dechlorination of Polychlorinated Dibenzo-*p*-dioxins and Dibenzofurans by a *Dehalococcoides*-Containing Culture

1. Introduction

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) are hydrophobic, insoluble compounds that are associated with the organic fractions of soils and sediments. Despite their hydrophobic nature, they are subject to a variety of processes such as equilibrium partitioning between aqueous, particle and gas phase; particle borne transport; photodegradation; and conversion through hydroxyl radical reactions (Hites 1990). The PCDD/Fs are also subject to biologically linked transformation reactions. Dechlorination of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) by microorganisms and biogenic materials has been demonstrated under both aerobic and anaerobic conditions (Wittich 1998; Adriaens & Grbić-Galić 1995; Beurskens et al. 1995; Barkovskii & Adriaens 1996; Ballerstedt et al. 1997; Albrecht et al. 1999; Fu et al. 1999; Yoshida et al. 2005; Vargas et al. 2001; Ahn et al. 1005). These transformation processes may represent an important factor in the fate of PCDD/Fs in the environment. Barabás et al. (Barabás et al. 2004[a], 2004[b]) used geostatistical analysis of historical contamination

and detected a reductive dechlorination fingerprint showing formation of 2,3,7,8-TCDD at the expense of 1,2,3,7,8-PeCDD in Passaic River, New Jersey, USA sediments. The reductive dechlorination of heavily chlorinated PCDD/Fs under anaerobic conditions followed by aerobic oxidation of the resulting lightly chlorinated congeners could transform the chlorinated PCDD/Fs to non chlorinated end products (Fu et al. 2001).

Microorganisms enriched or eluted from sediment, soil and sludge have been shown to dechlorinate a suite of PCDD/F congeners (Bunge et al. 2003, Fennell et al. 2004, Liu & Fennell 2007). One bacterial species, *Dehalococcoides* sp. strain CBDB1 grows using a PCDD as an electron acceptor (Bunge et al. 2003). Investigation of microbial biotransformation of PCDD/Fs provides information needed for attempts to enhance bioremediation in contaminated environmental matrices. Although we know transformation occurs, more information on the theoretical and practical aspects of the process is needed to answer fundamental questions pertinent to bioremediation feasibility studies and operational strategies. For example, integration of information such as identification of functioning indigenous and exogenous microorganisms, understanding the effectiveness of amendments to enhance biotransformation activities, development of methods to monitor the effectiveness of amendment implementation and resulting microbial activities, and establishing model frameworks to predict the fate and transport of PCDD/Fs *in situ* are needed for successful employment of bioremediation.

One limitation to a bioremediation approach is the lack of information on the dechlorination kinetics of PCDD/Fs. Although several studies on the microbial reductive dechlorination of PCDD/Fs have been reported (Beurskens et al. 1995; Ballerstedt et al. 1997; Bunge et al. 2003; Fennell et al. 2004), because of the difficulty and expense of

sample preparation and analyses, dechlorination rates have rarely been estimated. Published dechlorination rates ranged over several orders of magnitude. The transformation rate of spiked 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) by a mixed culture enriched from Saale River, Germany sediment was 60 nM/d (Ballerstedt et al. 1997), while dechlorination rates of octachlorodibenzo-*p*-dioxin (OCDD) in spiked and historical Passaic River matrices ranged from 0.007-0.075 nM/d (Albrecht et al. 1999). Since the concentrations of the responsible microorganisms were not known in those studies, the rates were not linked with biomass concentrations. The dechlorination rates of 1,2,3,4,6,7,9-heptaCDD were 0.01-0.17 nM/d and 0.004-0.014 nM/d by Aldrich humic acid and polymaleic acid, respectively (Fu et al. 1999). The discrepancy in rates may result from different experimental conditions, such as the physical-chemical properties of the PCDD/F congeners investigated, the properties of the matrices in which the PCDD/Fs were studied, the affinity of the microorganisms or enzymes that mediate the dechlorination reactions for PCDD/Fs, effects of the amendments applied to enhance dechlorination, biotransformation mechanisms, or other factors. For example, Ahn et al. (2005) reported half-lives for 1,2,3,4-TeCDD/F of 3 to 4 months when sediments were amended with additional aromatic chlorinated compounds, but in excess of 12 months under unenhanced conditions. Since factors affecting one specific dechlorination process may vary significantly, it is useful to obtained kinetic parameters for each specific system in order to simulate and predict the fate of PCDD/Fs within that system.

Previous studies have reported the ability of *Dehalococcoides ethenogenes* strain 195 to dechlorinate a variety of halogenated compounds (Fennell et al. 2004; Liu & Fennell 2007; Maymó-Gatell et al. 1997; Adrian et al. 2007; He et al. 2006), including

chloroethenes, selected chlorinated benzenes, polychlorinated biphenyls, chlorophenols, polybrominated diphenyl ethers, chlorinated naphthalenes and PCDD/Fs. However, dechlorination kinetics has not yet been examined. In this study, we report results from a new study of the dechlorination kinetics of 1,2,3,4-TeCDD by a mixed culture containing *Dehalococcoides ethenogenes* strain 195. *D. ethenogenes* strain 195 dechlorinates 1,2,3,4-TeCDD to 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) then to 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) in pure culture and in the mixed culture. We investigated the kinetics of 1,2,3,4-TeCDD dechlorination in medium-sediment mixtures amended with 1,2,3,4-TeCDD concentrations over several orders of magnitude. We also performed kinetic analysis of data from our previous studies of dechlorination of PCDD/Fs under different conditions (Fennell et al. 2004; Liu & Fennell 2007; Liu et al. Isotope manuscript [Chapter 5]). Dechlorination kinetic parameters obtained from controlled studies could be used in predictive modeling to help predict bioremediation outcomes and timeframes.

2. Materials and Methods

We previously reported dechlorination of 1,2,3,4-TeCDD and 1,2,3,4-TeCDF by pure culture *D. ethenogenes* strain 195 (Fennell et al. 2004) and dechlorination of 1,2,3,4-TeCDD (Fennell et al. 2004; Liu et al. Isotope manuscript [Chapter 5]) and 1,2,3,4,7,8-HxCDF (Liu & Fennell 2007) by a mixed culture containing *D. ethenogenes* strain 195. Complete methods for those studies were already reported in detail (Fennell et al. 2004;

Liu & Fennell 2007; Liu et al. Isotope manuscript [Chapter 5]). Here we describe the experimental methods for a study of 1,2,3,4-TeCDD at different initial concentrations.

2.1 Chemicals and Stock Solutions

The 1,2,3,4-TeCDD, 1,2,4-TrCDD, 1,3-DCDD, and 2,2',5-trichlorobiphenyl (2,2',5-TrCB) were purchased from AccuStandard (New Haven, CT). Tetrachloroethene (PCE) (99.9+%), trichloroethene (TCE) (99.5+%), and butyric acid (99+%) were obtained from Aldrich Chemical Company (Milwaukee, WI). The *cis*-1,2,-dichloroethene (*cis*-1,2-DCE) was purchased from Supelco, Bellefonte, PA. Vinyl chloride (VC) (99.5+%) was obtained from Fluka Chemie GmbH, Germany. Ethene and methane were purchased from Matheson Tri-Gas, Inc. The 1,2,3,4-TeCDD stock solutions were prepared at 6.21 mM (2000 mg/L) and 0.31 mM (100 mg/L) in toluene to allow precise amendment to cultures at the high and low concentration levels. A 114 mM butyric acid solution was made by dissolving neat butyric acid in deionized water.

2.2 Culture Maintenance

The source culture containing *D. ethenogenes* strain 195 was grown at 25°C on PCE and butyric acid using methods described previously (Fennell et al. 2004; Fennell 1998; Fennell & Gossett 1997). PCE and butyric acid were fed regularly to the semi-continuous flow reactor at concentrations of 110 μM and 440 μM, respectively. The ratio of equivalents of electron donor to electron acceptor was 2:1 (Fennell 1998; Fennell &

Gossett 1997). The source culture was operated at a nominal hydraulic retention time of 56 days.

2.3 Quantification of *D. ethenogenes* strain 195. [Analyses described in this section were performed by my colleagues Ms. Eun-Kyeu Son and Dr. Valdis Krumins of Rutgers University, Department of Environmental Sciences. Ms Son developed the method and procedures and performed some of the analyses; and Dr. Krumins performed some of the analyses].

Quantification of *D. ethenogenes* strain 195 in the mixed culture was performed by quantitative polymerase chain reaction (qPCR) analysis. A standard curve was obtained using 10-fold serial dilutions of plasmid DNA carrying a cloned 16S rRNA gene of *D. ethenogenes* strain 195. Sample DNA and 10-fold serially diluted 16S rRNA gene standards were amplified in parallel on an iQ5 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Each 25 μL of reaction volume contained 1 μL of sample or standard DNA, 1X iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 0.2 μM (each) *Dehalococcoides*-specific16S rRNA gene 1F forward (5'-GATGAACGCTAGCGGCG-3') and 259R reverse (5'-CAGACCAGCTACCGATCGAA-3') primers (Hendrickson et al. 2002; Duhamel et al. 2004). Thermocycling conditions were as follows: 5 min at 95 °C and 40 cycles of 30 sec at 95 °C, 30 sec at 55 °C and 30 sec at 72 °C.

2.4 Dechlorination of 1,2,3,4-TeCDD at Different Concentrations

For the current study we examined dechlorination of 1,2,3,4-TeCDD by the mixed culture over a wide range of initial concentrations. Experiments were carried out in 28 mL anaerobic culture tubes (18 × 150 mm, Bellco Glass, Inc. Vineland, NJ). Tubes were amended with 1,2,3,4-TeCDD at four different concentrations, 62 μM (20 ppm), 31 μM (10 ppm), 3.1 μM (1 ppm) and 0.31 μM (0.1 ppm), respectively. The procedure was as follows: 0.1 g dry sediment prepared as described previously (Liu & Fennell 2007), was weighed and added to each tube. The required volume of 1,2,3,4-TeCDD stock solution was dispensed to each tube to wet the dry sediment. The toluene was allowed to volatilize under a sterile N_2 purge, leaving behind a coating of 1,2,3,4-TeCDD on the sediment carrier. A 10 mL volume of culture was transferred to each tube under anoxic and sterile conditions resulting in a final nominal 1,2,3,4-TeCDD concentration at designated the concentration levels. The headspace was flushed with anoxic N_2/CO_2 (70%/30%). Each tube also received 63 μL 114 mM butyric acid as an electron donor and hydrogen source to achieve a final concentration of 688 μM, 4 μL of a 50 g/L fermented yeast extract solution and 50 μL vitamin solution (Fennell 1998) as nutrient sources. At each concentration level, three active cultures and two killed controls were prepared and sacrificed for analysis at each sampling time point. The control cultures were autoclaved for one hour on each of three consecutive days. The above treatments received only 1,2,3,4-TeCDD as the halogenated substrate. Besides the 1,2,3,4-TeCDD only treatments,

we set up two parallel treatments. PCE was spiked to serve as the sole halogenated substrate in one treatment while PCE and 1,2,3,4-TeCDD were both added in another treatment. The experimental protocol is shown in Table 4-1.

Table 4-1. Experimental protocols for kinetic studies of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) with and without addition of tetrachloroethene (PCE).

Tube set	Electron donor (Butyric acid)	Halogenated substrates	Electron Donor[a] (μequiv/tube)	Halogenated Compounds[b] (μequiv/tube)	Electron donor /Halogenated substrates (equiv/equiv)
1	688 μM	1,2,3,4-TeCDD at 62 μM	2752	496	5.5 :1
2	688 μM	1,2,3,4-TeCDD at 31 μM	2752	248	11:1
3	688 μM	1,2,3,4-TeCDD at 3.1 μM	2752	49.6	55.5:1
4	688 μM	1,2,3,4-TeCDD at 0.31 μM	2752	4.96	555:1
5	688 μM	PCE at 110 μM	2752	880	3:1
6	688 μM	PCE at 110 μM plus 1,2,3,4-TeCDD at 62 μM	2752	1376	2:1

[a]hydrogen equivalent basis (4 eq/mol)

[b]one equivalent per chlorine

2.5 Analytical Methods

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

The culture tubes were completely sacrificed at each time point for extraction. Samples were extracted and prepared for instrumental analysis as described previously (Vargas et al. 2001). After extraction and cleanup, PCDDs were analyzed using an Agilent 6890 gas chromatograph equipped with a 5973N mass selective detector (GC-MS) (Agilent Technologies, Inc. Santa Clara, CA) and a HP-5MS (Agilent Technologies, Inc. Santa Clara, CA, USA) column (60 m × 0.25 mm I.D.). PCDDs were detected based on the retention times of standards and their molecular ions (*m/z*: 322 for 1,2,3,4-TeCDD, 286 for 1,2,4,-TrCDD, 252 for 1,3-DCDD, and 256 for 2,2',5-TrCB). The response factors of each PCDD congener compared to the surrogate, 2,2',5-TrCB, were calculated over five concentration levels. The PCDDs were quantified based on the calculated response factors and presented as averages of duplicate or triplicate data points plus or minus one standard deviation. Results are presented as nominal concentration (μM) based on the total sediment culture slurry or as molar percent of individual congeners' makeup of the total moles of PCDD present. Presentation of PCDDs as mole percent

assumes that no anaerobic degradation of the dibenzo-*p*-dioxin structure occurred, and that the PCDDs underwent no significant reactions other than dechlorination.

The total carbon content of the dry sediment as a carrier for PCDD/Fs was determined on a Shimadzu TOC-V_{CSN} total organic carbon analyzer with a SSM-5000 solid sample analyzer as described by Li et al. (2007).

3. Calculation of Kinetic Parameters

3.1 First Order Reaction Kinetics

Dechlorination experiments were carried out over relatively long incubation periods and for most experiments the hydrogen donor, butyric acid, was added intermittently. Because biotransformation of PCDD/Fs could be limited by the electron acceptor (PCDD/F) concentration and electron donor (hydrogen) concentration, the dechlorination of PCDD/Fs might best be described as pseudo-first order. The first order reaction model for PCDD/F dechlorination can be written as

$$-\frac{dC_{PCDD/F}}{dt} = k_1 C_{PCDD/F} \quad (1)$$

and the concentration of the parent PCDD/F along the progress curve can be found through the integrated form of equation 1

$$C_{PCDD/F,t} = C_{PCDD/F,0} e^{-k_1 t} \quad (2)$$

where, k_1 is the pseudo-first order reaction rate constant for the dechlorination of the PCDD/Fs and $C_{PCDD/F,t}$ and $C_{PCDD/F,0}$ are the concentrations of PCDD/Fs at time t and

time 0. The values of k_1 were determined from the slope of the logarithmic form of equation 2:

$$-k_1 t = \ln\left(\frac{C_{PCDD/F,t}}{C_{PCDD/F,0}}\right) \quad (3)$$

The corresponding half life, $t_{½}$, was calculated as

$$t_{1/2} = ln2/k_1 \quad (4)$$

Measured PCDD/F concentrations were used for the calculations with the exception of the 3.1 μM tube study, for which we used the molar fraction as a surrogate for $C_{PCDD/F,t}$ to $C_{PCDD/F,0}$ ratio. In that set of cultures, the measured concentration of the parent compound did not exhibit a measurable decrease, although dechlorination products were formed.

3.2 Monod Type Kinetics

Dechlorination kinetics has also been modeled using a double Monod kinetic expression which considers not only the concentration of the halogenated compounds but also that of the electron donors whose availability may limit dechlorination rates (Fennell & Gossett 1998). The equation to describe the dechlorination of PCDD/Fs may be written as (Fennell & Gossett 1998):

$$\frac{dC_{PCDD/F}}{dt} = \frac{-k_{PCDD/F} X_{dechlor} C_{PCDD/F}}{K_{s(PCDD/F)} + C_{PCDD/F}} \times \frac{C_{w(H_2)}}{K_{S(H_2)} + C_{W(H_2)}} \quad (5)$$

where $k_{PCDD/F}$ is the maximum specific substrate utilization rate (μmol/cell·day); $X_{dechlor}$ is the concentration of the biomass of the dechlorinator (cells/L); $K_{S(PCDD/F)}$ is the half-velocity coefficient for PCDD/F transformation (μmol/L); $K_{S(H2)}$ is the half-velocity coefficient for H_2 use; and $C_{w(H2)}$ is the aqueous concentration of H_2.

Equation 5 incorporates the influence of the concentration of PCDD/F and H_2, and the dechlorinator cell concentration on the dechlorination kinetics of the PCDD/Fs. In our study, the equivalent amount of electron donor added to the culture was much greater than the amount of the equivalent of halogenated substrates (Table 4-1). However, it is important to consider the effect of electron donor limitation since the butyrate was added as multiple pulse additions over a long incubation period. The $K_{S(H2)}$ for the mixed culture was estimated to be 0.1 μmol/L (Fennell & Gossett 1998; Smatlak & Gossett 1996). Previous studies with the mixed culture indicated that the addition of butyrate to the culture at a concentration of 440 μM supplied hydrogen in excess of 10^{-4} atm (0.078 μmol/L, aqueous concentration) over a period of at least 12 hours while butyrate persisted (Fennell & Gossett 1997, 1998). Thereafter the hydrogen concentration decreased to about 10^{-5} atm (0.0078 μmol/L, aqueous concentration) where it remained stable. Thus, in our cultures, the concentration of H_2 could realistically limit kinetics of dechlorination of the PCDD/Fs. Monod parameters obtained by analysis of our data could be corrected by solving for the expected range of the value of the expression [$C_{w(H2)}$ / ($K_{S(H2)}$+$C_{w(H2)}$)] under conditions immediately following butyrate addition ([$C_{w(H2)}$ / ($K_{S(H2)}$+$C_{w(H2)}$)] = 0.44) and those after butyrate was depleted ([$C_{w(H2)}$ / ($K_{S(H2)}$+$C_{w(H2)}$)] = 0.07). If we neglect this factor for the initial analyses, the concentration of the PCDD/Fs

and the kinetic parameters $k_{PCDD/F}$ and $K_{S(PCDD/F)}$ can be determined from a simplification of Equation 5:

$$\frac{dC_{PCDD/F}}{dt} = \frac{-k_{PCDD/F}X_{dechlor}C_{PCDD/F}}{K_{s(PCDD/F)}+C_{PCDD/F}} \quad (6)$$

$X_{dechlor}$ is the biomass concentration of *D. ethenogenes* strain 195. The mixed culture contained 2 x 10^8 16S rRNA *Dehalococcoides* gene copies per mL of mixed culture, or 2 x 10^8 *Dehalococcoides* cells per mL, assuming one 16S rRNA gene per cell (Seshadri et al. 2005). We do not know if the dechlorination of PCDD/Fs by strain 195 is an energy yielding process or co-metabolic. If it is a co-metabolic process, the concentration of $X_{dechlor}$ would change only through decay processes. If the PCDD/Fs are growth substrates for strain 195, the yield could be assumed to be similar for growth on PCE. Maymó-Gatell et al. (1997) measured a protein yield for strain 195 during the dechlorination of PCE to be 4.8 ± 0.3 g of protein per mole of chloride released. An assessment in terms of cell number yield was about 1.6 × 10^{14} cells per mole chloride released. This PCE yield is similar to that reported by Bedard et al. (2007) for *Dehalococcoides* JN culture growing on PCBs. The yield measured by qPCR was 9.25 x 10^{14} ± 0.04 x 10^{14} *Dehalococcoides* cells per mol of chlorine removed. In a recent study on the dechlorination of trichlorinated dibenzo-*p*-dioxins, the yield of *Dehalococcoides* species, for growth on 1,2,4-TrCDD and 1,2,3-TrCDD were estimated as 2.8 x 10^8 ± 0.6 x 10^8 and 2.5 x 10^8 ± 0.7 x 10^8 copies per µmol chloride (Ewald et al. 2007), close to the estimation obtained for strain 195 growth on PCE. 1,2,3,4-TeCDD was dechlorinated to 1,2,4-TrCDD and further to 1,3-DCDD by strain 195, however, when tested alone, 1,2,4-TrCDD was not dechlorinated by strain 195 (data not shown). It is therefore reasonable to believe that 1,2,4-TrCDD was not a growth substrate for strain 195. The dechlorination of

1,2,3,4-TeCDD at an initial concentration of 62 μM proceeded to a removal rate of 32% at the end of incubation. The calculated chloride release was therefore 0.2 μM. If we assume a yield of 1.6×10^{14} cells per mole chloride released for dechlorination of PCDD/Fs by strain 195 and we assume that the dechlorination of 1,2,3,4-TeCDD provided energy for the growth of strain 195, the estimated increase in cell number would be 0.32×10^8 cells. The cell number in the mixed culture by qPCR was 2×10^8 cells/mL. Therefore, the total cell increase during the dechlorination of 1,2,3,4-TeCDD would not exceed 2% of the biomass present at the estimated initial concentration level of 62 μM 1,2,3,4-TeCDD. Similarly the estimation of cell increases at the 1,2,3,4-TeCDD concentrations of 31 μM, 3.1 μM, and 0.31 μM would be about 0.5%, 0.06% and 0.001% of the biomass present, respectively. The cellular increase for 5 μM 1,2,3,4,7,8-HxCDF (neglecting growth on intermediates) would likewise be about 0.17% of the biomass present, or less. These calculations indicate that the possible growth resulting from the dechlorination of PCDD/Fs is negligible. In summary, whether the PCDD/F congeners added are growth substrates or not, $X_{dechlor}$ would vary mainly according to the decrease caused by decay processes during the dechlorination of the PCDD/F. For the short term experiments involving PCDD/F congeners, we therefore assumed $X_{dechlor}$ to be a constant in equation 6.

A linearized form of equation 6 may be obtained by rearranging the equation as

$$\frac{dt}{dC_{PCDD/F}} = -\frac{K_{S(PCDD/F)}}{k_{PCDD/F}X_{dechlor}} \cdot \frac{1}{C_{PCDD/F}} - \frac{1}{k_{PCDD/F}X_{dechlor}} \qquad (7)$$

The kinetic parameters $K_{S(PCDD/F)}$ and $k_{PCDD/F}$ may then be estimated by plotting $dt/dC_{PCDD/F}$ over $1/C_{PCDD/F}$. The ratio of the slope/intercept of the resulting linear

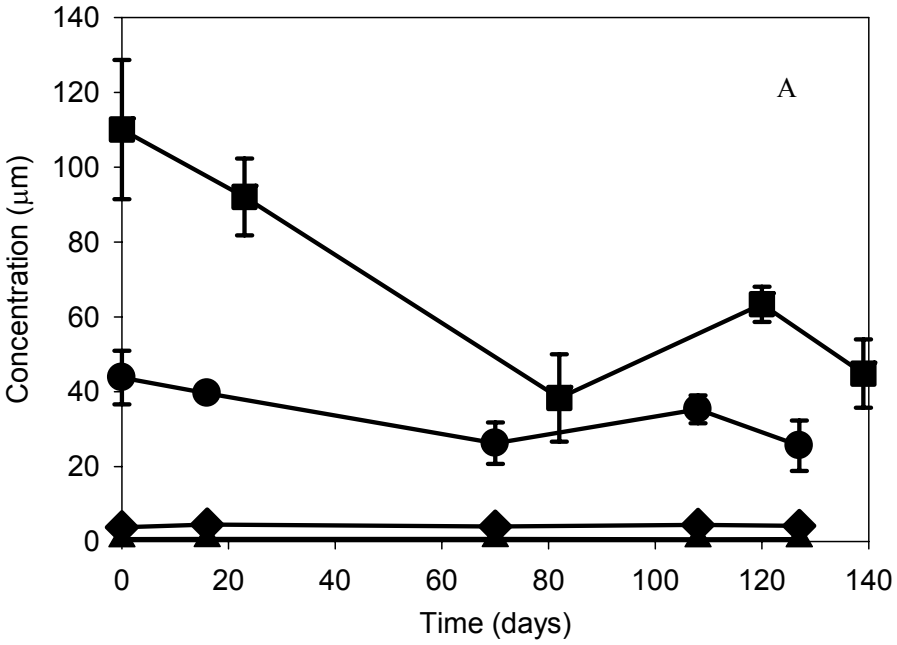
regression line is $K_{S(PCDD/F)}$. The value of $k_{PCDD/F}$ could be estimated from the intercept of the line, knowing the concentration of biomass $X_{dechlor}$.

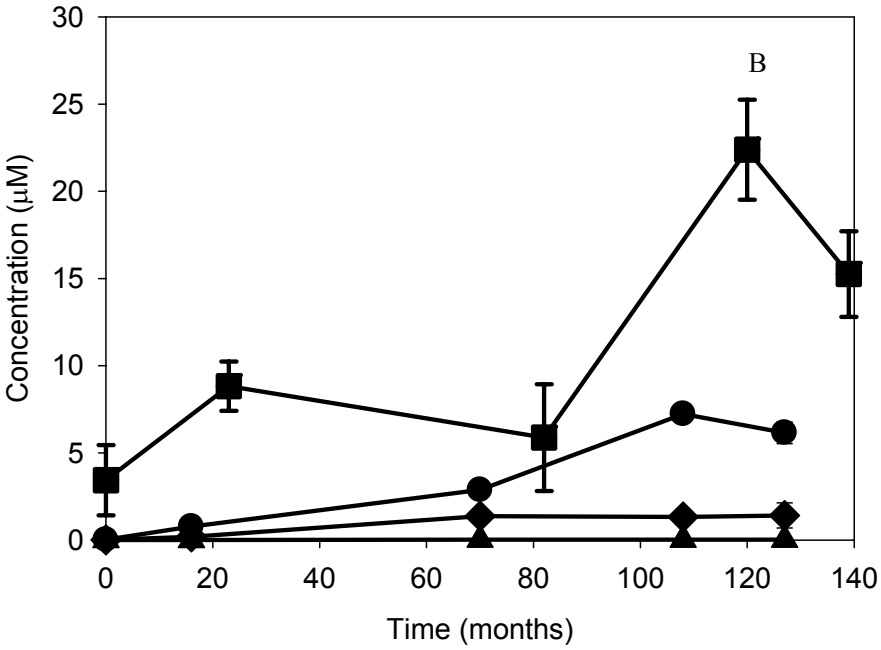
4. Results

4.1 Dechlorination of 1,2,3,4-TeCDD at Different Concentrations

Dechlorination of 1,2,3,4-TeCDD occurred in all active tubes spiked with 1,2,3,4-TeCDD at all concentration levels (Figure 4-1A). There was no lag period prior to dechlorination of 1,2,3,4-TeCDD. As shown previously (Fennell et al. 2004), 1,2,3,4-TeCDD was dechlorinated to 1,2,4-TrCDD, further to 1,3-DCDD (Figure 4-1B, C). The 1,2,4-TrCDD and 1,3-DCDD dechlorination products were observed at all concentration levels except at 0.31 μM, where dechlorination of 1,2,3,4-TeCDD only proceeded to 1,2,4-TrCDD. The concentrations of 1,2,3,4-TeCDD, 1,2,4-TrCDD and 1,3-DCDD and their mole fractions at the end of the experiment are listed in Table 4-2. At the initial 1,2,3,4-TeCDD concentration of 62 μM, the mole percents of PCDDs in the system were 67.6 ± 3.0% for 1,2,3,4-TeCDD, 23.1 ± 0.3% for 1,2,4-TrCDD and 9.3 ± 2.6% for 1,3-DCDD at the end of the incubation. Similarly, the mole percents were 79.2 ± 3.7%, 19.5 ± 3.1%, and 1.3 ± 0.7% at the initial 1,2,3,4-TeCDD concentration of 31 μM and 75.3 ± 8.2%, 24.2 ± 8.0%, and 0.3 ± 0.2% at 3.1 μM for the substrate, intermediate and product. However, the results at the initial 1,2,3,4-TeCDD concentration of 0.31 μM exhibited a different pattern: only 3.9 ± 0.8% of 1,2,3,4-TeCDD was transformed to 1,2,4-TrCDD. No further dechlorination was detected. At the end of the incubation period, the three

PCDDs possessed more or less similar mole fractions at all concentration levels except 0.31 μM. This suggests that the dechlorination reaction was a first order reaction.





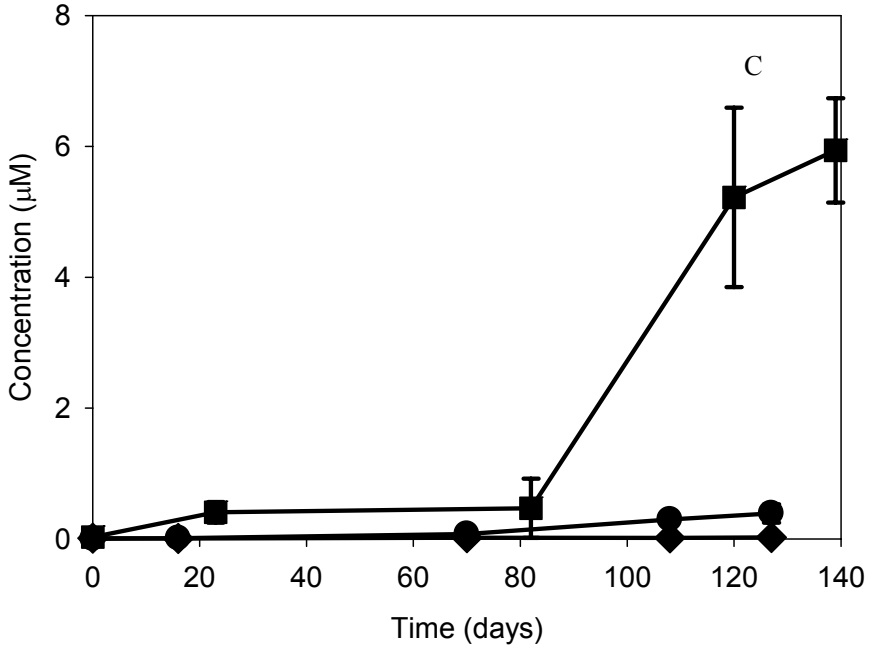


Figure 4-1. Dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (A) and formation of 1,2,4-TrCDD (B) and 1,3-DCDD (C) at initial 1,2,3,4-TeCDD concentrations of 62 μM (■), 31 μM (●), 3.1 μM (♦), and 0.31 μM (▲).

Table 4-2. Dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) to the daughter products 1,2,4-trichlorodibenzo-*p*-dioixn (1,2,4-TrCDD), and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) at different initial 1,2,3,4-TeCDD concentrations.

Initial 1,2,3,4-TeCDD Conc. (μM)	**1,2,3,4-TeCDD**	**1,2,4-TrCDD**	**1,3-DCDD**	**1,2,3,4-TeCDD**	**1,2,4-TrCDD**	**1,3-DCDD**
	Concentrations (μM)			Mole Percents (%)		
62 μM	44.83±9.15	15.26±2.46	5.94±0.80	67.6 ± 3.0	23.1 ± 0.3	9.3 ± 2.6
31 μM	25.57±6.74	6.15±0.62	0.39±0.14	79.2 ± 3.7	19.5 ± 3.1	1.3 ± 0.7
3.1 μM	4.16±0.66	1.41±0.72	0.02±0.02	75.3 ± 8.2	24.2 ± 8.0	0.3 ± 0.2
0.31 μM	0.63±0.03	0.03±0.00	N.D.	96.1 ± 0.8	3.9 ± 0.8	N.D.

N.D. = not detected

4.2 Estimation of Pseudo-First Order Reaction Rate Constants

Dechlorination of 1,2,3,4-TeCDD at initial concentrations of 62 μM, 31 μM, and 3.1 μM illustrated characteristics of a first order reaction in that similar dechlorination extent was achieved over a wide range of concentrations. Therefore, the absolute values of the PCDD/F concentrations were plotted based on equation 3. The pseudo-first order reaction rate constants were estimated to be 0.006 ± 0.0026 d^{-1}, 0.0033 ± 0.0016 d^{-1}, 0.0023 ± 0.0006 d^{-1}, and 0.0004 ± 0.0004 d^{-1}, for initial 1,2,3,4-TeCDD concentrations of 62 μM, 31 μM, 3.1 μM, and 0.31 μM, respectively. The respective half lives from the higher initial 1,2,3,4-TeCDD concentration to lower ones were 3.9, 7.0, 10.1, and 57.8 months,

respectively. Results showed that the reaction constants obtained from each concentration level were similar except at 0.31 μM level (Table 4-3). Thus dechlorination of 1,2,3,4-TeCDD seemed to follow first order kinetics over the initial concentration range from 3.1 μM to 62 μM. However, at an initial concentration of 0.31 μM, the extent of dechlorination was much less relative to the results determined from cultures initiated at the higher initial concentrations.

Table 4-3. Pseudo-first order reaction rate constants and half lives of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) at different initial concentrations.

Initial 1,2,3,4-TeCDD Concentration	**1,2,3,4-TeCDD first order rate constant k_1 (day^{-1})**	**Half life (months)**
62 μM	0.006 ± 0.0026	3.9
62 μM (with PCE)	0.0025 ± 0.0017	9.2
31 μM	0.0033 ± 0.0016	7.0
3.1 μM	0.0023 ± 0.0006	10.1
0.31 μM	0.0004 ± 0.0004	57.8

4.3 Dechlorination Kinetic Parameters

The data from the three relatively high initial 1,2,3,4-TeCDD concentrations, 62 μM, 31 μM and 3.1 μM, were used to estimate the dechlorination kinetic parameters $K_{S(TeCDD)}$ and k_{TeCDD}. The dechlorination rate at each concentration level was estimated by the linear regression of the concentration versus time plot, to be -0.43 ± 0.15 μM/d, -0.11 ± 0.05

μM/d, -0.0017 ± 0.0033 μM/d, and -0.0002 ± 0.0002 μM/d for the higher to lower initial concentrations, respectively (Table 4-4). The dechlorination rates were also calculated based on the mole percents and used for the linear regression since this analysis of the results showed more consistency than the absolute dechlorination rates (Table 4-4). According to equation 7, dt/dC_{TeCDD} for each concentration was plotted against the reciprocal of the corresponding initial concentration (Figure 4-2). The half velocity coefficient $K_{S(TeCDD)}$ was estimated from the ratio of the slope to the intercept of the resulting regression line, y = -496.8x – 1.709. The $K_{S(TeCDD)}$ was estimated to be 291 μM. This estimated half velocity coefficient for 1,2,3,4-TeCDD ($K_{S(TeCDD)}$) was far greater than the concentration levels of 1,2,3,4-TeCDD employed in the study. Under this condition, equation 6 could be approximated as

$$\frac{dC_{PCDD/F}}{dt} = \frac{-k_{PCDD/F}X_{dechlor}C_{PCDD/F}}{K_{S(PCDD/F)}} \qquad (8)$$

which resembles the form of a first order reaction. Thus, it may not be surprising to observe a pseudo-first order reaction dechlorination of 1,2,3,4-TeCDD at the concentration levels employed in this study.

Similarly, $k_{TeCDD}{\cdot}X_{dechlor}$ was estimated to be 1/1.709. As the biomass concentration of the dechlorinator *D. ethenogenes* strain 195 has been determined to be 2×10^8 cells/mL and it was considered constant during the dechlorination, k_{TeCDD} was then estimated to be 2.93×10^{-12} μmole/ cell·day.

Table 4-4. Dechlorination kinetic parameters of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) at all concentrations.

Initial 1,2,3,4-TeCDD **Concentration**	dC_{TeCDD}/dt **(μM/day)**[a]	dC_{TeCDD}/dt **(μM/day)**[b]	$K_{S(TeCDD)}$ **(μM)**	k_{TeCDD} **(μmole/cell·day)**
62 μM	-0.43 ± 0.15	-0.1240 ± 0.0149	291	2.93×10^{-12}
31 μM	-0.11 ± 0.05	-0.0513 ± 0.0017		
3.1 μM	-0.0017 ± 0.0033	-0.0062 ± 0.0016		
0.31 μM	-0.0002 ± 0.0002	$-5.9 \times 10^{-5} \pm 1.1 \times 10^{-5}$		

a. Calculation based on absolute concentrations.

b. Calculation based on mole percents.

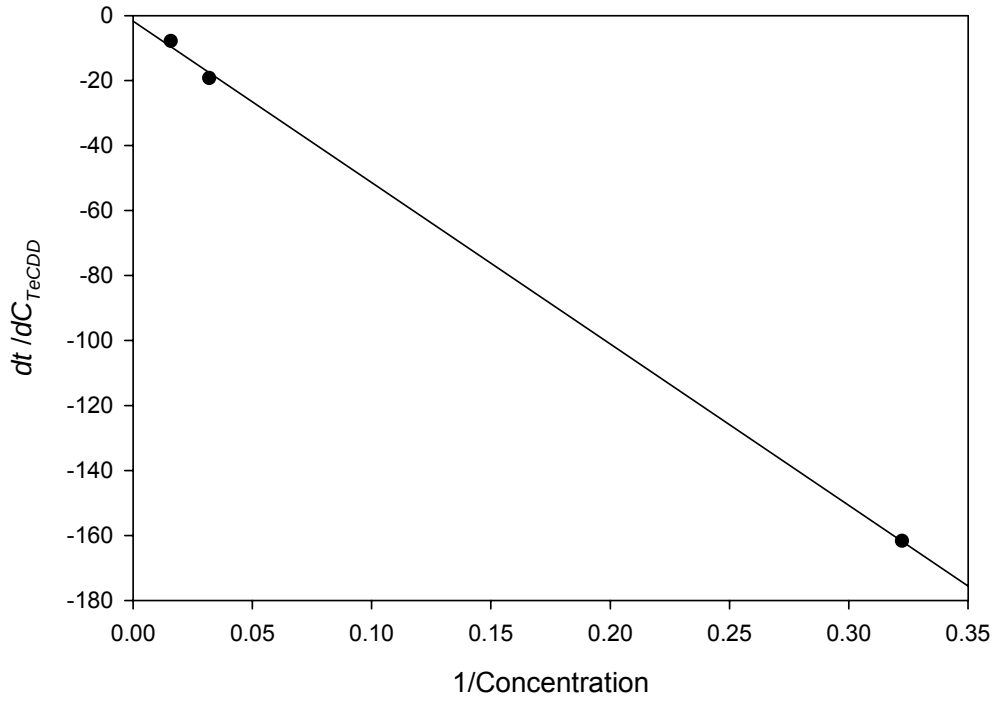


Figure 4-2. A plot of dt/dC_{TeCDD} versus 1/C with linear regression.

4.4 Effects of PCE on 1,2,3,4-TeCDD Dechlorination

The effect of addition of PCE was investigated for the initial concentration of 1,2,3,4-TeCDD at 62 μM. The first order reaction rate constant k_1 was 0.0025 ± 0.0017 d^{-1}, which is in the same order of magnitude as the value obtained when 1,2,3,4-TeCDD was the sole halogenated substrate at the same concentration (Table 4-3).

When 1,2,3,4-TeCDD and PCE both present in the system, there are two governing dechlorination kinetics:

$$\frac{dC_{TeCDD}}{dt} = \frac{-k_{TeCDD}X_{dechlor}C_{TeCDD}}{K_{s(TeCDD)}+C_{TeCDD}} \times \frac{C_{w(H_2)}}{K_{S(H_2)}+Cw(H_2)} \quad (5)$$

and

$$\frac{dC_{PCE}}{dt} = \frac{-k_{PCE}X_{dechlor}C_{PCE}}{K_{s(PCE)}+C_{PCE}} \times \frac{C_{w(H_2)}}{K_{S(H_2)}+Cw(H_2)} \quad (9)$$

The half velocity coefficient of PCE has been determined as 0.5 μM, which is much less than the initial PCE concentration in the system, 110 μM (Fennell & Gossett 1998; Smatlak & Gossett 1996). In this case, PCE is not the rate-limiting factor. So the dechlorination rate of PCE is determined by the concentration of the electron donor: H_2. *D. ethenogenes* strain 195 is known to dechlorinate PCE sequentially to ethene, where all chloroethenes but VC are growth substrates. Strain 195 is able to dechlorinate 1,2,3,4-TeCDD although it is yet to be determined if this dechlorination provides energy for its growth. When both PCE and 1,2,3,4-TeCDD are present, two scenarios might exist. There might be competition between the dechlorination of PCE and 1,2,3,4-TeCDD for the available biomass and electron donors. The ratio of the equivalent of electron donor

to electron acceptors (1,2,3,4-TeCDD+PCE) was 2:1, therefore the supply of electron donor was in excess in the system. However, since the hydrogen concentration in the system was likely normally below the $K_{S(H2)}$, the concentration of H_2 could limit dechlorination of the PCDD/Fs, as explained in the calculation section (3.2). It is known that the reductive dehalogenase-PceA is the enzyme responsible for the dechlorination of PCE to TCE (Magnuson et al. 1998). However, the mechanism of dechlorination of 1,2,3,4-TeCDD is unknown. If the same enzyme works on both halogenated substrates, the competition for the enzyme would affect the dechlorination of both compounds. Therefore, competitions for electron donor and biomass could occur. In the second scenario, the existence of PCE might impose a beneficial impact on the dechlorination of 1,2,3,4-TeCDD. The dechlorination of PCE provides energy for the growth of strain 195. Therefore during dechlorination of PCE, the concentration of strain 195 will increase, thus the available pool of biomass for dechlorination of 1,2,3,4-TeCDD might increase as well. In summary, the presence of PCE could potentially impact the dechlorination either negatively or positively.

The first order reaction rate constant obtained from the culture set amended with an initial 1,2,3,4-TeCDD concentration of 62 μM, along with PCE addition, was 0.0025 ± 0.0017 d^{-1},or about half the reaction rate of 0.006 ± 0.0026 d^{-1} observed when 1,2,3,4-TeCDD was the sole halogenated substrate at the same initial concentration. Although the reaction rates are close, the results suggest that the presence of PCE does impose a negative effect on the dechlorination of 1,2,3,4-TeCDD. This was clearly observed during the early stages of the experiment, at which time the transformation of 1,2,3,4-TeCDD to 1,2,4-TrCDD was faster when PCE was not present. Later, the concentration

of 1,2,3,4-TeCDD decreased in a parallel pattern in the systems with and without PCE addition (Figure 4-3). It seems that PCE affected the dechlorination of 1,2,3,4-TeCDD most during the early stages of the test but less as the dechlorination of 1,2,3,4-TeCDD proceeded. The initial delay in the cultures amended with PCE might be explained by the competition of the dechlorinators (strain 195) between different substrates. We speculate that perhaps after the dechlorination of PCE produced higher concentrations of dechlorinators, the competition was eased.

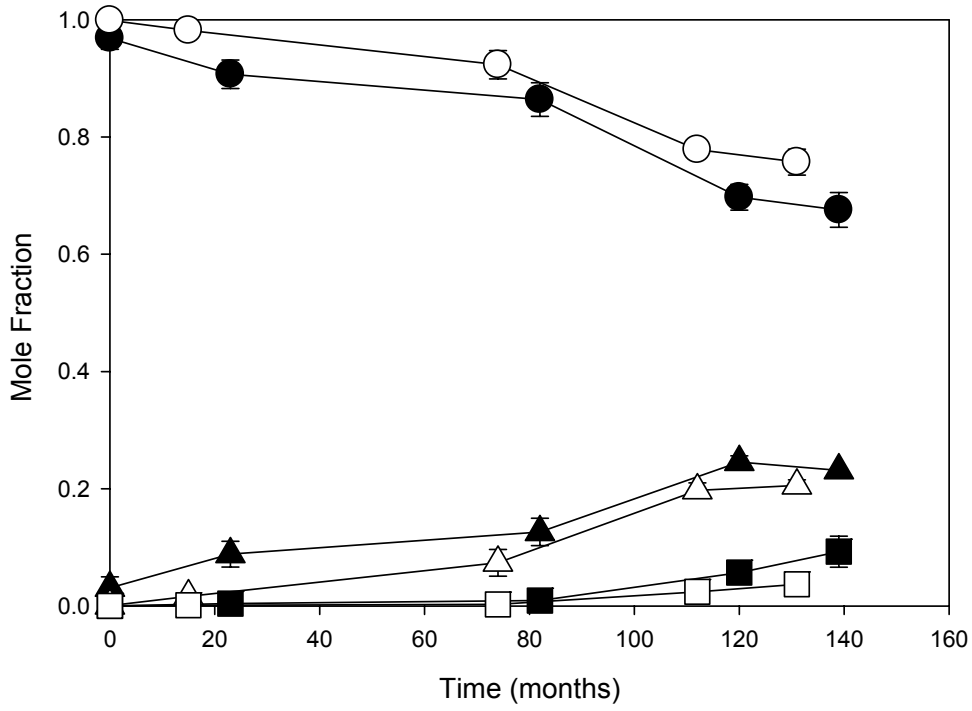


Figure 4-3. Comparison of the dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (●) and formation of 1,2,4-trichlorodibenzo-*p*-dioixn (1,2,4-TrCDD) (▲) and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (■) without (closed symbols) and with (open symbols) tetrachloroethene (PCE) addition.

4.5 Dechlorination Rates of 1,2,3,4,7,8-HxCDF, 1,2,3,4-TeCDD and 1,2,3,4-TeCDF

Pseudo-first order reaction rate constants (k_1) were determined for all previous data collected for *D. ethenogenes* strain 195 in pure and mixed culture (Fennell et al. 2004; Liu & Fennell 2007; Liu et al. Isotope manuscript [Chapter 5]). The data are shown collectively in Table 4-5. We reported that this culture dechlorinated 1,2,3,4,7,8-HxCDF to 1,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 1,2,4,7,8-(PeCDF), and, when co-amended with 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB), further to two tetrachlorodibenzofuran (TeCDF) congeners, identified as 1,3,7,8-TeCDF and 1,2,4,8-TeCDF (Liu & Fennell 2007). After 195 days, the treatment amended with 1,2,3,4-TeCB contained 57.6 ± 1.5 mol % 1,2,3,4,7,8-HxCDF; 32.2 ± 0.7 mol % PeCDFs; and 5.8 ± 0.4 mol % 1,3,7,8-TeCDF and 4.4 ± 0.4 mol % 1,2,4,8-TeCDF. In the bottles spiked only with 1,2,3,4,7,8-HxCDF, the mole percents at day 195 were 87.2 ± 0.7%, 12.1 ± 0.4%, and 0.3 ± 0.2% and 0.3 ± 0.1%, for 1,2,3,4,7,8-HxCDF, the PeCDF congeners and the two TeCDF congeners, respectively. Similarly, the final mole percents in the treatment amended with PCE were 84.1 ± 4.4%, 15.5 ± 4.2%, 0.3 ± 0.1%, and 0.2 ± 0.0% for the parent compound, intermediates and final products. In cultures amended with 1,2,3,4,7,8-HxCDF alone, the first order rate constant was 0.0031 ± 0.0012 d^{-1}, for 1,2,3,4,7,8-HxCDF plus PCE the rate was 0.0028 ± 0.0006 d^{-1} , and the rate for 1,2,3,4,7,8-HxCDF plus 1,2,3,4-tetrachlorobenzene was 0.0047 ± 0.0010 d^{-1} (Table 4-5). Addition of 1,2,3,4-TeCB enhanced both the rate and the extent of dechlorination of 1,2,3,4,7,8-HxCDF.

5. Discussion

5.1 Bioavailability of PCDDs to Dechlorinating Bacteria

When dealing with the biotransformation of hydrophobic chemicals like PCDD/Fs, bioavailability may impact kinetics of bacterial transformation. When discussed in the context of microbial transformation, bioavailability is referred to the availability of compounds for microbial degradation (Harms 1997). Hydrophobic compounds like PCDD/Fs tend to be sorbed to the organic matter in the soil or sediment. To be available to the microorganisms, they might go through a series of physical-chemical processes (Harms 1997), including desorption from the organic matter, dissolution to the aqueous phase, and diffusion driven by the concentration gradient, prior to becoming accessible to the microbe. The microbial uptake of PCDD/Fs creates a concentration gradient in the proximity of microorganisms, therefore a flux exists from the place where PCDDs were originally present to the accessible range of the microorganisms driven by the gradient. Since substrate dissolved in the aqueous phase is readily available to the microorganisms, it is considered true that the bioavailability is to a large extent determined by the aqueous solubility of compounds. However, the results from this study did not support this principle. The aqueous solubility of 1,2,3,4-TeCDD at 25°C is 0.63 μg/L (2 nM) (Shiu et al. 1988), which is far less than all the nominal concentrations (0.31 μM to 62 μM) employed in this study. In the 1,2,3,4-TeCDD amended culture, 1 gram of dried sediment was added as the carrier of 1,2,3,4-TeCDD. It is reasonable to assume that most of the 1,2,3,4-TeCDD would be associated with the organic matter in the sediment particles, the colloidal particles (including microorganisms), and the glass wall of the vessels. The

physical-chemical properties of 1,2,3,4-TeCDD have been determined as follows by Shiu et al (1998): aqueous solubility is 0.63 μg/L at 25°C; log K_{ow} is 5.77 at 25°C; vapor pressure is 0.000275 Pa; and Henry's law constant is 3.77 Pa·m^3/mol. These data illustrate that 1,2,3,4-TeCDD is a very hydrophobic compound with low solubility and low vapor pressure, indicating that its main sink in our system would be the solid phase. The dried sediment introduced to our system was determined to have a total carbon concentration of 53,783 ppm. We assume that most of the total carbon is organic carbon. The partitioning of 1,2,3,4-TeCDD between the organic matter and the aqueous phase could be estimated as (Schwarzenbach et al. 1993):

$$K_{om} = \frac{C_{om}}{C_w} \quad (10)$$

where C_{om} is the concentration of 1,2,3,4-TeCDD in the organic matter (mol/kg$_{om}$); Cw is the aqueous concentration of 1,2,3,4-TeCDD (mol/L); and K_{om} is the organic matter-water partitioning coefficient. The value of log K_{om} is related to log K_{ow} by an empirical equation obtained for chlorinated hydrocarbons as *(*Schwarzenbach et al. 1993; Karickhoff 1981*)*:

$$\log K_{om} = 0.88 \cdot \log K_{ow} - 0.27 \quad (11)$$

From the known K_{ow} value of 1,2,3,4-TeCDD, log K_{om} was estimated based on equation 11 to be 4.81. K_{om} could be used to further estimate the distribution of 1,2,3,4-TeCDD between the aqueous phase and the organic matter in the sediment assuming the aqueous concentration of 1,2,3,4-TeCDD reached the saturation solubility, C_w of 0.63 μg/L and the corresponding C_{om} was about 40.7 mg/kg. The amount of organic matter in the sediment added to the cultures was 0.0054 g. Thus we estimated that the total 1,2,3,4-

TeCDD sorbed to the organic matter in the sediment was 0.22 μg. The total amount of 1,2,3,4-TeCDD in the aqueous phase was 0.63 μg/L × 0.01 L = 0.0063 μg. The total amount of 1,2,3,4-TeCDD dissolved in the aqueous phase and sorbed to the organic matter was about 0.22 ug, which takes about 22%, 2.2%, 0.22% and 0.11% of the total 1,2,3,4-TeCDD in the system at the concentration levels of 0.31 μM, 3.1 μM, 31 μM and 62 μM, respectively. This estimation showed that even if the aqueous concentration of 1,2,3,4-TeCDD reaches saturation solubility, most of the 1,2,3,4-TeCDD in the system is not in the aqueous phase or sorbed to the organic matter. The rest of the 1,2,3,4-TeCDD might be sorbed to the glass wall or to the colloidal phase. Strictly speaking, the concentration of 1,2,3,4-TeCDD in equation 5 should be the aqueous concentration if we assume that dissolved 1,2,3,4-TeCDD is readily available for microbial dechlorination. If this holds true, no matter what the initial nominal concentration of 1,2,3,4-TeCDD is, the dechlorination of 1,2,3,4-TeCDD should have similar reaction rates for all the concentration levels since the rate would be governed by the aqueous concentration of the compound. However, the results showed different dechlorination rates at each nominal concentration level, and the dechlorination rate was approximately in proportion to the corresponding initial nominal concentration. That is, pseudo-first order kinetics was observed. This implies that the microorganisms were able to dechlorinate 1,2,3,4-TeCDD not only directly available from the aqueous phase but also available from other phases, e.g. lipids in cell membranes. The bioavailability of PCDDs has been discussed at length as a reason for the slow dechlorination processes of PCDDs in the environment. The hydrophobic properties of PCDDs limit their presence in the aqueous phase, which as an accepted paradigm limits the access of microorganisms to the compounds as well.

However, this study illustrated that a higher dechlorination rate was achieved in proportion to the higher initial nominal concentration level of 1,2,3,4-TeCDD when all the other experimental conditions remained the same. Similar results were reported by Ballerstedt et al (2004) where different dechlorination rates of 1,2,4-TrCDD to 1,3-DCDD by a mixed culture were reported for a variety of initial concentrations of 1,2,4-TrCDD at 1, 10, 40, and 250 μM. The product formation rate increased with the increase in the substrate concentrations from 0.05 to 5.4 μM/d.

5.2 Implications of Kinetic Parameters

This study systematically examined the dechlorination kinetics of 1,2,3,4-TeCDD under different conditions. We observed that the dechlorination followed a first order kinetics, which means different reaction rates were observed at different concentration levels. This particularly explains the huge discrepancy among the calculated rates from previous studies, which only gave a calculated dechlorination rate at specific concentration levels (Ballerstedt et al. 1997; Albrecht et al. 1999; Fu et al. 1999). Extra care needs to be taken when comparing the dechlorination rates under different experimental conditions. Instead, presentation of dechlorination with half lives might be a better approach if the dechlorination has the characteristics of a first order reaction.

The kinetic parameters of dechlorination of 1,2,3,4-TeCDD was calculated as $K_{S(TeCDD)}$ of 291 μM and k_{TeCDD} of 2.93 × 10^{-12} μmole/cell·day. The half velocity coefficient $K_{S(TeCDD)}$ indicates the affinity of the dechlorinator to the substrate. The $K_{S(PCE)}$

is 0.5 μM (Fennell & Gossett 1998; Smatlak & Gossett 1996) for the same dechlorinator in our system, *Dehalococcoides ethenogenes* strain 195. This shows that the affinity for 1,2,3,4-TeCDD and PCE of the same dechlorinator was quite different. The affinity for PCE of strain 195 was much higher than its affinity for 1,2,3,4-TeCDD. The 1,2,3,4-TeCDD is more hydrophobic with far lower aqueous solubility. The molecular structure of 1,2,3,4-TeCDD may also result in more difficulties for microbial processes. This could be confirmed by the much slower dechlorination process PCDDs usually undergo compared with that of PCE dechlorination.

This study examined the kinetics of dechlorination of a chosen PCDD congener and presented the dechlorination kinetic parameters. The reaction rates of a PCDF congener under different conditions was presented as well. The results illuminated different governing factors on the dechlorination of PCDD/F congener. The outcomes might explain the discrepancies in literature regarding the dechlorination rates for PCDD/Fs. More importantly, a positive message was obtained that the dechlorination of PCDDs might not be limited by their aqueous solubility, which could have important implications in the bioremediation of PCDD/F contaminated matrices. However, caution should be used in the application of the results since different experimental systems could result in different kinetic parameters as discussed previously (Smatlak & Gossett 1996).

Table 4-5. First order reaction rate constants and half lives of selected polychlorinated dibenzo-*p*-dioxin and dibenzofurans (PCDD/Fs) during dechlorination by *D. ethenogenes* strain 195.

Study and Substrate	PCDD/F Nominal Conc. (μM)	PCDD/F Solubility (nM)	Halogenated Co-Substrate	Temp (°C)	*k* (day^{-1}) ± Std Error	Half life (month)	Ref.
Kinetics Study							This Study
1,2,3,4-TeCDD	62	2		28	0.006±0.0026	3.9	
1,2,3,4-TeCDD	62		PCE	28	0.0025±0.0017	9.2	
1,2,3,4-TeCDD	31			28	0.0033±0.0016	7	
1,2,3,4-TeCDD	3.1			28	0.0023±0.0006[a]	10	
1,2,3,4-TeCDD	0.31			28	0.0004±0.0004	58	
Stable Isotope Fractionation Study							Liu et al. Isotope manuscript [Chapter 5]
1,2,3,4-TeCDD	31			28	0.0092±0.0009	2.5	
1,2,3,4-TeCDD	31		PCE	28	0.01±0.0016	2.5	
HxCDF Study							Liu & Fennell 2007
1,2,3,4,7,8-HxCDF	5			28	0.0031±0.0012	7.5	
1,2,3,4,7,8-HxCDF	5		PCE	28	0.0028±0.0006	8.3	
1,2,3,4,7,8-HxCDF	5		TeCB	28	0.0047±0.001	4.9	
Mixed Culture							Fennell et al. 2004
1,2,3,4-TeCDD	31		PCE	34	0.04±0.009	0.58	
Pure Culture							Fennell et al. 2004
1,2,3,4-TeCDD	31		PCE	28	0.0058±0.0029	4	
1,2,3,4-TeCDF	13		PCE	28	0.004±0.0027	5.4	

a. calculated using molar fraction

Chapter 5

Carbon Stable Isotope Fractionation of Chloroethenes and Polychlorinated Dibenzo-*p*-Dioxins by a Mixed Culture Containing *Dehalococcoides ethenogenes* Strain 195

1. Introduction

In situ biotransformation may contribute to the natural attenuation of persistent contaminants in the environment. The stimulation of *in situ* biotransformation can be used to treat groundwater, soils and aquatic sediments. Monitoring strategies are needed to evaluate the success of *in situ* biotransformation and to determine whether the enhancement strategies are effective. Documentation of the existence and effectiveness of *in situ* microbial biotransformation of pollutants can be time consuming and expensive and requires enormous effort for installation of monitoring points, sampling, and analysis to record pollutant concentration changes over time. Thus innovative tools are needed to quantify *in situ* transformation.

One promising tool for assessment of biological transformation of environmental contaminants is Compound Specific Isotope Analysis (CSIA). In CSIA the ratio of the

relative abundance of the heavy and light isotopes of a given element is determined in an individual compound. The concept of CSIA relies on kinetic isotope fractionation during a microbial transformation reaction. The kinetic isotope effect (KIE) is caused by different reaction rates for a compound containing the heavy isotope versus the light isotope at the location of the reaction. Since more energy is needed to break the chemical bond constituted by heavy isotopes, the reaction is normally slower for the molecule containing the heavy isotope than for the light isotope of the same element. This typically results in an enrichment of heavy isotopes in the residual substrate fraction. Biologically mediated stable isotope fractionation has been examined under different conditions in the laboratory and field for various contaminants including benzene, toluene, ethylbenzene and xylenes (BTEX) (Meckenstock et al. 1999; Hunkeler et al. 2001[b]); methyl-tertiary-butyl ether (MTBE) (Gray et al. 2002; somsamak et al. 2005); polynuclear aromatic hydrocarbons (PAHs) (Richnow et al. 2003; Yanik et al. 2003); phenol and benzoate (Hall et al. 1999); polychlorinated biphenyls (Drenzek et al. 2001); and chlorinated ethenes (Hunkeler et al. 1999; Bloom et al. 2000; Sherwood Lollar et al. 2001; Nijenhuis et al. 2005). Recent literature reviews were provided by Schmidt et al. (2004) and Meckenstock et al. (2004).

Carbon stable isotope fractionation of chlorinated ethenes has been monitored in order to verify and estimate the extent of *in situ* biodegradation in field studies and can be used to validate a bioremediation effort. CSIA may be used as both a qualitative and a quantitative measurement to monitor *in situ* biodegradation processes (Sherwood Lollar et al. 2001; Nijenhuis et al. 2005; 2007).

CSIA could be of particular use for examining transformation of more complex environmental pollutants whose biotransformation is difficult to assess in complex environments. One such class of chemicals is the polychlorinated dibenzo-*p*-dioxins (PCDDs). PCDDs accumulate in soils, sediments and biota due to their high hydrophobicity, low solubility and low volatility, and they are ubiquitous in the environment (Czuczwa & Hites 1984; Hites 1990; Wagrowski & Hites 2000). Contamination of soil and sediment by PCDDs is a serious environmental problem (Bopp et al. 1991; Koistinen et al. 1995). PCDDs generally exist in the environment at very low concentrations, however, they are of great public concern because of the toxicity of the 2,3,7,8-substituted congeners (Van den Berg et al. 2006) and their potential for bioaccumulation (Cai et al. 1994).

Lightly chlorinated PCDDs may be biotransformed or mineralized under aerobic conditions (Wittich 1998), while highly chlorinated PCDDs undergo reductive dechlorination under anaerobic conditions (Adriaens et al. 1995; Beurskens et al. 1995; Ballerstedt et al. 1997). Abiotic reactions with humic substances may also be an important fate process (Fu et al. 1999). Recently two bacterial strains have been identified which dechlorinate PCDDs under anaerobic conditions, — *Dehalococcoides* sp. strain CBDB1 (Bunge et al. 2003) and *Dehalococcoides ethenogenes* strain 195 (Fennell et al. 2004).

D. ethenogenes strain 195 dechlorinates tetrachloroethene (PCE) to vinyl chloride (VC) and ethene. It grows on tetrachloroethene (PCE), trichloroethene (TCE), *cis*-dichloroethene (DCE) and 1,1-DCE (Maymó-Gatell et al. 1997, 1999), and selected chlorinated benzenes (Fennell et al. 2004). It has been shown that *D. ethenogenes* strain

195 also dechlorinates some polychlorinated biphenyls (PCBs), chlorinated naphthalenes, and PCDDs (Fennell et al. 2004). Specifically, strain 195 dechlorinates 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) to 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD), and subsequently to 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD).

The objective of this study was to analyze stable isotope fractionation during the dechlorination of PCDDs, in order to evaluate the possibility of documenting environmental transformation of PCDDs by CSIA. PCDDs have a low solubility in water and most PCDDs are expected to be sorbed to soil or sediment material in the environment. This limits their bioavailability. This limited bioavailability may be a critical governing factor for dechlorination kinetics and may reduce the extent of isotope fractionation. To examine the potential for isotope fractionation of PCDDs under conditions similar to those that would be encountered in the environment, we conducted experiments using sediment-containing batch cultures. The dissolution of PCDDs from sediment particles was included in the experimental approach so that the concept of isotope fractionation as an indicator for reductive dechlorination processes could be more applicable for real environmental systems. We compared the carbon stable isotope fractionation upon sequential dechlorination of PCE and 1,2,3,4-TeCDD by a mixed culture containing *D. ethenogenes* strain 195. Ability to quantify and understand the isotope fractionation pattern of reactant and metabolite during sequential dechlorination processes may have potential to serve as a proxy for *in situ* biotransformation.

2. Methodology

2.1 Chemicals

1,2,3,4-TeCDD, 1,2,4-TrCDD, 1,3-DCDD, and 2,2',5-trichlorobiphenyl (2,2',5-TrCB) were purchased from AccuStandard (New Haven, CT). PCE (99.9+%), TCE (99.5+%), *cis*-1,2-DCE and butyric acid (99+%) were obtained from Aldrich Chemical Company (Milwaukee, WI). Vinyl chloride (VC) (≥99.97%) was obtained from Linde AG (Leuna, Germany).

2.2 Culture Cultivation

A mixed culture containing *D. ethenogenes* strain 195 was grown at 34°C for PCE studies or at 25°C for PCDD studies, on PCE and butyric acid using methods described previously (Fennell et al. 2004; Fennell et al. 1997).

2.3 Experimental Setup [The PCE experiment described here were conducted by Ms. Danuta Cichocka and Dr. Ivonne Nijenhuis in the UFZ Centre for Environmental Research, Leipzig, Germany.]

We examined carbon stable isotope fractionation during PCE dechlorination in batch experiments. Cultures in 250 mL vials, containing 100 mL medium were prepared by inoculating with 2% volume:volume vol:vol of culture from late exponential growth

phase on 500 μM PCE. Cultures were amended with PCE (500 μM) as an electron acceptor and 2.75 mM butyric acid as an electron donor and hydrogen source (Fennell et al. 1997) and were incubated in a rotary shaker at 34°C. At the beginning of the experiment and at various time points, 0.5 mL gas phase samples were taken, and concentrations of chlorinated ethenes were determined. Simultaneously, an aliquot of 0.5 mL headspace sample of each culture was removed and immediately analyzed for carbon isotope composition.

1,2,3,4-TeCDD experiments were carried out in 160-mL serum bottles in batch mode. One gram of dry sterile sediment was added to each bottle. The sediment was completely wetted by addition of 0.5-mL of a 6211 μM 1,2,3,4-TeCDD (2000 mg 1,2,3,4-TeCDD /L) toluene stock solution. The toluene was allowed to volatilize under a sterile N_2 purge, leaving behind a coating of 1,2,3,4-TeCDD on the sediment carrier. Culture (100 mL) was transferred to each bottle under anoxic and sterile conditions which should have been equivalent to a nominal 1,2,3,4-TeCDD concentration of 31 μM (10 mg/L). We routinely recovered only about 6 μM of PCDDs, indicating that some of the added compound was not recovered by our sampling and extraction method. The sediment to media ratio was 0.01 (w/v).. Taking the solubility of 0.63 μg/L for 1,2,3,4 TeCDD at 25°C (Shiu et al. 1988) in account about 0.0063 % of the spiked 1,2,3,4-TeCDD was dissolved and most was presumably sorbed to the sediment particles and colloidal organic matter, and perhaps to the glass surface. Three sets of triplicate treatments were set up. In addition to 1,2,3,4-TeCDD, each bottle received 440 μM butyric acid as an electron donor and hydrogen source and 40 μL of a 50 g/L fermented yeast extract solution (Fennell et al. 1997) as a nutrient source, periodically. One set of triplicate bottles was amended with

110 μM PCE, as an additional halogenated substrate, periodically. One set of triplicate bottles was autoclaved to serve as a killed control. The bottles were shaken at 120 rpm at 28°C on a platform shaker.

2.4 Analytical Methods

At the UFZ, ethene and chlorinated ethenes were quantified as described previously (Nijenhuis et al. 2005), using a Varian Chrompack CP-3800 gas chromatograph (Middelburg, the Netherlands) with flame ionization detection with a 30 m x 0.53 mm GS-Q column (J&W Scientific, Waldbronn, Germany). The temperature program used was as follows: 1 min at 100°C, 50°C/min to 225°C and held for 2.5 min. The FID was operated at 250°C and helium was used as carrier gas (0.69 $x10^5$ Pa; 11.5 mL/min). This method allowed the separation of methane, ethene, VC, 1,1-, *trans*- and *cis*-DCE, TCE and PCE. The sampling was automated using an HP 7694 headspace autosampler (Hewlett Packard, Palo Alto, USA), and 0.5 mL headspace samples were added to 10 mL autosampler vials flushed with helium, closed with a Teflon coated butyl rubber septum and crimped.

Two-mL samples of well mixed culture/sediment mixture were removed for PCDD analyses. Samples were extracted and prepared for instrumental analysis as previously described (Vargas et al. 2001). Briefly, after centrifugation, the solid phase was extracted with 2:1 (vol:vol) toluene:acetone solution and toluene, successively, with 2,2',5-trichlorobiphenyl as a surrogate. Interfering organic compounds were removed by

passing the solvent phase through a 2 mL glass pipette filled with Florisil (Sigma-Aldrich, St. Louis, MO) and eluting with toluene. PCDDs were analyzed using an Agilent 6890 gas chromatograph equipped with a 5973N mass selective detector (GC-MS) (Agilent Technologies, Inc. Santa Clara, CA) and a HP-5MS (Agilent Technologies, Inc. Santa Clara, CA, USA) column (30 m × 0.25 mm I.D.). PCDDs were detected based on the retention times of standards and their molecular ions (*m/z*: 322 for 1,2,3,4-TeCDD, 286 for 1,2,4,-TrCDD, 252 for 1,3-DCDD, and 256 for 2,2',5-TrCB). A qualifying ion was monitored to assure the correct identification of each congener (320 for 1,2,3,4-TeCDD, 288 for 1,2,4-TrCDD, 254 for 1,3-DCDD, 186 for 2,2',5-TrCB). The PCDD concentrations are presented as averages of triplicate data points plus or minus one standard deviation (1σ). The mass balance of PCDDs discussed as mole percent assumed that no anaerobic mineralization of the dibenzo-*p*-dioxin structure occurred, and that the PCDDs underwent no significant reactions other than dechlorination.

The isotope composition of chlorinated ethenes and ethene was analyzed using a gas chromatography combustion isotope ratio mass spectrometer (GC-C-IRMS) as described previously (Nijenhuis et al. 2007). Aliquots (500 µL) of the headspace samples were injected into a gas chromatograph (Agilent 6890; Palo Alto, USA) in split mode (split 1:3) using a split/splitless injector at 250°C. For chromatographic separation a RTX-Q-Plot column (30 m x 0.32 mm x 10 µm, Restek, Bellefonte USA) was used. The temperature program was as follows: initial temperature was set to 40°C for 5 min, then was increased at a rate of 20°C/min to 150°C, and next increased at a rate of 5°C/min to a final temperature of 250°C, which was held for 3 min. All samples were analyzed at least three times. Generally, using this method, the standard deviation was <0.5 δ unit.

Carbon stable isotope analysis of PCDDs was performed using a GC-C-IRMS. An Agilent 6890 gas chromatograph was connected to Finnigan MAT 252 mass spectrometer via a Finnigan GC-C/TC III Interface *(5)*. A ZB-5 column (60 m × 0.32 mm I.D. with 0.25 μm thickness) (Phenomenex, Torrance, CA.) was used to separate the individual PCDD congeners in the samples. The temperature program was as follows: 100°C for 1 min, increased to 220°C at 15°C/min; held for 5 min; then increased to 320°C at 6°C/min; and held for 10 min. The injector was held at 280°C and operated in a pulsed splitless mode with a 0.8 min purge time, and a 3.10 x10^5 Pa purge pressure. The purge flow was 10 mL/min and the pulse time was 1 min. Helium was the carrier gas at a constant flow rate of 1.5 mL/min. At a minimum, triplicate measurements by GC-C-IRMS were performed for each sample. The CSIA data from the triplicate bottles are reported as averages of triplicate injections for each time point from each replicate plus or minus one standard deviation.

2.5 Carbon Stable Isotope Calculations

Carbon isotope ratios are reported in standard δ notation in parts per thousand (‰) relative to an international reference standard:

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

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

The Rayleigh model was used to calculate the isotope fractionation by correlating changes in concentration to changes in isotope composition of a substrate (Rayleigh 1896).

$$\ln(\frac{R_t}{R_0}) = (\alpha - 1)\ln(\frac{C_t}{C_0}) \quad (2)$$

R_t and R_0 are the isotopic compositions (ratio of $^{13}C/^{12}C$) of the substrate at time t and time 0, and C_t and C_0 are the concentrations of ^{12}C in the substrate at time t and time 0. α is the carbon isotope fractionation factor relating changes in concentration to changes in isotope composition.

In a system where the concentration of the heavy isotope is negligible, for example as ^{13}C is in a natural system, C_t/C_0 may be approximated as the total concentration of ^{13}C plus ^{12}C in the substrate at time t and time 0., i.e. the measured concentration of the substrate.

The isotope fractionation is often reported as an isotope enrichment factor, ε,

$$\varepsilon = (\alpha - 1) \times 1000 \quad (3)$$

In a closed system, the mass balance on the isotopes of carbon in different compounds in the system is expressed as

$$R_{Total,t} = \sum R_{i,t} \times f_{i,t} = \sum R_{i,0} \times f_{i,0} \quad (4)$$

where $R_{Total,t}$ is the carbon isotope ratio of the total pool of parent compound and its metabolites in the system at time t. Theoretically, $R_{Total,t}$ should be constant over the course of the reaction. $R_{i,0}$ is the carbon isotopic composition of a chemical i at time 0

and $f_{i,0}$ is the fraction of chemical i at time 0. Similarly, $R_{i,t}$ and $f_{i,t}$ are its carbon isotopic composition and fraction at time t.

Measured R values are the average isotope composition of a specific chemical. Thus the isotope fractionation factors calculated from the average isotope composition reflect the overall fractionation of an element in the specific chemical during the reaction. The primary kinetic isotope fractionation upon a bond cleavage can affect the isotope composition of the molecule to a great extent. However if the isotope of interest is located at positions other than the reactive site, it will only result in a secondary isotope fractionation which is much less than the primary isotope fractionation. Consequently isotopes in the non-reactive positions do not contribute to the primary kinetic isotope fractionation. The intrinsic isotope fractionation normalizes the observed isotope fractionation of a molecule to the kinetic isotope effect of bond cleavage by subtracting isotope dilution of non-reactive positions which are not altered during the reaction as well as the intramolecular competition of substituents in the reactive position (Elsner et al. 2005).

The intrinsic enrichment factor could be calculated as (Morasch et al. 2004)

$$\varepsilon_{intrinsic} = \varepsilon_{bulk} \times n \qquad (5)$$

where n is the number of the atoms of interest in one molecule, n=12 for PCDDs and 2 for chloroethenes, respectively. The ε_{bulk} is the $\boldsymbol{\varepsilon}$ calculated from experimental data. This approach can be used to estimate the kinetic isotope effect (KIE) upon bond cleavage to compare isotope fractionation of various chemicals (Elsner et al. 2005).

2.6 Analysis of Variance

Analysis of variance (ANOVA) was performed to compare the $\delta^{13}C$ values of 1,2,3,4-TeCDD and its dechlorination products 1,2,4-TrCDD and 1,3-DCDD. The analyses were performed with Microsoft Excel. All $\delta^{13}C$ values obtained at each time point over the study (time 0 to 153 days) were included in the analysis.

3. Results and Discussion

3.1 Dechlorination

The mixed culture containing *D. ethenogenes* strain 195 dechlorinated PCE to VC and ethene via the intermediate products TCE and *cis*-DCE (Fig 5-1 A). The VC and ethene present at day 1 was carryover from the inoculum. Dechlorination commenced on day 4, when the first product of the reaction, TCE, appeared. On day 8 both TCE and *cis*-DCE were detected at low concentrations, however these intermediate products underwent further, rapid dechlorination. At day 8 VC production increased rapidly and the concentration of ethene was slightly increased over the background level. Ethene concentration increased on day 14, when no other substrates except for VC were present in the medium. PCE was dechlorinated from an initial concentration of 430 μM to about 90 μM in 11 days. By day 14 PCE was completely dechlorinated. At the completion of the experiment on day 17, VC and ethene were at 230 μM and 130 μM, respectively. During the experiment, a loss approximately 70 μmol/L of chlorinated ethenes was

observed. This loss was likely due to the sampling strategy used since at each time point at least 1 mL of headspace was removed. Additional losses due to leaking of gas during incubation cannot be excluded.

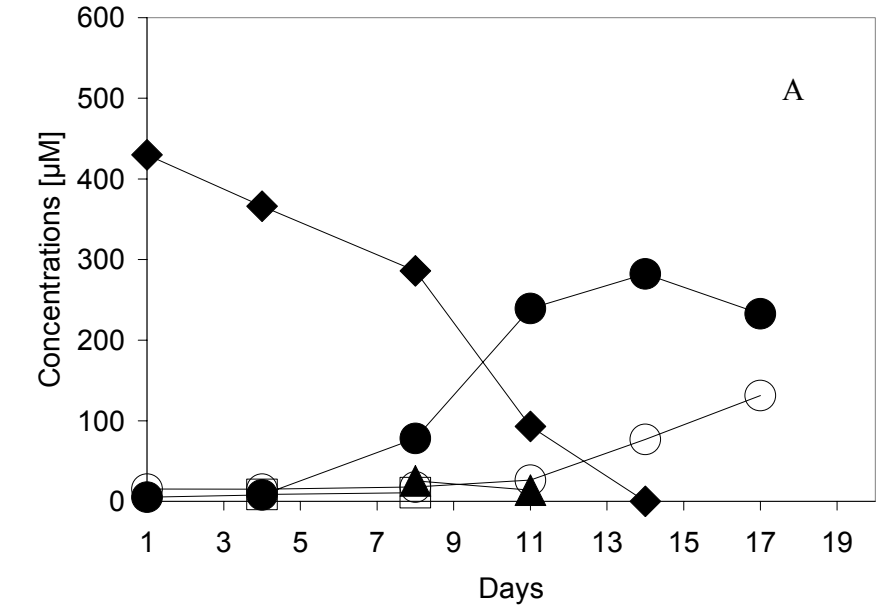
The mixed culture containing *D. ethenogenes* strain 195 dechlorinated 1,2,3,4-TeCDD both in the presence and absence of PCE. The transformation of 1,2,3,4-TeCDD to 1,3-DCDD via 1,2,4-TrCDD in two replicate cultures without PCE addition is shown in Figures 2A and 2C. No 1,2,4-TrCDD or 1,3-DCDD were observed in the autoclaved control (Figures 5-2A and C). A slight lag time was observed in the bottles with 1,2,3,4-TeCDD when PCE was added as a co-substrate (see supporting information, Figure 5-S2 C,E and G). In the bottles without PCE addition, by day 153, the mole percents of 1,2,3,4-TeCDD, 1,2,4-TrCDD and 1,3-DCDD were 24 ± 3.8%, 42 ± 0.6% and 33 ± 3.4%, respectively. In the bottles with PCE addition, the mole percents of 1,2,3,4-TeCDD, 1,2,4-TrCDD and 1,3-DCDD were 23 ± 4.5%, 57 ± 3.2% and 19 ± 1.3%, respectively. The two sets exhibited similar overall extents of dechlorination of 1,2,3,4-TeCDD, however the rates of product formation were slightly different. In culture bottles amended with both 1,2,3,4-TeCDD and PCE, PCE was dechlorinated to VC and ethene (data not shown).

3.2 Carbon Stable Isotope Fractionation of PCE

Carbon stable isotope fractionation was observed during the dechlorination of the chlorinated ethenes (Figure 5-1B). The $\delta\ ^{13}C$ values of chlorinated ethenes and ethene

ranged from -48.0 ± 0.2‰ to -2.7 ± 0.4‰. The δ ^{13}C ranges for each chloroethene and ethene were: -27.1 ± 0.4‰ to -16.5 ± 0.8‰ for PCE; -32.9 ± 0.5‰ to -21.9 ± 0.2‰ for TCE; -38.0 ± 0.2‰ to -2.7 ± 0.4‰ for VC; and -48.0 ± 0.2‰ to -28.2 ± 0.3‰ for ethene. The first dechlorination step from PCE to TCE was associated with isotope fractionation leading to a depletion of ^{13}C in the product (-32.9‰) and enrichment of ^{13}C in the substrate (-26.3‰) as shown on day 4. The Rayleigh equation (eq 2) was used to quantify the PCE fractionation and gave an enrichment factor (ε) of -7.1 ± 0.5 ‰ (Figure 5-1C). The quantification of the subsequent fractionation steps, for example from TCE to DCE, by the Rayleigh equation was not possible because TCE is an instantaneously formed product which is further dechlorinated to DCE. Qualitatively, TCE became enriched in ^{13}C in the second dechlorination step (day 8) as indicated by an enrichment of ^{13}C (-21.9‰) compared to the substrate PCE (-23.5‰). At this day a very small amount of DCE was also detected, however, it did not accumulate and determination of its carbon isotope composition was not possible. VC, the subsequent dechlorination product, was significantly depleted in ^{13}C (-38‰) compared to TCE and probably DCE. By day 11 VC became enriched again (-29.8‰) and significantly depleted ethene appeared (-39.8‰) suggesting a strong isotope fractionation in the final dechlorination step. Afterwards (day 14) ethene became even more depleted (-48‰) and then it started to become slightly enriched again (-40‰). An isotope balance should be achieved when all chlorinated compounds are dechlorinated and the final product, ethene, reached the isotope composition exhibited by the first substrate (PCE) at the beginning of experiment. This was not observed because the experiment was stopped before the last dechlorination step completed. The VC and ethene present at day 1 were transferred with the inoculum,

which was derived from a culture in the very late dechlorination phase in which PCE, TCE and *cis*-DCE typically were already degraded. Thus the VC and ethene present in the inoculum were transformation products of the previously spiked PCE and therefore they were enriched in ^{13}C compared to the freshly spiked PCE at the beginning of the experiment. The isotope composition of ethene (-28.2‰) was close to the isotope composition of the PCE used as a substrate which is usually around -27 to 28‰ indicating that the isotope balance in the previous experiment was nearly closed and thus the dechlorination reaction completed. The ethene formed in this experiment was highly depleted in ^{13}C indicating a significant isotope fractionation during its formation.


A
Concentrations [µM]
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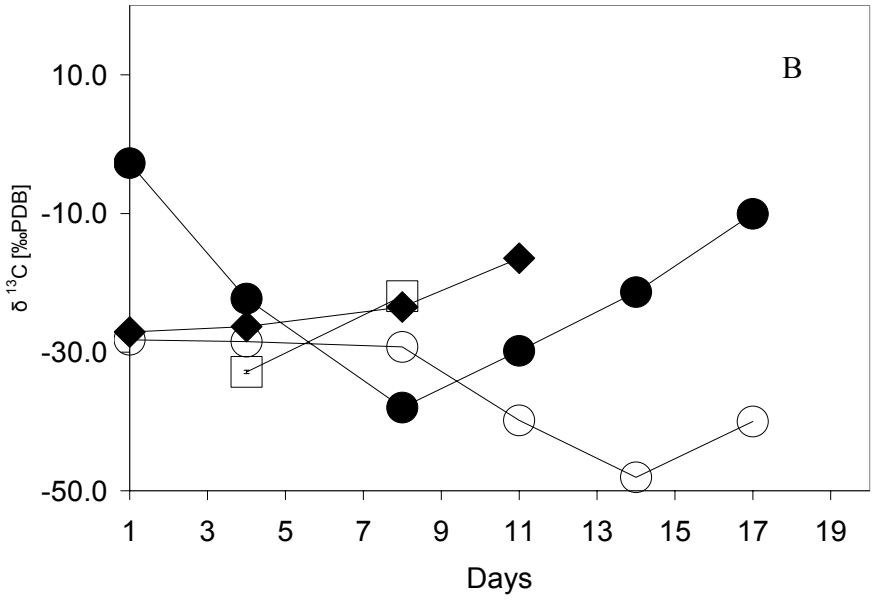
500
400
300
200
100
0
1
3
5
7
9
11
13
15
17
19
Days


B
δ 13C [‰PDB]
10.0
-10.0
-30.0
-50.0
1
3
5
7
9
11
13
15
17
19
Days

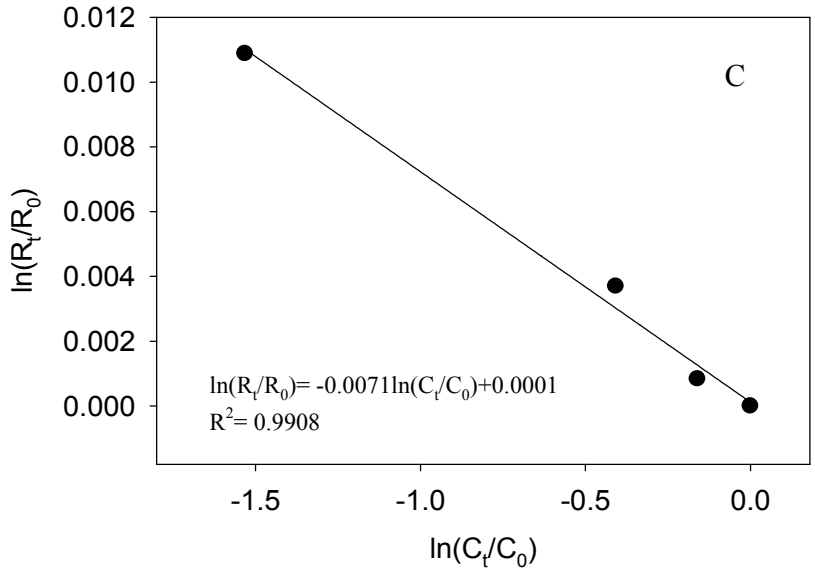


Figure 5-1. Change in concentrations (A) and carbon stable compositions (B) of tetrachloroethene (PCE) (♦) and its products trichloroethene (TCE) (□), *cis*-1,2-dichloroethene (*cis*-DCE) (▲), vinyl chloride (VC) (●) and ethene (○) during the reductive dechlorination of PCE by a mixed culture containing *Dehalococcoides ethenogenes* strain 195. The PCE data were plotted based on the Rayleigh model in (C).

3.3 Carbon Stable Isotope Fractionation of PCDDs

The isotope compositions of 1,2,3,4-TeCDD and its dechlorination products are shown in Figures 5-2B and 5-2D for two replicate cultures without PCE addition. The averages and ranges of isotope compositions of PCDDs for both treatments are listed in Table 5-1. By comparing the averages and ranges of δ values, it is apparent that 1,2,4-TrCDD was

consistently isotopically enriched in ^{13}C compared to the substrate, 1,2,3,4-TeCDD. The 1,3-DCDD was depleted in ^{13}C relative to the substrate, 1,2,3,4-TeCDD and the intermediate, 1,2,4-TrCDD. Similar patterns were observed for triplicate bottles, although the isotopic ranges for each compound varied slightly (see Supporting Information Figure 5-S2). The carbon isotopic compositions of total PCDDs in the system varied from -28.6‰ to -26.6‰. ANOVA was performed on the entire data set to determine if the isotopic compositions of the parent and daughter congeners were significantly different. The *p* values were much less than 0.01 (Table 5-1) confirming that the difference in isotope compositions between different congeners was significant at the 99% confidence level.

Table 5-1. Average carbon stable isotope compositions of polychlorinated dibenzo-*p*-dioxins (PCDDs), isotope balance, and p value from analysis of variance (ANOVA).

δ values of PCDDs in different treatments (‰)		1,2,3,4-TeCDD only	1,2,3,4-TeCDD +PCE
1,2,3,4-TeCDD	Average	-28.5 ± 0.8	-29.2 ± 0.6
	Range	-30.2 ± 0.8 ~ -26.8 ± 1.5	-29.0 ±0.2 ~ -27.2 ± 0.3
1,2,4-TrCDD	Average	-26.8 ± 1.0	-26.8 ± 0.5
	Range	-28.8 ± 0.6 ~ -25.4 ± 0.1	-27.9 ± 0.5 ~ -26.2 ± 0.4
1,3-DCDD	Average	-29.5 ± 0.6	-28.1 ± 0.5
	Range	-30.9 ± 0.8 ~ -28.8 ± 0.4	-30.1 ± 2.0 ~ – 28.0 ± 0.3
Isotope balance		-27.7 ± 0.7	-27.5 ± 0.4
p value from ANOVA		2.57E-12	1.68E-16

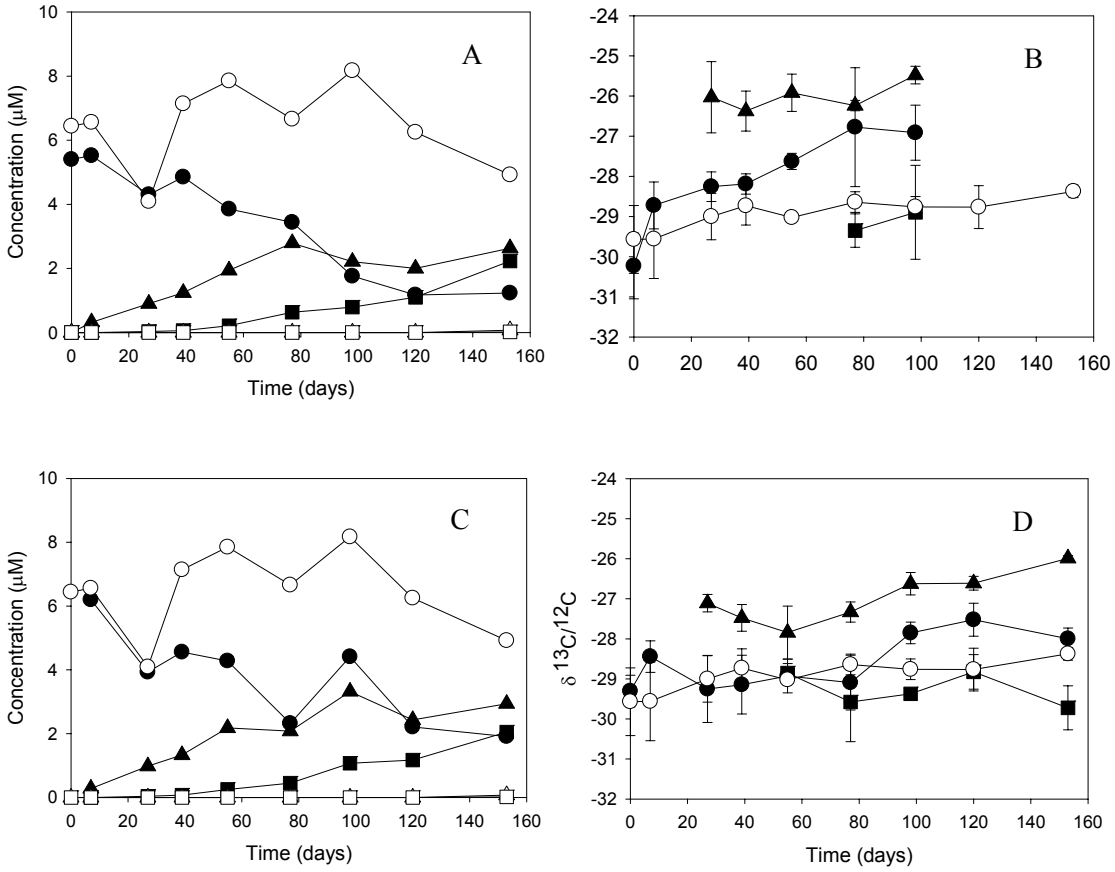
Interestingly, the product 1,2,4-TrCDD of the first dechlorination step was found to be enriched in ^{13}C compared to the substrate 1,2,3,4-TeCDD. Conversely, 1,3-DCDD the product of second dechlorination step was depleted in ^{13}C compared to the precursor 1,2,4-TeCDD. This pattern was found in all replicates (see supporting information). The enrichment of ^{13}C in the product is an uncommon pattern and is unlikely to be a result of the carbon-chlorine bond cleavage at the aromatic ring. Possibly the subsequent dehalogenation step fractionated the metabolite to a greater extent in our experiments.

A greater magnitude of isotope fractionation for dechlorination of 1,2,4-TrCDD to 1,3-DCDD than for 1,2,3,4-TeCDD to 1,2,4-TrCDD may explain the observed isotope pattern. A very similar pattern was found in a recent study of 1,2,4 and 1,2,3 TrCDD degradation by a *Dehalococcoides* containing mixed culture (Ewald et al. 2007). The first transformation step to DCDDs was associated with a lower isotope fractionation than the subsequent step to monochlorodibenzo-*p*-dioxins. A similar phenomenon was observed in the dechlorination of PCE to TCE in this study. TCE became enriched in ^{13}C (-21.9‰) compared to the substrate PCE (-23.5‰) because of the isotope fractionation occurring during the second dechlorination step where TCE was further dechlorinated to *cis*-DCE. The enrichment factor, ε, for 1,2,3,4-TeCDD was estimated from the Rayleigh model (eq 2). Graphical analysis for results from two culture replicates amended with 1,2,3,4-TeCDD as the sole halogenated compound are shown in Figure 5-2E. The ε values were -2.9 ± 0.7 ‰ and -1.1 ± 0.4 ‰, respectively. The analysis of the isotope fractionation by the Rayleigh model relating change in the isotope composition and concentration of the reactant for all other replicates show significant variation and uncertainty ranging from even inverse isotope fractionation pattern to a relatively low

fractionation (Figure 5-S3 and Table 5-S1). For example the Rayleigh analysis for the third replicate culture amended with 1,2,3,4-TeCDD as the sole halogenated compound yielded a slightly inverse carbon isotope fractionation (net positive ε). Similarly the isotope discrimination in the replicate cultures which received PCE in addition to 1,2,3,4-TeCDD was low (see Supporting Information Figure 5-S3). The different isotope compositions of reactant and metabolite qualitatively indicate substantial isotope fractionation during reductive dechlorination, however, the high variability of carbon isotope enrichment factors (ε) as calculated by the Rayleigh approach may indicate certain kinetic limitations previous to the isotope sensitive step which is most likely the carbon – chlorine bond cleavage. Kinetic limitations previous to the isotope sensitive step (Northtop 1981) like dissolution of PCDDs as well as uptake by the organisms may lower the magnitude of isotope fractionation that could be calculated by the Rayleigh approach. Taking a maximum solubility of 0.63 µg/l of 1,2,3,4 TeCDD into account (Shiu et al. 1988), the majority of the spiked substrate is likely sorbed to the sediment material and other suspended or dissolved organic matter and perhaps to the glass surface and, thus, has limited bioavailability. As a fraction of the medium with sediment material containing the 1,2,3,4 TeCDD was analyzed at each time point, the isotope composition of non reacted material which was not bioavailable could be superimposed to some extent on the measured isotope composition. For determination of a precise Rayleigh enrichment factor, the substrate should be readily bioavailable and thus in isotopic equilibrium in all compartments of the experiment (i.e., adsorbed, dissolved, and within the cell). Thus, the extent of isotope fractionation measured in our sediment-culture experiments is only an estimate but the highest enrichment factor we observed may come

close to the biological fractionation that actually occurs within the cell. However the fractionation may indeed be higher if the substrate were readily bioavailable. The solubility of the first metabolite 1,2,4-TrCDD is about 10 time higher than the 1,2,3,4-TeCDD substrate concentration, implying a higher bioavailability. This may contribute to a relatively pronounced discrimination of ^{13}C during the subsequent dechlorination step of 1,2,4-TrCDD to 1,3-DCDD and contribute to the surprising enrichment of ^{13}C in 1,2,4-TrCDD in the course of the experiment.

Our results showed the complications inherent in interpreting enrichment factors with respect to biochemical mechanisms according to the transition state theory, however the results can be used to elucidate kinetic limitations of transformation processes in biological systems (Northrop 1981).


A
B
C
D
Concentration (μM)
Time (days)
δ 13C/12C

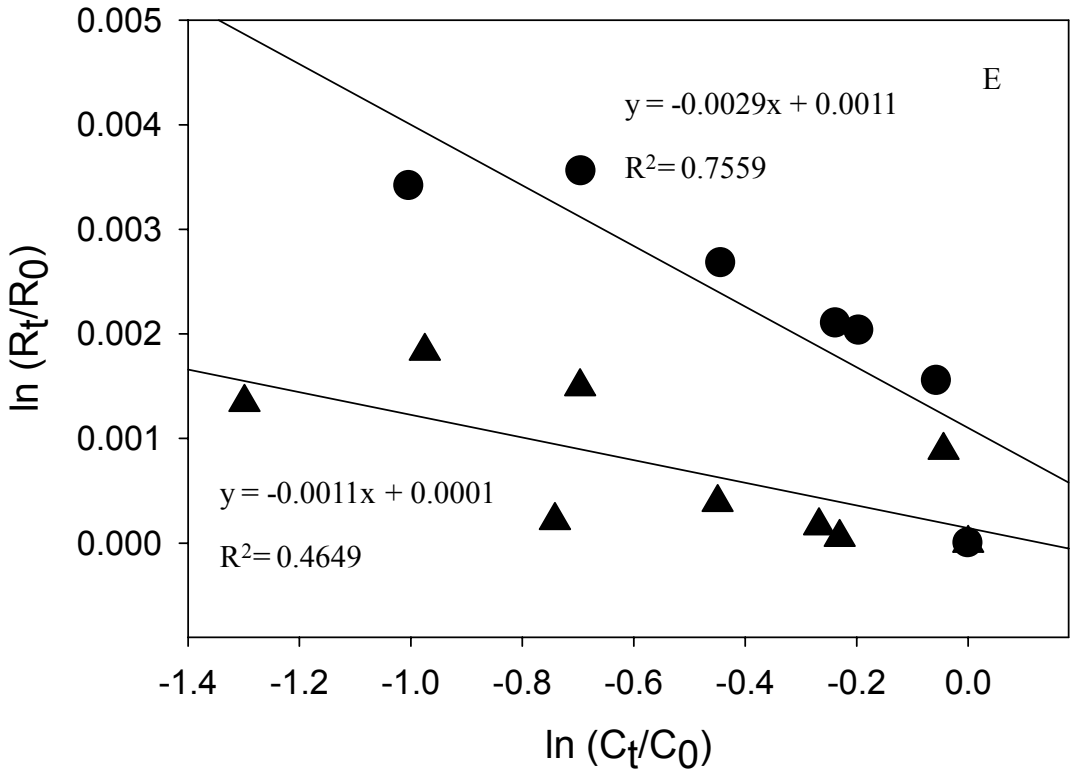


Figure 5-2. Change in concentrations (A, C) and carbon isotope compositions (B, D) of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (●) and its products 1,2,3-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) (▲) and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (■) during reductive dechlorination by a mixed culture containing *Dehalococcoides ethenogenes* strain 195 amended with 1,2,3,4-TeCDD as the sole halogenated substrate in replicate 1 (A, B) and replicate 2 (C, D). Open symbols show results from the killed controls. Data from replicate 1 (●) and replicate 2 (▲) were plotted based on the Rayleigh model in (E) with regression results in solid and dash lines respectively.

3.4 Intrinsic Isotope Enrichment Factors, $\varepsilon_{intrinsic}$

The interpretation of the enrichment factor for characterizing the chemical mechanism of carbon chlorine bond cleavage is limited when non isotope fractionating steps control the overall kinetics of a biochemical reaction. Nontheless, taking this concern into account the $\varepsilon_{intrinsic}$ were calculated in order to compare biochemical fractionation upon the C-Cl bond cleavage in PCDDs with reductive dechlorination reactions of other compounds. The PCDDs contain 12 carbon atoms, which imposes a dilution effect on the magnitude of observed kinetic isotope fractionation. This effect was corrected by calculating intrinsic enrichment factors (eq 5) as shown in Table 2. The intrinsic enrichment factor of PCE was -14.2 ± 1.0 ‰, while the intrinsic enrichment factors of 1,2,3,4-TeCDD were -34.8 ± 8.8 ‰ and –13.0 ± 5.3 ‰, for the two replicate cultures without PCE co-amendment (Table 5-2).

The values of $\varepsilon_{\text{intrinsic}}$ were of the same magnitude for PCE and the PCDDs, which suggests the consistency of the carbon isotope fractionation of the reductive C-Cl bond cleavage under the mediation of *D. ethenogene*s strain 195. The isotope discrimination upon 1,2,3-TrCDD dechlorination by a *Dehalococcoides* containing mixed culture (Ewald et al. 2007) was of the same order of magnitude as we observed in our mixed culture containing *D. ethenogenes* strain 195 (Table 5-2.). Dechlorination of 1,2,4-TrCDD in the study of Evald et al. was also associated with isotope discrimination but

the magnitude was significantly lower and could not be evaluated with the Rayleigh approach (Ewald et al. 2007).

Griebler et al. (2004) investigated carbon isotope fractionation of trichlorobenzenes during dechlorination by *Dehalococcoides* sp. strain CBDB1 and calculated intrinsic enrichment factors. Our values are comparable to those obtained from their study (Table 5-2). Both studies investigated carbon isotope fractionation during the reductive dechlorination of chlorinated compounds by *Dehalococcoides* and obtained very similar $\varepsilon_{intrinsic}$ for various compounds, which suggests the magnitude of the carbon isotope fractionation during the reductive C-Cl bond cleavage is similar and may share a similar mechanism.

The maximum kinetic isotope effect according to semiclassical calculation is -54.0 ‰ (Elsner et al. 2005; Huskey 1991). The $\varepsilon_{intrinsic}$ values for 1,2,3,4-TeCDD, PCE and chlorinated benzenes are slightly lower (Table 5-2). Although these biological reactions were typically associated with other kinetically non-fractionating steps, e.g., sorption-desorption, the calculated values fall in the range expected for cleavage of the C-Cl bond (Elsner et al. 2005).

Table 5-2. Carbon stable isotope enrichment factors, ε, and intrinsic carbon stable isotope enrichment factors, $\varepsilon_{intrinsic}$, observed during the reductive dechlorination of 1,2,4- and 1,2,3-trichlorobenzenes, 1,2,3- and 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,3-TrCDD and 1,2,4-TrCDD), tetrachloroethene (PCE) and 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD).

Compound	Microorganisms	ε ‰	$\varepsilon_{intrinsic}$ ‰	Reference
1,2,4-Trichlorobenzene	*Dehalococcoides* sp. strain CBDB1	-3.2	-19.5	(Griebler et al.2004)
1,2,3-Trichlorobenzene	*Dehalococcoides* sp. strain CBDB1	-3.5	-22.1	(Griebler et al.2004)
	Dehalococcoides sp. strain CBDB1 with methyl viologen	-3.4	-20.8	(Griebler et al.2004)
1,2,3 TrCDD	*Dehalococcoides mixed culture*	-0.92	-11.0 ± 2.2	(Ewald et al. 2007)
1,2,4 TrCDD	*Dehalococcoides mixed culture*	> 0.05		(Ewald et al. 2007)
PCE	*Dehalococcoides ethenogenes* strain 195	-7.1 ± 0.5	-14.2 ± 1.0	This study
1,2,3,4-TeCDD replicate 1	Mixed culture containing *Dehalococcoides ethenogenes* strain 195	-2.9 ± 0.7	-34.8 ± 8.8	This study
1,2,3,4-TeCDD replicate 2	Mixed culture containing *Dehalococcoides ethenogenes* strain 195	-1.1 ± 0.4	-13.0 ± 5.3	This study

3.5 Comparison to Other Fractionation Studies

Carbon stable isotope fractionation has been observed previously for biodegradation of many organic compounds. We investigated carbon stable isotope fractionation during the dechlorination of 1,2,3,4-TeCDD and PCE. Isotope signatures of PCDDs from both treatments and from replicate bottles showed similar patterns in that 1,2,4-TrCDD was enriched and 1,3-DCDD was depleted in ^{13}C compared to the educt, 1,2,3,4-TeCDD. There were significant differences between the isotope compositions of 1,2,3,4-TeCDD, 1,2,4-TrCDD and 1,3-DCDD according to the ANOVA results (Table 5-1). The ^{13}C isotope enrichment of 1,2,4-TrCDD compared with its parent compound was surprising. However, comparable fractionation patterns were reported for dechlorination of chloroethenes and TrCDD by *Dehalococcoides* containing cultures (this study; Ewald et al. 2007). Bloom et al. (2000) detected enriched ^{13}C in *cis*-DCE relative to TCE in the transformation of TCE to *cis*-DCE. Nijenhuis et al. (2005) described a similar pattern in the biotransformation of PCE to *cis*-DCE via TCE by *Desulfitobacterium* sp. strain PCE-S and *Sulfurospirillum multivorans.* TCE became enriched in ^{13}C compared with PCE in the course of the degradation experiment probably due to larger carbon isotope fractionation in subsequent dechlorination steps (Nijenhuis et al. 2005). In this study, isotopic fractionation during PCE dechlorination followed this pattern where TCE became enriched in ^{13}C relative to PCE. The second daughter product instantaneously formed upon dechlorination of the first daughter, TCE, become strongly enriched at the end of the experiment suggesting significant isotope fractionation during the

dechlorination reaction of TCE. The isotope composition of 1,2,4-TrCDD was thus controlled by the two sequential dechlorination steps of 1,2,3,4-TeCDD. The transformation of 1,2,4-TrCDD to 1,3-DCDD also influences the carbon isotope patterns and this contributes to the characteristic isotope composition of the metabolites which we observed during the 1,2,3,4 TeCDD transformation. Additionally, the possibly limited bioavailability of 1,2,3,4-TeCDD in our sediment slurry experiment affected the isotope fractionation of the parent to some extent. However, our experiments may indicate how an isotope composition pattern of lightly chlorinated PCDDs in sediments may develop as reductive dechlorination processes proceed. A similar isotope fractionation pattern was observed during experiments with TrCDD (Ewald et al. 2007). The reasonthat subsequent dechlorination steps exhibited a higher fractionation extent is not clear, We speculate that the bioavailability of the metabolite is greater than that of the parent compound. A lower concentration and higher water solubility of the metabolite as well as its formation in the vicinity of the dehalogenase may contribute to its higher bioavailability, which may reduce kinetic limitation due to sorption-desprption processes, uptake or transport within the cell all of which are known to reduce or dampen the isotope fractionation effect (Nijenhuis et al, 2005*)*. Further research is needed for a mechanistic understanding of isotope fractionation during sequential dechlorination reactions, however as observed for sequential PCE dechlorination the less chlorinated compounds become more strongly fractionated than their precursors.

Interestingly, Drenzek et al. (2001) did not observe significant isotope fractionation during reductive dechlorination of 2,3,4,5-tetrachlorobiphenyl (2,3,4,5-TeCB) to 2,3,5-trichlorobiphenyl (2,3,5-TrCB), compounds with similarities to the PCDDs. The isotope

compositions of 2,3,4,5-TeCB and 2,3,5-TrCB remained nearly constant during the dechlorination process. The daughter product was slightly heavier than the educt, with an average difference of 0.3‰ in their isotope compositions and the $\delta\ ^{13}C$ values were significantly different at the 95% confidence level by *t*-test. The differences we observed during PCDD dechlorination were more pronounced. There was an average difference of 1.6‰ between 1,2,4-TrCDD and 1,2,3,4-TeCDD and -2.8‰ between 1,3-DCDD and 1,2,4-TrCDD, in cultures without PCE addition. For the set with PCE addition, the differences were 1.3‰ and -2.4‰, respectively. These differences were significant at the 99% confidence level. This clearly indicated that the dechlorination reactions were associated with isotope fractionation and showed that reductive dechlorination can alter the carbon isotope composition of compounds which have 12 carbon atoms at a detectable level.

3.6 Environmental Implications

To our knowledge, this study is only the second which investigated the isotope fractionation of PCDDs during biological reductive dechlorination (Ewald et al. 2007). Microbial reductive dechlorination of the more highly chlorinated congeners to congeners with 1 to 3 chlorines has been well documented in sediment enrichments (Bunge et al. 2001; Vargas et al. 2001; Fu et al. 2001). Specific bacterial strains have also been shown to reductively dechlorinate some PCDD congeners (Bunge et al. 2003; Fennell et al. 2004). Documenting reductive dechlorination of PCDDs *in situ* has proven to be quite difficult. Confirmation of pathways of dechlorination and increases in concentrations of

dechlorination daughter products may be difficult to demonstrate because of the large numbers of congeners that may be present, and because regulatory requirements result in quantification of only the highly toxic 2,3,7,8-substituted congeners in most environmental samples. Since lightly chlorinated congeners are more soluble than highly chlorinated congeners, they are more prone to movement from the sediment to the water column and to volatilization (Lohmann et al. 2003). However, since the lightly chlorinated congeners (mono-, di- and tri-chlorinated) are far less toxic than the more highly chlorinated congeners, these compounds are not routinely analyzed in environmental samples, with only a few exceptions (Lohmann et al. 2000, 2003). It is unclear whether significant reductive dechlorination of historical PCDDs has occurred *in situ* in contaminated sediments. Barabás et al. (2004[a,b]) detected a dechlorination fingerprint using geostatistical analysis of historical PCDD sediment concentrations. Observation of carbon isotope fractionation resulting from reductive dechlorination of PCDDs could provide an additional method for analysis of this process in the environment. The characteristic changes of isotope compositions of PCDDs related to reductive dechlorination could be obtained in the laboratory and applied to the field by extrapolation. However, this procedure should be undertaken with caution since the fate of PCDDs in the environment is complex and under the influence of multiple physical, chemical and biological processes. Unlike chloroethenes, the investigation of the isotope fractionation during transformation of PCDDs is in an initial stage. More research is needed to expand the knowledge base—e.g., are the fractionation patterns observed related to the activity of specific enzymes, cometabolic versus respiratory transformation, or differences in rates of reaction? This study illustrated that isotope fractionation

occurred during the microbial reductive dechlorination of PCDDs and the enrichment factors quantified for this process were similar to those for other chlorinated compounds undergoing reductive dechlorination. The unique isotope pattern illustrated here might be used as an evidence for biotransformation processes.

4. Supporting Information

4. 1 Calculation of the Kinetic (or Intrinsic) Isotope Effect

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

$$KIE = \frac{{}^{l}K}{{}^{h}K} \tag{S1}$$

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

The isotope fractionation factor α can be defined as

$$\alpha = \frac{{}^{h}K}{{}^{l}K} \tag{S2}$$

Thus

$$\alpha = \frac{1}{KIE} \tag{S3}$$

Further,

$$\varepsilon = (\frac{1}{KIE} - 1) \times 1000 \quad \text{(S4)}$$

The ε calculated from equation S4 is the intrinsic enrichment factor which reveals the real difference between the reaction rates of molecules containing different isotope species on the reactive site.

However, the enrichment factor ε calculated based on the Rayleigh equation from experimental data is the averaged isotope effect of the total molecule. Elsner et al. (2005) elaborated factors that mask the intrinsic kinetic isotope effect as the dilution effect from nonreactive position where the isotope presents in the molecule other than the reactive site, the intramolecular competition of isotopes in indistinguishable reactive positions, and the influence of rate limiting steps prior to bond breakage/commitment to catalysis.

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

$$\varepsilon_{reactive\ position} = n/x \bullet \varepsilon_{bulk} \quad \text{(S5)}$$

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

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

$$\varepsilon_{intrinsic} = z \bullet \varepsilon_{reactive\ position} \quad \text{(S6)}$$

where z is the number of indistinguishable reactive positions in one molecule that the isotope of interest might occupy. For the primary isotope effect in non-concerted reactions, $z = x$. Therefore, equation S12 may also be expressed as

$$\varepsilon_{intrinsic} = n \bullet \varepsilon_{bulk} \quad \text{(S7)}$$

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

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

For 1,2,3,4-TeCDD, $n = 12$ and $x = 1$. There is no intramolecular competition in the molecule of 1,2,3,4-TeCDD, thus the intrinsic enrichment factor of 1,2,3,4-TeCDD is $12\times \varepsilon_{bulk}$.

The Streitwieser semiclassical limits for isotope effects at 25°C is 1.057 (Elsner et al. 2005; Huskey 1991) for C-Cl bond breakage, which is a theoretical prediction of the maximum KIE, denoted as KIE_M. Based on eq S4, the maximum isotope enrichment factor is calculated as -53.9‰.

As Elsner et al. (2005) discussed, realistic values with an assumption of the transition state at 50% bond breakage would yield a predicted KIE value of 1.03. Similarly, the corresponding isotope enrichment factor is -29.1‰, comparable to the absolute values for $\varepsilon_{intrinsic}$ obtained in this study (Table 5-2).

4.2 Linearity of GC-C-IRMS Response for PCDDs

The linearity of the detector response of the GC-C-IRMS system was assessed for 1,2,3,4-TeCDD over a detector output range of 80 to 1400 mV (Figure 5-S1). A linear regression of the detector response had a slope of 0.82, indicating that the nonlinearity of the instrument during the analysis of 1,2,3,4-TeCDD was about 0.8 ‰ per 10-fold increase in the detector response between 80 and 1400 mV. This systematic error is higher than that reported for other compounds. For example, the detector response for toluene exhibited a nonlinearity of 0.1‰ per 10-fold increase in the detector response between 45 and 3500 mV (Richnow et al. 2003). This could be a result of the poor combustibility of PCDDs related to their high molecular weight and stable molecular structure. These characteristics may result in higher error during GC-C-IRMS measurement of the stable carbon isotope ratios of the PCDDs. The values were not corrected for linearity and isotope values were reported for measurements where concentrations were of the same order of magnitude and thus comparable wherever possible.

4.3 Carbon Stable Isotope Fractionation of PCDDs

Besides the two replicates presented in the paper, the remainder of the PCDD data are presented here, including three replicates from the treatment with PCE addition and

replicate 3 from the treatment with 1,2,3,4-TeCDD as the sole halogenated compound (Figure 5-S2). The data were regressed based on Rayleigh model (Figure 5-S3).

The enrichment factors and intrinsic enrichment factors are listed in Table 5-S1. The results show that the enrichment factors of 1,2,3,4-TeCDD during the reductive dechlorination were very low and their variability is large. This is understandable considering several factors. The physical-chemical properties of PCDDs make them difficult candidates for isotope analysis. As discussed previously, they have a stable structure and poor combustibility, which may result in great error in isotope analysis. The compound specific isotope analysis requires good separation in the components of samples, which sometimes is hard to fulfill in practice. It also requires that the compound of interest in the samples reach a certain concentration to obtain reasonably precise measurements. Although it has been shown with variability, carbon isotope fractionation was observed in the reductive dechlorination of 1,2,3,4-TeCDD. The unique pattern of the isotope compositions of the PCDDs and the quantitative analysis with Rayleigh equation confirmed carbon isotope fractionation during 1,2,3,4-TeCDD dechlorination.

4.4 Carbon Stable Isotope Mass Balance

In a closed system, the mass balance on the isotopes of carbon in different compounds in the system is expressed as

$$R_{Total,t} = \sum R_{i,t} \times f_{i,t} = \sum R_{i,0} \times f_{i,0} \qquad \text{(S8)}$$

where $R_{Total,t}$ is the carbon isotope ratio of the total pool of parent compound and its metabolites in the system at time t. Theoretically, $R_{Total,t}$ should be constant over the course of the reaction. $R_{i,0}$ is the carbon isotopic composition of a chemical *i* at time 0 and $f_{i,0}$ is the fraction of chemical *i* at time 0. Similarly, $R_{i,t}$ and $f_{i,t}$ are its carbon isotopic composition and fraction at time *t*. The isotope mass balance was calculated as averages of the three replicates in the treatments with and without PCE addition (Figure 5-S4).

Table 5-S1. Carbon stable isotope enrichment factor,ε, and intrinsic carbon stable isotope enrichment factor, $\varepsilon_{\text{intrinsic}}$, observed during the reductive dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) in a mixed culture containing *Dehalococcoides ethenogenes* strain 195 with and without addition of tetrachloroethene (PCE).

Compound	ε (‰)	$\varepsilon_{intrinsic}$ (‰)	r^2
1,2,3,4-TeCDD replicate 1	-2.9 ± 0.7	-34.8 ± 8.8	0.244
1,2,3,4-TeCDD replicate 2	-1.1 ± 0.4	-13.0 ± 5.3	0.465
1,2,3,4-TeCDD replicate 3	0.4 ± 0.3	4.2 ± 4.2	0.125
1,2,3,4-TeCDD+PCE replicate 1	-0.1 ± 0.3	-1.4 ± 4.1	0.016
1,2,3,4-TeCDD+PCE replicate 2	-0.4 ± 0.2	-4.7 ± 2.5	0.344
1,2,3,4-TeCDD+PCE replicate 3	-0.1 ± 0.2	-0.6 ± 2.1	0.013

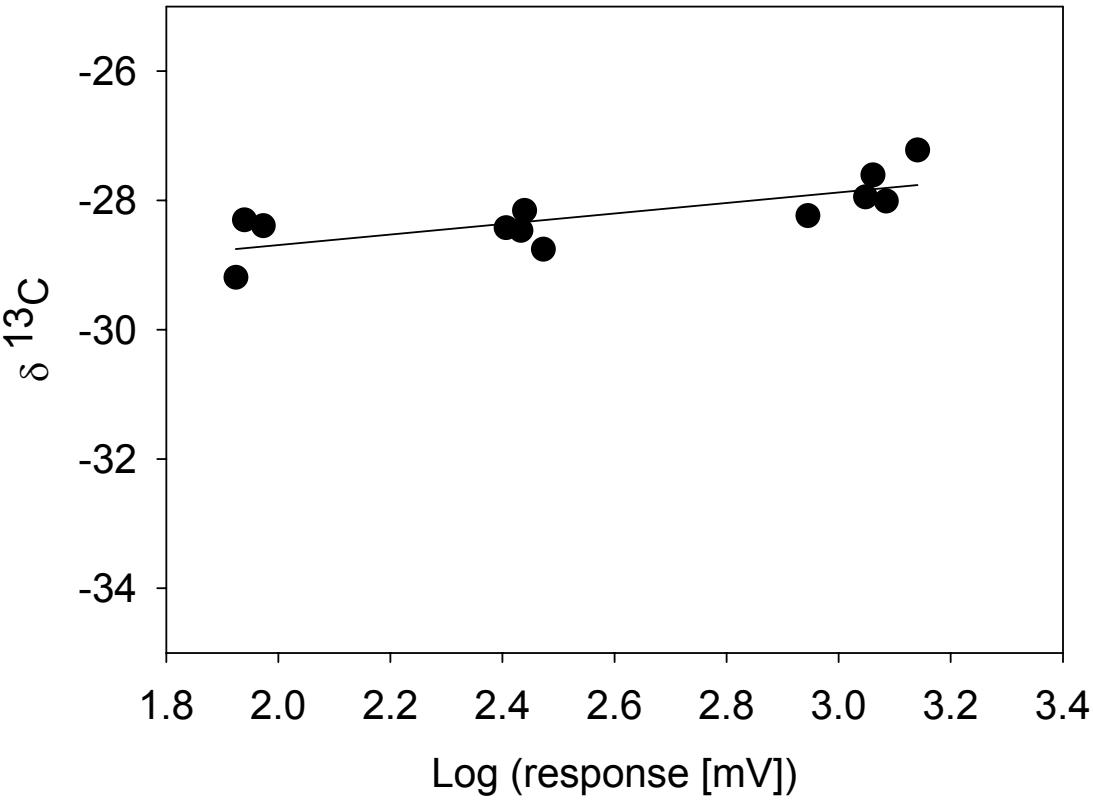
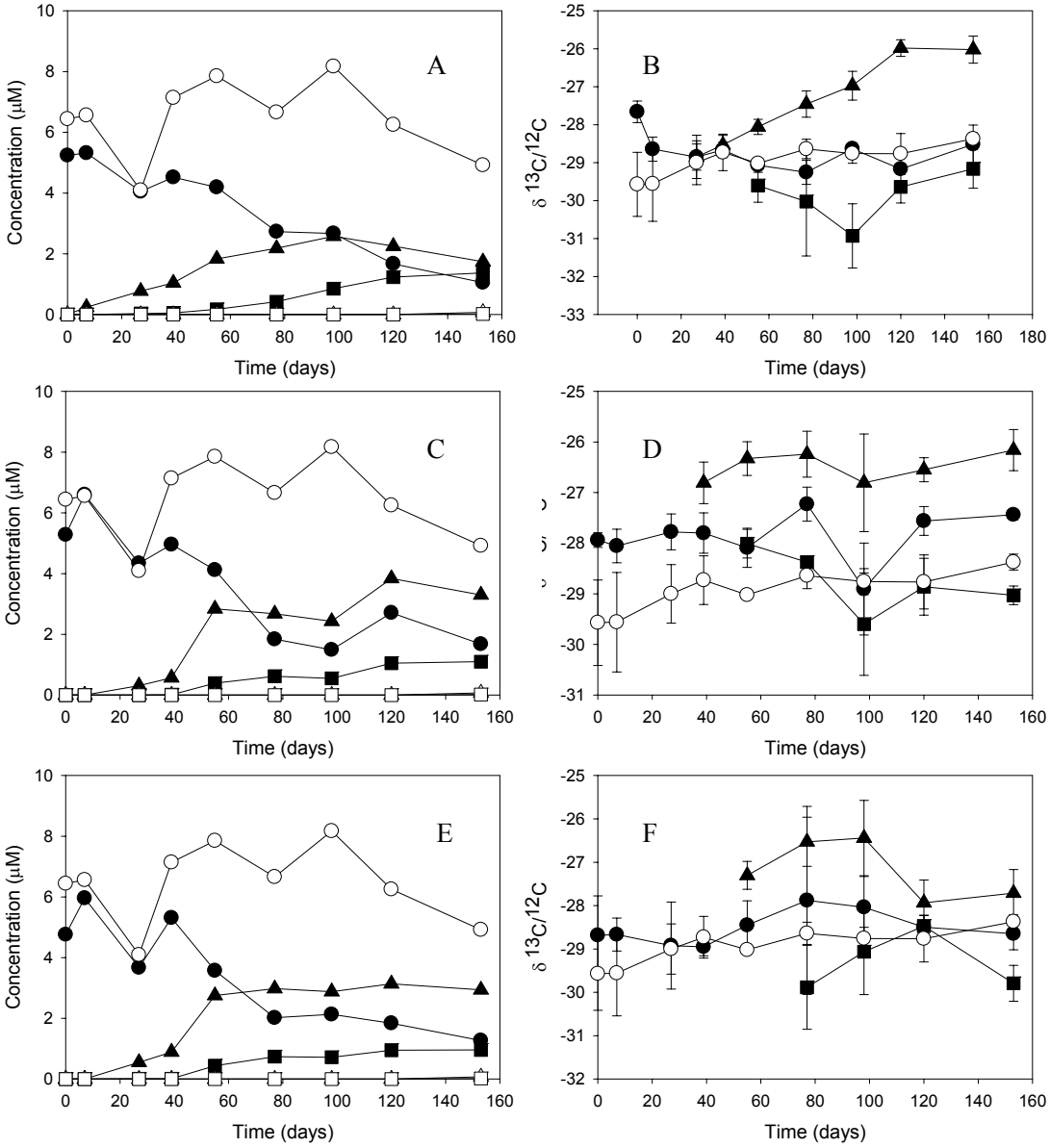


Figure 5-S1. Linearity of the detector response of the GC-C-IRMS system for the 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) standard over a detector output range of 80 to 1400 mV.


A
B
C
D
E
F
Concentration (μM)
δ 13C/12C
Time (days)

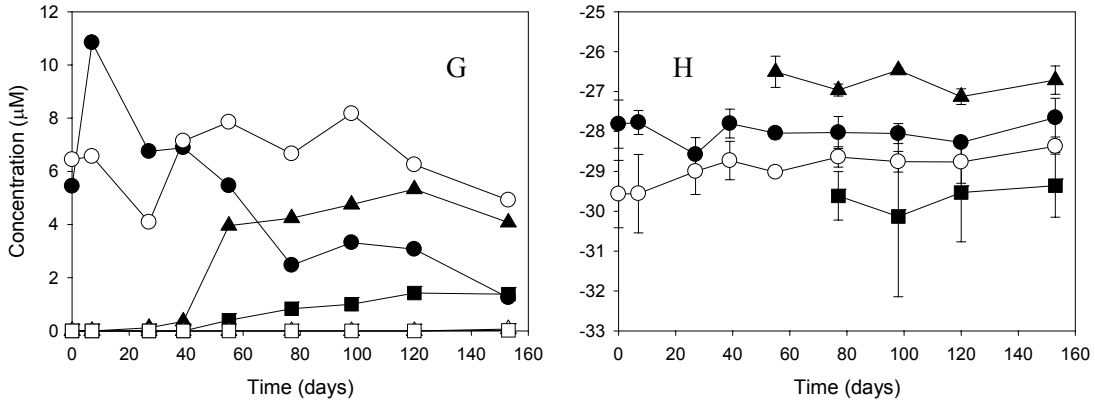


Figure 5-S2. Change in concentrations (A, C, E, G) and carbon isotope compositions (B, D, F, H) of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (●) and its products 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) (▲) and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (■) during reductive dechlorination by the mixed culture containing *Dehalococcoides ethenogenes* strain 195 in replicate 3 (A, B) from treatment with 1,2,3,4-TeCDD as the sole halogenated substrate and replicate 1 (C, D), 2 (E, F) and 3 (G, H) from the treatment with PCE addition. Open symbols are results from autoclaved controls while closed symbols are results from active samples.

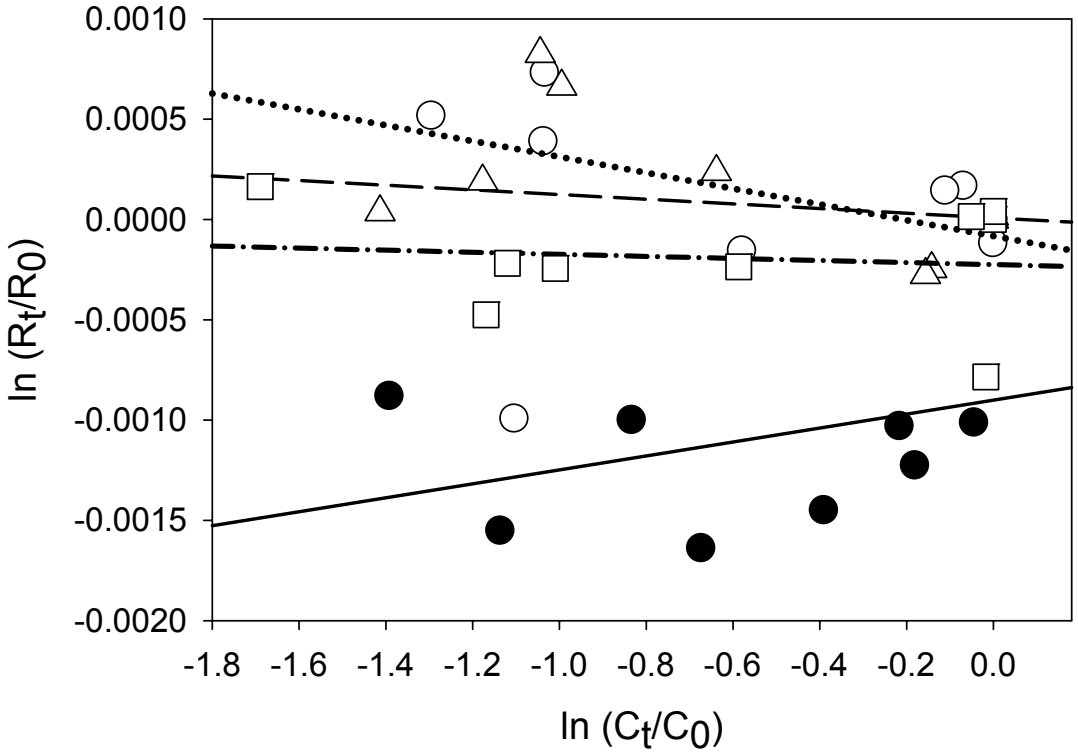


Figure 5-S3. Rayleigh model regression of replicate 3 (●) in solid line from treatment with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) as the sole halogenated substrate, and replicate 1 (○) in dashed line, replicate 2 (Δ) in dotted line and replicate 3 (□) in dash-dot line from the treatment with PCE addition.

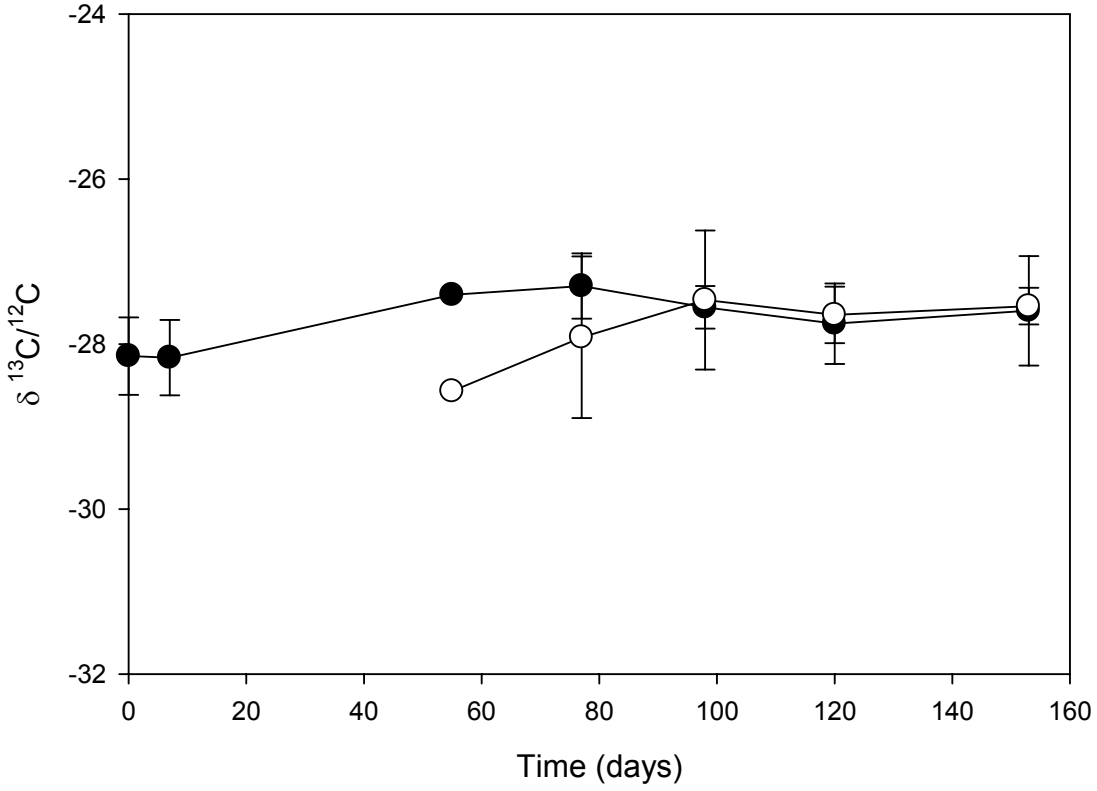


Figure 5-S4. The isotope mass balance of the treatment with tetrachloroethene (PCE) (●) and without PCE (○) addition.

Chapter 6

Strategies for Enhancing PCDD/F Dechlorination in Sediments: Haloprimers and Bioaugmentation Enhance the Dechlorination of PCDD/Fs

1. Introduction

The accumulation of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) in sediment has been reported worldwide (Bopp et al. 1991; Kjeller & Rappe 1995; Verta et al. 2007). In the environment, sediment acts as a sink for PCDD/Fs, especially the highly chlorinated ones (Hites 1990) because of their high hydrophobicity, low solubility, low vapor pressure, and the corresponding high affinity for organic matter. PCDD/Fs may undergo reductive dechlorination by microorganisms and biogenic materials in the anoxic sediment environment. The reductive dechlorination of PCDD/Fs has been observed in several laboratory studies of enrichment cultures or microorganisms eluted from sediments (Adriaens & Grbić-Galić 1994; Adriaens et al. 1995; Beurskens et al. 1995). Both the dechlorination of historical PCDD/Fs and freshly spiked PCDD/Fs were observed in microcosms prepared with contaminated sediments (Barkovskii & Adriaens 1996; Albrecht et al. 1999).

Dechlorination of PCDD/Fs is a slow process with a time frame of months or even years. The slow rates may be attributable to several factors. The intrinsic physical-

chemical properties of the PCDD/Fs including high octanol-water partitioning coefficients (K_{ow}) and exceedingly low solubilities in water correlate to their preferential association with the sediment rather than the water phase (Harms 1998). Their strong sorption to sediments may reduce their availability to microorganisms, including dechlorinators. The lack of available electron donors, co-factors or other nutrients for microorganisms in the environment could impede the dechlorination. Furthermore, the environmental concentrations of PCDD/Fs is typically part per trillion to part per billion, thus providing only very low substrate concentrations to support the growth of microorganisms (Harms 1998).

Two *Dehalococcoides* species, *Dehalococcoides* sp. strain CBDB1 and *Dehalococcoides ethenogenes* strain 195 have been shown to dechlorinate PCDD/Fs (Bunge et al. 2003; Fennell et al. 2004). Growth by dehalorespiration of PCDDs has been shown only for *Dehalococcoides* sp. strain CBDB1 (Bunge et al. 2003). The direct or implicated involvement of environmental strains of *Dehalococcoides* spp. in the dehalogenation of PCDD/Fs (Ballerstedt et al. 2004; Yoshida et al. 2005; Ahn et al. 2007 Manuscript) and closely related compounds, the polychlorinated biphenyls (PCBs) (Fagervold et al. 2005; Yan et al. 2006; Bedard et al. 2007) may indicate their importance in the biotransformation of these compounds in the environment.

Previous studies have shown that the extent and pattern of dechlorination of both PCBs and PCDD/Fs is affected by amendments such as electron donors or halogenated compounds as priming agents (haloprimers) (Albrecht et al. 1999; Bedard et al. 1998; Vargas et al. 2001; Ahn et al. 2005). The addition of haloprimers was shown by Bedard et al. (1998) and DeWeerd and Bedard (1999) to enhance dechlorination of

polychlorinated biphenyls (PCBs). Albrecht et al. (1999) reported that octachlorodibenzo-*p*-dioxin (OCDD) was dechlorinated to 2-monchlorodibenzo-*p*-dioxin (2-MCDD) in sediments from Passaic River, New Jersey, USA after amendment with a haloprimer, 2-monobromodibenzo-*p*-dioxin (2-MBDD), and hydrogen. A recent investigation of the microbial community eluted from those Passaic River sediments also showed that the addition of the haloprimer affected both the dechlorination extent and pattern (Fu et al. 2005). 1,2,3,4,6,7,8-Heptachlorodibenzo-*p*-dioxin (1,2,3,4,6,7,8-HpCDD) was spiked under methanogenic and sulfidogenic conditions with or without the amendment of 2-MBDD. It was observed that the presence of 2-MBDD increased dechlorination extent up to 10% and decreased the formation of 2,3,7,8-substituted congeners, apparently enhancing preferential lateral dechlorination over peri dechlorination. Dechlorination under methanogenic conditions proceeded from 1,2,3,4,6,7,8-HpCDD to MCDDs, while dechlorination stopped at dichlorodibenzo-*p*-dioxins (DCDDs) under sulfidogenic conditions. A microbial consortium enriched from Arthur Kill, New Jersey, USA sediment using a mixture of 2-, 3-, and 4-bromophenol exhibited enhanced dechlorination of 1,2,3,4-TeCDD compared to unenriched sediments (Vargas et al. 2001). Ahn et al. (2005) examined the effect of haloprimers including bromophenols (BPs), chlorophenols (CPs), 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB), 2,3,4,5-tetrachloroanisole (2,3,4,5-TeCA), 2,3,4,5-tetrachlorophenol (TeCP), 2,3,5,6-tetrachlorobenzoate (TeCBA), and 2',3',4'-trichloroacetophenone (TrCAP) on dechlorination of 1,2,3,4-TeCDD and 1,2,3,4-tetrachlorodibenzofuran (1,2,3,4-TeCDF) in marine and estuarine sediments. Results showed that haloprimers with more analogous

structure to PCDD/Fs, such as 1,2,3,4-TeCB and 2,3,4,5-TeCA, enhanced the dechlorination of 1,2,3,4-TeCDD more significantly than BPs and CPs.

Direct addition of haloprimers such as 1,2,3,4-TeCB to sediments *in situ* to enhance bioremediation is problematic, because of the potentially negative environmental impact of the haloprimers themselves. However, some of these compounds could be used for *ex situ* application in confined treatment or for producing active bioaugmentation cultures that may then be added to sediments. Furthermore, studies of haloprimer effect on PCDD/F dechlorination may lead to breakthroughs with respect to understanding the nature of PCDD/F dechlorinating bacteria.

In this study, we examined the effectiveness of a variety of amendments for the dechlorination of a spiked model PCDD congener, 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) in microcosms prepared with sediments from three contaminated sites: River Kymijoki, Finland; Gulf Island Pond, Maine, USA; and Lake Roosevelt, Washington, USA. We amended the microcosms with a single or combined addition of electron donor and haloprimers, and with bioaugmentation by a mixed culture containing *Dehalococcoides ethenogenes* strain 195 (River Kymijoki only). Specifically, *D. ethenogenes* strain 195 dechlorinates 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) to 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD), and subsequently to 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (Fennell et al. 2004). We also added different haloprimers to the mixed culture to see if they improved the dechlorination of PCDD/Fs. Results from this study could provide useful information to direct the remedial operations in PCDD/Fs-contaminated sediments.

2. Materials and Methods

2.1 Chemicals

The 1,2,3,4-TeCDD, 1,2,4-TrCDD, 1,2,3-trichlorodibenzo-*p*-dioxin (1,2,3-TrCDD), 1,3-DCDD, 2,3-dichlorodibenzo-*p*-dioxin (2,3-DCDD), 1,4-dichlorodibenzo-*p*-dioxin (1,4-DCDD), 1-monochlorodibenzo-*p*-dioxin (1-MCDD), 2-monochlorodibenzo-*p*-dioxin (2-MCDD), dibenzo-*p*-dioxin (DD) and 2,2',5-trichlorobiphenyl (2,2',5-PCB) were purchased from AccuStandard, Inc (New Haven, CT). The 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB), 2',3',4'-trichloroacetophenone (2', 3', 4'-TrCAP), and 2-chlorophenol (2-CP) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Sediment Samples

Sediments were obtained from River Kymijoki, Finland, Gulf Island Pond, Maine, USA and Lake Roosevelt, Washington, USA. River Kymijoki is heavily contaminated by pulp and paper mill effluents and from the manufacturing of chloro-alkali chemicals. The sediments are highly contaminated with chlorinated phenols, PCDD/Fs, and polychlorinated diphenyl ethers (PCDEs) that originated from production of a chlorophenolic wood preservative, Ky-5 (Isosaari et al. 2002). Gulf Island Pond is a 15 mile long impoundment in the Androscoggin River, above which there are three large paper companies with discharge into the river (Anonymous). Lake Roosevelt is on the Columbia River and was formed by the construction of the Grand Coulee Dam. It has been reported to be contaminated with trace elements discharged as slag materials from a

smelter (USGS). Sediments were generously provided by Dr. Matti Verta (Finnish Environment Institute, Helsinki, Finland) for River Kymijoki, Mr. Barry Mower (Maine, Department of Environmental Protection) for Gulf Island Pond, and Dr. Steve Cox (U.S. Geological Survey) for Lake Roosevelt. Sediments were packed into sterile glass jars, sealed, and stored at 4°C until used.

2.3 Medium and Microcosm Preparation

Dechlorination experiments in the sediments were carried out in 100 mL volumes in 160-mL serum bottles. One microcosm study was set up for Gulf Island Pond and Lake Roosevelt sediments, and two separate microcosm studies were performed for River Kymijoki sediments. Microcosm set up was performed as described previously (Liu & Fennell 2007). Briefly, one gram of dry sterile sediment was added to each bottle followed by spiking of 1,2,3,4-TeCDD stock solution in toluene as a coating on the dry sediment. Toluene was allowed to dry under a sterile N_2 purge, leaving a coating of 1,2,3,4-TeCDD on the dry sediment. The resulting nominal concentration of 1,2,3,4-TeCDD (assuming no partitioning and uniform distribution throughout the microcosm volume) was 15.5 μM. The second microcosm study performed for River Kymijoki sediments utilized a nominal 1,2,3,4-TeCDD concentration of 50 μM.

Sediment microcosms were prepared using a 20% (vol:vol) sediment and 80% (vol:vol) medium slurry. The medium used for microcosm set up and maintenance of a mixed culture containing *Dehalococcoides ethenogenes* strain 195 was prepared as described previously (Fennell 1998). The medium contained 0.2 g/L NH_4Cl, 0.072 g/L

K_2HPO_4, 0.055 g/L KH_2PO_4, 0.2 g/L $MgCl_2 \cdot 6H_2O$, and 0.1 g/L $FeCl_2 \cdot 4H_2O$. The medium was brought to boil in a flask then purged with N_2 for thirty minutes. After cooling, one mL of 500 g/L $Na_2S \cdot 6H_2O$ stock solution was added. The purging gas was changed from N_2 to 70%/30% N_2/CO_2 and $NaHCO_3$ was added to a final concentration of 2.5 g/L. Stock solutions of vitamins and trace metals were added as previously described (Fennell et al. 2004; Fennell 1998). Sediments were added to the medium under a 70%/30% N_2/CO_2 purge to make slurries. A 100 mL volume of the well mixed slurry was then dispensed to each 1,2,3,4-TeCDD spiked 160 mL serum bottle under a 70%/30% N_2/CO_2 gas purge. Bottles were capped with gray PTFE coated butyl rubber septa and crimped with aluminum caps.

The experimental protocol for the setup of the first microcosm study is shown in Table 6-1. All microcosms received 1,2,3,4-TeCDD. Triplicate killed controls were prepared by autoclaving microcosms for 45 min on three consecutive days. The live controls only received 1,2,3,4-TeCDD and sediment slurry. Sodium lactate and sodium propionate were added as electron donors to the designated treatments with a final concentration of 500 μM each. Haloprimers 1,2,3,4-TeCB and 2',3',4'-TrCAP were prepared in methanol stock solutions which were spiked to achieve a final concentration of 25 μM in the microcosms. A 0.8 mL volume of a saturated aqueous solution of tetrachloroethene (PCE), a known growth substrate for *D. ethenogenes* strain 195 was added as a haloprimer to obtain a final concentration of 10 μM. A mixed culture containing *D. ethenogenes* strain 195 was maintained with butyric acid and PCE at 2:1 electron equivalent ratio at 25°C (Fennell et al. 2004; Fennell 1998). One mL of the culture was added to the bioaugmented treatments. The mixed culture contained 2×10^8

Dehalococcoides cells per mL (see Chapter 4). All the stock solutions were prepared under anaerobic and sterile conditions. Serum bottles were incubated at 28°C for 3 months in the dark, while shaken at 100 rpm. These microcosms were sampled for initial and final conditions.

A second set of microcosms were set up with River Kymijoki sediments (designated as River Kymijoki set II (Table 6-1)). This microcosm set was sampled more frequently than the first in order to better delineate the progress of dechlorination in response to different biostimulants. Microcosms were prepared with the same procedures as described above. The haloprimers 2-CP and 1,2,3,4-TeCB were added at a final concentration of 500 μM and 25 μM, respectively. Electron donors were not added to this set of microcosms because results from the previous experiment showed that the addition of electron donor did not stimulate the dechlorination relative to the live control. The mixed culture was added at 2 mL per bottle to the bioaugmentation treatments. Serum bottles were incubated at 28°C for 126 days in the dark, while shaken at 100 rpm. These microcosms were sampled initially and at 36, 79, and 126 days.

The effect of PCE and 1,2,3,4-TeCB, both growth substrates for strain 195, on the dechlorination of PCDD/Fs by the strain 195-containing mixed culture was determined in previously described experiments (Liu & Fennell 2007; Chapter 4, Liu et al. Isotope manuscript [Chapter 5]). Experiments were carried out with 1,2,3,4-TeCDD or 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) as the PCDD/F congeners and PCE or 1,2,3,4-TeCB as the haloprimers.

Table 6-1. Experimental protocols for sediment microcosm study (the number of replicates is shown for each treatment).

Treatments	River Kymijoki	Gulf Island Pont	Lake Roosevelt	River Kymijoki Set II
Killed control	2	3	3	3
Live control	2	3	3	3
Electron donor (Ed)[a]	2	3	3	
Ed[a] + 1,2,3,4-TeCB[b]	3	3	3	
Ed[a] + 2',3',4'-TrCAP[c]	3	3	3	
Ed[a] + Bioaugmentation[d]	3			
Ed[a] + Bioaugmentation[d] +1,2,3,4-TeCB[b]	3			
Ed[a] + Bioaugmentation[d] +PCE[e]	3			
2-CP[f]				3
1,2,3,4-TeCB[b]				3
Bioaugmentation[d]				3
2-CP[f] + Bioaugmentation[d]				3
1,2,3,4-TeCB[b] + Bioaugmentation[d]				3

a. Electron donor was a mixture of sodium lactate and sodium propionate at 500 μM each.

b. 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) at 25 μM.

c. 2',3',4'-trichloroacetophenone (2',3',4'-TrCAP) at 25 μM.

d. 1 mL mixed culture that contained 2 x 10^8 *Dehalococcoides* per mL was added to each bottle.

e. 0.8 mL of a saturated tetrachloroethene (PCE) aqueous solution was added to give a final PCE concentration of 10 μM.

f. 2-chlorophenol (2-CP) added at 500 μM.

2.4 Analytical Methods

Two mL of well mixed sediment slurry was removed from the microcosms at each time point. The samples were extracted and prepared for gas chromatography coupled to mass spectrometry (GC-MS) analysis for the PCDD/Fs and 1,2,3,4-TeCB, as described previously (Liu & Fennell 2007). Filtered supernatant from the sediment slurry samples was used for 2-CP analysis with high performance liquid chromatography (LC-10AS; Shimadzu Corp., Kyoto, Japan) as described previously (Ahn et al. 2003).

3. Results

3.1 Dechlorination of 1,2,3,4-TeCDD in Biostimulated Microcosms

After three months incubation, sediment microcosms from all three sites dechlorinated 1,2,3,4-TeCDD to various extents depending upon amendments. The molar percents of the 1,2,3,4-TeCDD and dechlorination daughter products detected at the end of three months are shown in Table 6-2.

Table 6-2. Molar percents (%) of major polychlorinated dibenzo-*p*-dioxin (PCDD) congeners in the sediment microcosms at the end of the incubation period.

Sediments /Amendments	1,2,3,4-TeCDD	1,2,4-TrCDD	1,2,3-TrCDD	1,3-DCDD	2,3-DCDD	1,4-DCDD	2-MCDD
River Kymijoki (Incubated for 3 months)							
Killed control	100			0	0		0
Live	23.6			1.9	2.5		71.0
Electron donor (Ed[a])	23.4			1.4	2.5		72.4
Ed[a] + 1,2,3,4-TeCB[b]	18.6			2.5	1.9		77.0
Ed[a] + Bioaugmentation[d]	19.3			1.2	2.6		77.0
Ed[a] + Bioaugmentation[d] + 1,2,3,4-TeCB[b]	11.8			6.2	3.5		78.5
Ed[a] + Bioaugmentation[d] + PCE[e]	18.3			15.8	7.9		58.0
Gulf Island Pond (Incubated for 3 months)							
Killed control	100	0		0	0		0
Live control	48.5	1.6		7.6	14.2		28.0
Electron donor (Ed[a])	48.9	1.2		6.7	15.0		28.2
Ed[a] + 1,2,3,4-TeCB[b]	12.8	0		1.2	1.1		84.9
Ed[a] + 2',3',4'-TrCAP[c]	11.6	0		4.6	11.0		82.8
Lake Roosevelt (Incubated for 3 months)							
Killed control	100	0				0	
Live control	99.7	0.3				0	
Electron donor (Ed[a])	99.2	0.8				0	
Ed[a] + 1,2,3,4-TeCB[b]	87	2.8				10.2	
Ed[a] + 2',3',4'-TrCAP[c]	83.2	16.8				0	

Table 6-2 Continued

River Kymijoki (set II) (Incubated for 126 days)						
Killed control	100.0	0.0	0.0	0.0	0.0	0.0
Live control	44.7	0.0	0.4	2.7	3.3	48.8
2-CP[f]	44.6	0.1	0.3	2.4	2.4	50.3
1,2,3,4-TeCB[b]	40.7	0.1	0.2	3.8	0.7	54.5
Bioaugmentation[d]	46.7	0.3	0.2	2.9	2.2	47.7
Bioaugmentation[d] + 2-CP[f]	52.7	0.4	0.2	4.6	3.9	38.2
Bioaugmentation[d] + 1,2,3,4-TeCB[b]	49.2	1.4	0.1	7.2	0.9	41.1

a. Electron donor was a mixture of sodium lactate and sodium propionate at 500 μM each.

b. 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) at 25 μM.

c. 2',3',4'-trichloroacetophenone (2',3',4'-TrCAP) at 25 μM.

d. 1 mL mixed culture that contained 2 x 10^8 *Dehalococcoides* per mL was added to each bottle.

e. 0.8 mL of a saturated tetrachloroethene (PCE) aqueous solution was added to give a final PCE concentration of 10 μM.

f. 2-chlorophenol (2-CP) added at 500 μM.

In the microcosms prepared with sediments from River Kymijoki, extensive dechlorination was observed for all active treatments, with only 12% - 24% of 1,2,3,4-TeCDD remaining after 3 months. No dechlorination was observed in the killed controls. The 1,2,3,4-TeCDD was transformed mostly to 2-MCDD with the formation of small amounts of 1,3-DCDD and 2,3-DCDD (Figure 6-1A). The only exception was the amendment with electron donor, bioaugmentation and PCE, in which less extensive dechlorination was observed when compared to microcosms that received an amendment of electron donor and bioaugmentation alone. Although the transformation of 1,2,3,4-

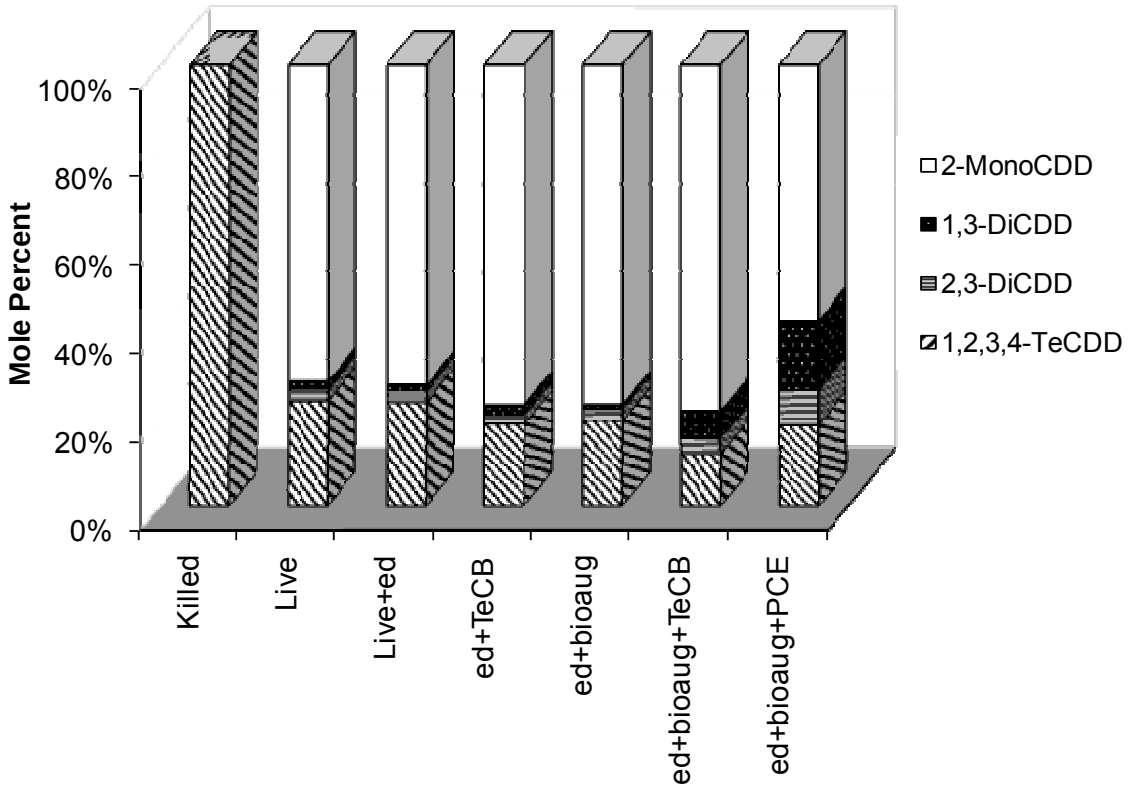
TeCDD was similar in these two treatments, the microcosm set amended with PCE had a greater residual of 1,3-DCDD and 2,3-DCDD and less 2-MCDD. The best performance was observed in microcosms amended with electron donor, bioaugmentation, and 1,2,3,4-TeCB. This treatment had slightly lower 1,2,3,4-TeCDD and higher 2-MCDD mole percentages relative to other amendments after 3 months. Even the live control, which only received the sediment and the 1,2,3,4-TeCDD, exhibited very active dechlorination. This indicated the presence of appropriate electron donors and indigenous microorganisms capable of dechlorinating 1,2,3,4-TeCDD.

In the microcosms prepared with Gulf Island Pond sediments, dechlorination of 1,2,3,4-TeCDD was observed in all active treatments (Figure 6-1B). No dechlorination was observed in the killed controls. The two microcosms sets with the haloprimers, 1,2,3,4-TeCB and 2',3',4'-TrCAP, showed the most extensive dechlorination, with 85% and 83%, respectively, of the 1,2,3,4-TeCDD transformed to 2-MCDD. The 1,3-DCDD and 2,3-DCDD were detected at small amounts (< 5 mol %) as intermediates. No TrCDD metabolites were detected. The extent of dechlorination in the microcosms amended with electron donor and in live controls was similar with 49 mol % of 1,2,3,4-TeCDD remaining in the microcosms after 3 months. The mole percents of 1,3-DCDD and 2,3-DCDD were 8% and 14% for live controls, and 7% and 15% for amendments with electron donors. A small amount of 1,2,4-TrCDD was formed in both treatments. The dechlorination pattern from all active treatments was consistent, with the detection of 1,2,4-TrCDD, 1,3-DCDD, and 2,3-DCDD as intermediates and the final product of 2-MCDD. The intermediate, 1,2,4-TrCDD, was not observed in the amendments with haloprimers, probably because of the extensive dechlorination. The live controls

receiving only 1,2,3,4-TeCDD achieved similar extent of dechlorination as the microcosms receiving electron donors. This demonstrated that sediments from this site are probably not electron donor-limited and thus the addition of electron donor might not be necessary. On the contrary, the addition of haloprimers greatly enhanced the dechlorination.

Far less dechlorination was observed in the microcosms prepared from Lake Roosevelt sediments (Figure 6-1C). A very small amount (< 1 mol%) 1,2,4-TrCDD was formed in the live control and electron donor-amended microcosms. The addition of haloprimers enhanced the dechlorination somewhat. Microcosms amended with electron donor and 2', 3', 4'-TrCAP formed approximately 20 mol% 1,2,4-TrCDD. Notably, the dechlorination pattern was different from the pathways observed for the two other sites in that 1,4-DCDD was formed at the expense of 1,2,4-TrCDD in microcosms amended with electron donor and 1,2,3,4-TeCB.

(A)


100%
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Killed
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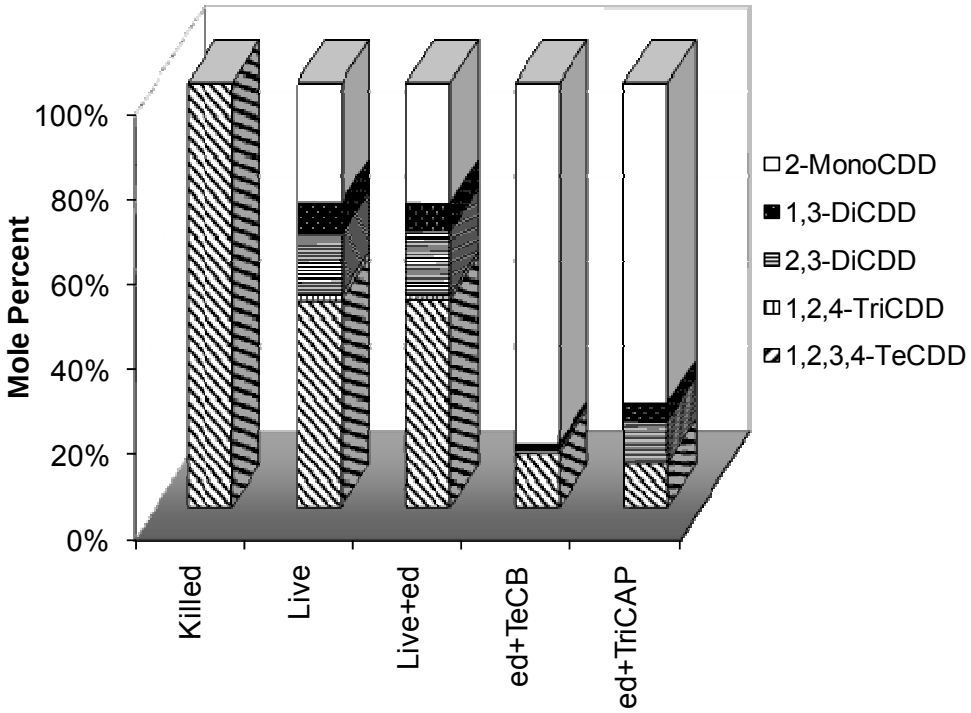
ed+bioaug+PCE
2-MonoCDD
1,3-DiCDD
2,3-DiCDD
1,2,3,4-TeCDD

(B)


100%
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1,2,3,4-TeCDD

(C)

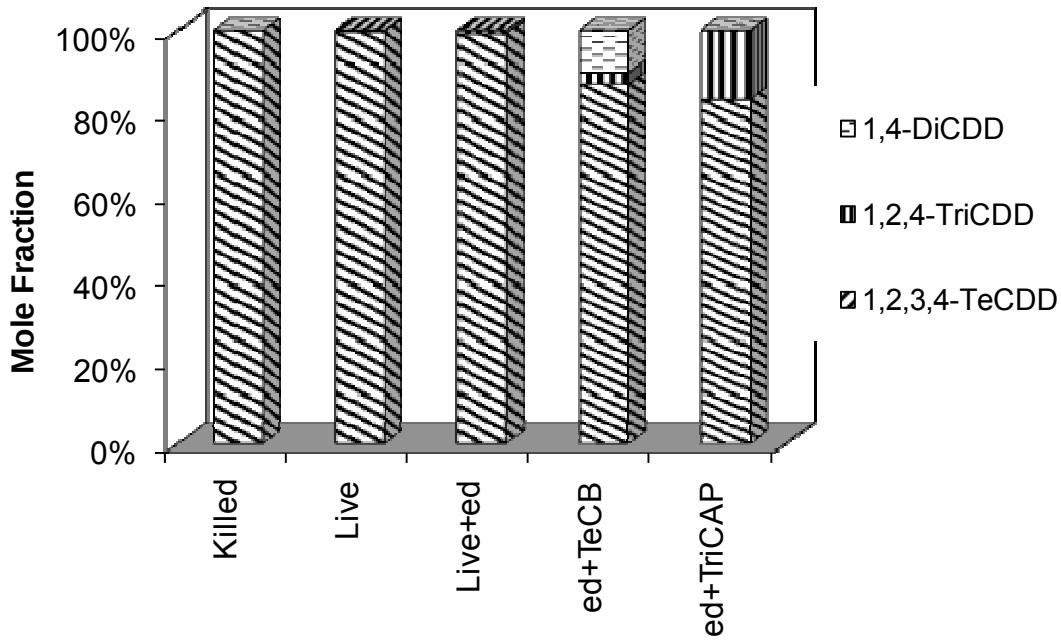


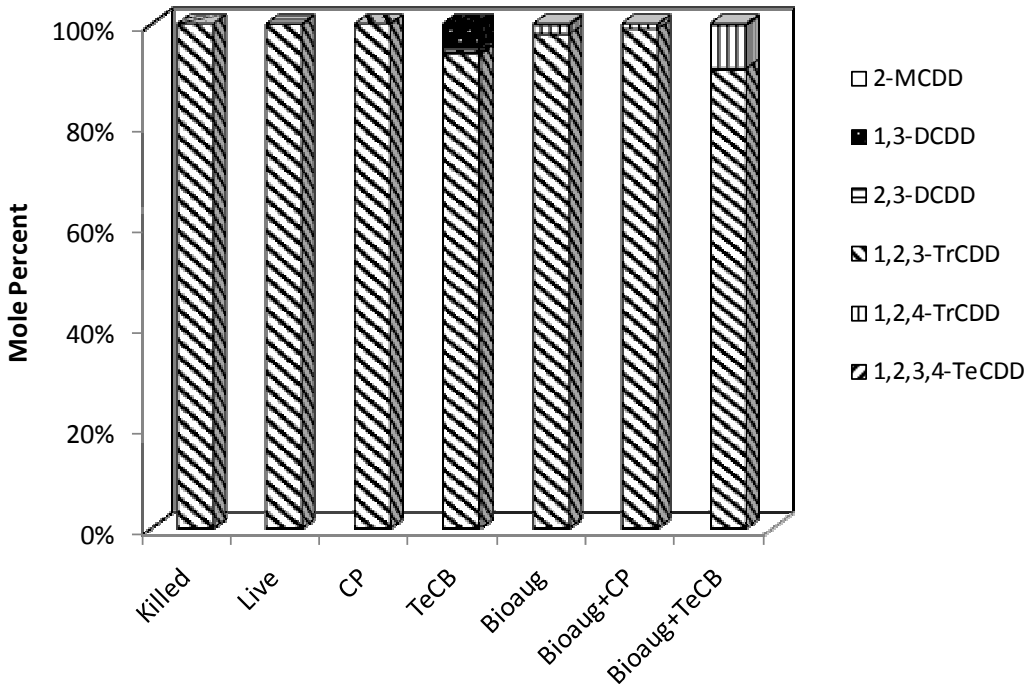
Figure 6-1. Dechlorination of 1,2,3,4-terachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) to 1,2,3-trichlorodibenzo-*p*-dioxin (1,2,3-TrCDD), 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD), 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD), 2,3-dichlorodibenzo-*p*-dioxin (2,3-DCDD), 1,4-dichlorodibenzo-*p*-dioxin (1,4-DCDD), and/or 2-monochlorodibenzo-*p*-dioxin (2-MCDD) in sediments from River Kymijoki (A), Gulf Island Pond (B), and Lake Roosevelt (C) under different conditions after three months of incubation. The meaning of acronyms are as follows: Killed – autoclaved control; Live – live control; Live+ed – amendment with electron donors; ed+TeCB – amendment with electron donors, and 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB); ed+bioaug – amendment with electron donors, and bioaugmentation; ed+bioaug+TeCB – amendment with electron donors, bioaugmentation, and (1,2,3,4-TeCB); ed+bioaug+PCE – amendment with electron donors, bioaugmentation, and tetrachloroethene (PCE); and ed+TrCAP – amendment with electron donors and 2',3',4'-trichloroacetophenone (2',3',4'-TrCAP).

3.2 Dechlorination of 1,2,3,4-TeCDD with River Kymijoki Sediment (Set II)

In the second set of microcosms prepared with River Kymijoki sediments (set II), the sampling interval was shorter. Samples were taken at days 0, 36, 79, and 126. On day 36 (Fig 6-2A), the dechlorination of 1,2,3,4-TeCDD in amendments with 1,2,3,4-TeCB alone and bioaugmentation plus 1,2,3,4-TeCB showed the greatest amount of dechlorination product formation. In microcosms amended with 1,2,3,4-TeCB alone, 1,3-DCDD was the primary daughter product. In the bioaugmented microcosms amended with 1,2,3,4-TeCB, 1,2,4-TrCDD was the primary daughter product. A lesser amount of dechlorination products were detected in microcosms that were bioaugmented either alone or plus 2-CP, after 36 days. Dechlorination was negligible in the killed controls, live controls, and 2-CP amended microcosms. By day 79 (Fig 6-2B), the amendment with bioaugmentation and 1,2,3,4-TeCB exhibited significant dechlorination—far more advanced compared to other treatments. More than 40 mol% of the 1,2,3,4-TeCDD was transformed to 2-MCDD, with 1,2,4-TrCDD, 1,2,3-TrCDD, 1,3-DCDD, and 2,3-DCDD detected as intermediates. The abundance of 1,2,4-TrCDD and 1,3-DCDD were dominant relative to the 1,2,3-TrCDD and 2,3-DCDD. This pathway matched that of *D. ethenogenes* strain 195 which dechlorinates 1,2,3,4-TeCDD to 1,2,4-TrCDD, and subsequently to 1,3-DCDD (Fennell et al. 2004). All other active treatments exhibited dechlorination, although the extent varied. No dechlorination was detected in the killed controls. The dominant formation of 2,3-DCDD was observed in the

microcosms with bioaugmentation alone and bioaugmentation plus 2-CP. However, in microcosms amended with 1,2,3,4-TeCB the pathway also proceeded preferentially through 1,3-DCDD. On day 126 (Fig 6-2C), all active microcosms illustrated similarly extensive dechlorination, as observed from the first microcosm study with River Kymijoki sediment. The DCDDs, 1,3-DCDD and 2,3-DCDD, were detected at low levels (< 10 mol%). Trace 1,2,4-TrCDD and 1,2,3-TrCDD were detected as well. It was worth noting that in the two microcosm sets amended with 1,2,3,4-TeCB, i.e., 1,2,3,4-TeCB alone and bioaugmentation plus 1,2,3,4-TeCB; 1,3-DCDD was the dominant DCDD congener. This was consistent with the results from day 79.

(A)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Killed
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Bioaug
Bioaug+CP
Bioaug+TeCB
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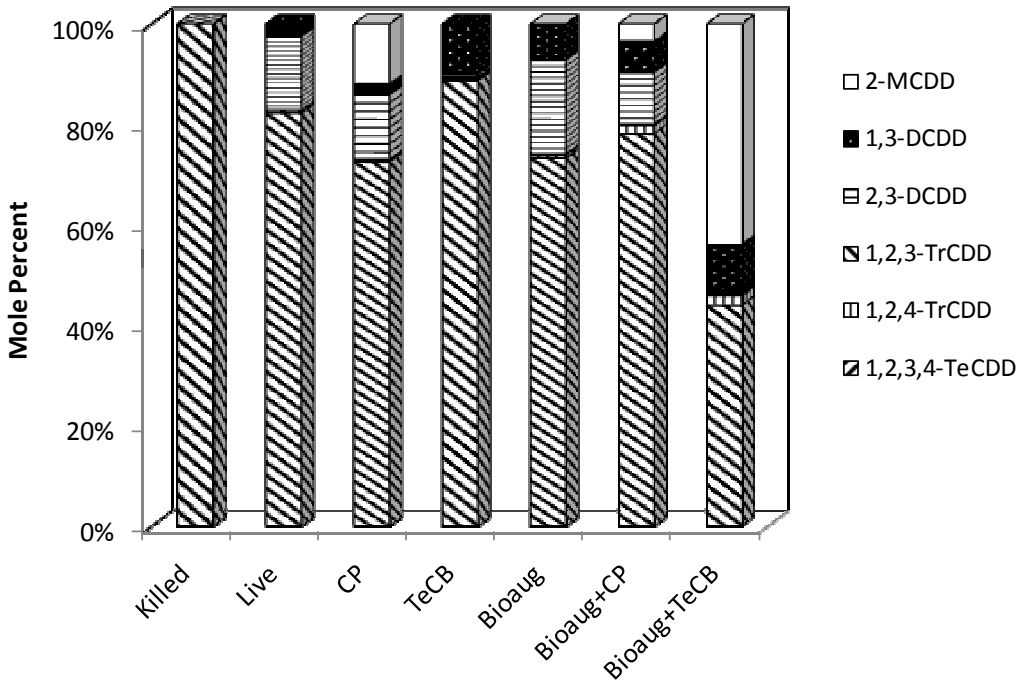
2,3-DCDD
1,2,3-TrCDD
1,2,4-TrCDD
1,2,3,4-TeCDD

(B)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Bioaug
Bioaug+CP
Bioaug+TeCB
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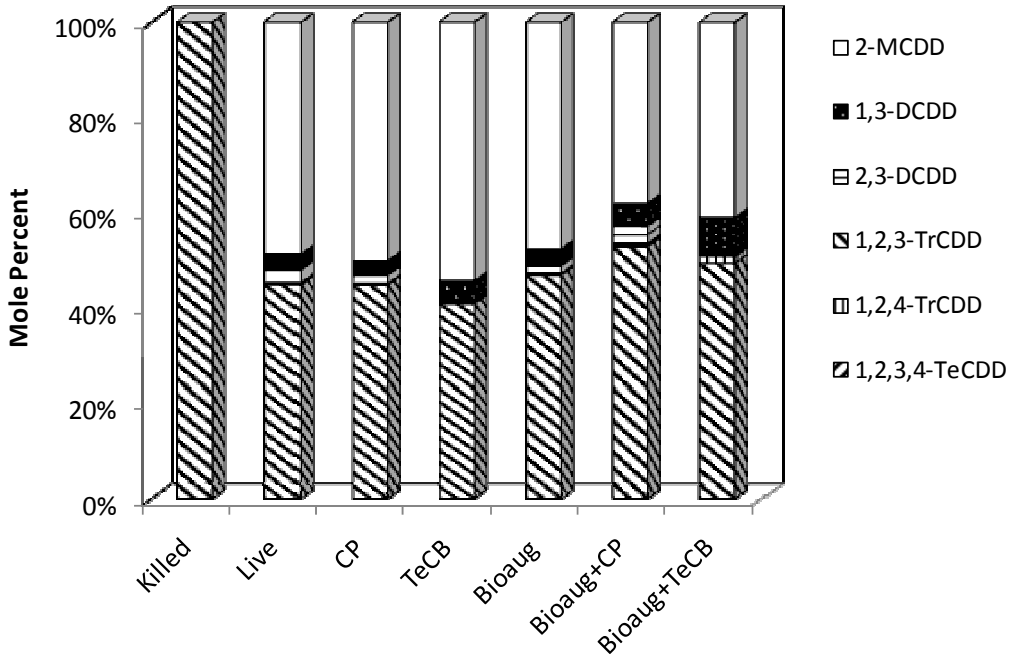
1,3-DCDD
2,3-DCDD
1,2,3-TrCDD
1,2,4-TrCDD
1,2,3,4-TeCDD

(C)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2-MCDD
1,3-DCDD
2,3-DCDD
1,2,3-TrCDD
1,2,4-TrCDD
1,2,3,4-TeCDD

Figure 6-2. Dechlorination of 1,2,3,4-terachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) to 1,2,3-trichlorodibenzo-*p*-dioxin (1,2,3-TrCDD), 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD), 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD), 2,3-dichlorodibenzo-*p*-dioxin (2,3-DCDD), and/or 2-monochlorodibenzo-*p*-dioxin (2-MCDD) under different conditions in River Kymijoki sediments, after incubation for 36 days (A), 79 days (B), and 126 days (C). The meaning of acronyms are as follows: Killed – autoclaved control; Live – live control; CP – amendment with 2-chlorophenol (2-CP); TeCB – amendment with 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB); bioaug – amendment with bioaugmentation; bioaug+2-CP – amendment with bioaugmentation and 2-CP; bioaug+TeCB – amendment with bioaugmentation, and 1,2,3,4-TeCB.

3.3 Dechlorination of PCDD/Fs by the Mixed Culture Containing *D. ethenogenes* sp. Strain 195 during Amendment with Haloprimers

Dechlorination of 1,2,3,4-TeCDD and 1,2,3,4,7,8-HxCDF was observed under different conditions by the mixed culture containing *D. ethenogenes* strain 195 (Liu & Fennell 2007;Liu et al. Isotope manuscript [Chapter 5]). PCE did not stimulate the dechlorination of 1,2,3,4-TeCDD as expected. The two sets with and without PCE addition exhibited similar overall extents of dechlorination of 1,2,3,4-TeCDD at the end of incubation (Figure 6-3), however, up until day 40, a slight lag in 1,2,4-TrCDD production was observed in the treatment with PCE addition, while dechlorination started without delay in the treatment without PCE. More of the final product, 1,3-DCDD was formed in the treatment without PCE than the one with PCE. Similar results were observed in the kinetic study (Chapter 4) where the rate of dechlorination of 1,2,3,4-TeCDD was faster in cultures amended with 1,2,3,4-TeCDD alone, versus cultures where 1,2,3,4-TeCDD and PCE were added together.

PCE did not stimulate the dechlorination of 1,2,3,4,7,8-HxCDF (Liu & Fennell 2007; Chapter 4). Rates and extent of dechlorination were similar for cultures spiked with 1,2,3,4,7,8-HxCDF and those spiked with PCE plus 1,2,3,4,7,8-HxCDF (See Chapter 4, Table 4-5. In contrast to PCE, 1,2,3,4-TeCB enhanced the dechlorination rate of 1,2,3,4,7,8-HxCDF approximately 3-fold (see Chapter 4, Table 4-5).

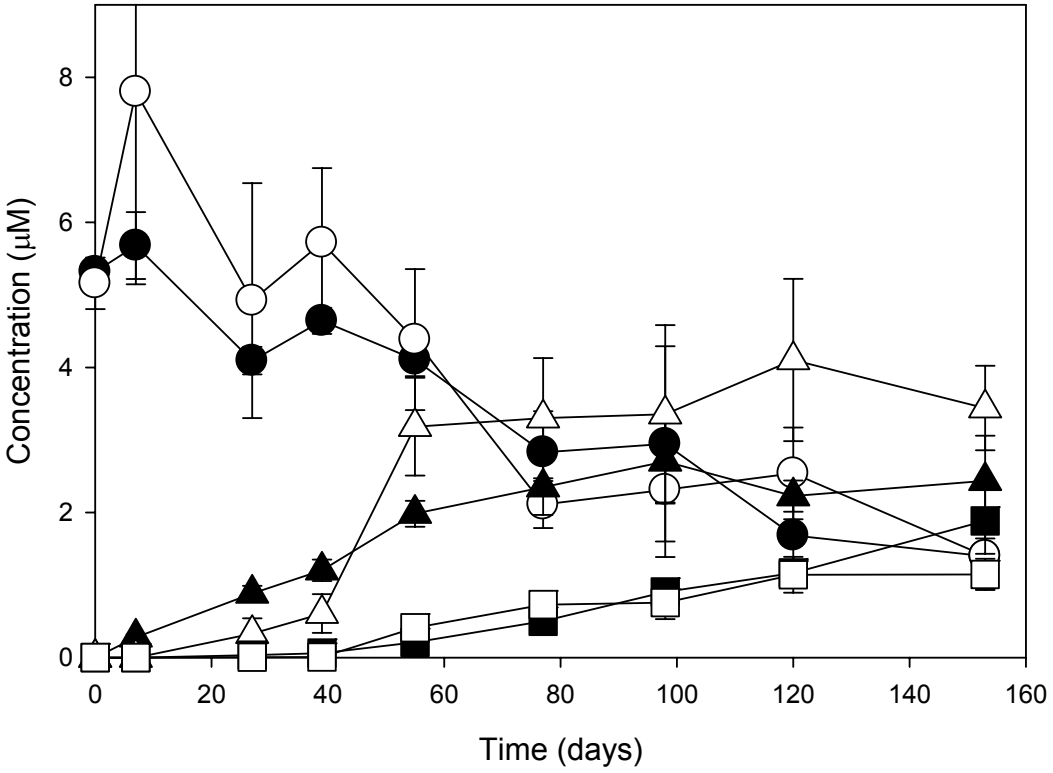

Figure 6-3. The dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (●) and formation of 1,2,4-trichlorodibenzo-p-dioxin (1,2,4-TrCDD) (▲) and 1,3-dichlorodibenzo-p-dioxin (1,3-DCDD) (■) by a mixed culture containing *Dehalococcoides ethenogenes* strain 195 without (closed symbols) and with (open symbols) PCE addition.

4. Discussion

Different amendments including electron donor, haloprimers, and bioaugmentation were applied to enhance the dechlorination of PCDD/F congeners in sediment slurries from three sites and in a mixed culture containing *D. ethenogenes* strain 195. Previous studies have shown that such amendments have various effects on the dechlorination of chlorinated compounds. The addition of "haloprimers" or alternate halogenated compounds was shown by Bedard et al. (1998) and DeWeerd and Bedard (1999) to enhance dechlorination of polychlorinated biphenyls (PCBs). Albrecht et al. (1999) demonstrated that OCDD was dechlorinated to 2-MCDD after amendment with 2-monobromodibenzo-*p*-dioxin and hydrogen. Dechlorination of 1,2,3,4-TeCDD in sediment enriched with bromophenols as haloprimers was faster than in sediments not amended with bromophenols (Vargas et al. 2001). Further investigation by Ahn et al. (2005) explored the efficiency of a variety of haloprimers with analogous structure to PCDD/Fs. These compounds, 1,2,3,4- TeCB, 2,3,4,5-tetrachloroanisole (2,3,4,5-TeCA), 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP), and 2',3',4'-TrCAP, all enhanced dechlorination of 1,2,3,4-TeCDD more extensively than did bromophenols, chlorophenols, or 2,3,5,6-tetrachlorobenzoate. In this study, amendment with haloprimers enhanced the dechlorination of 1,2,3,4-TeCDD in sediments from Gulf Island Pond and Lake Roosevelt. The microcosms of River Kymijoki sediment showed extensive dechlorination in all active treatments after 3 months of incubation. However, in the second set of River Kymijoki sediments, when the sampling interval was shortened,

addition of the haloprimer 1,2,3,4-TeCB alone and 1,2,3,4-TeCB plus bioaugmentation with *D. ethenogenes* strain 195 clearly enhanced the dechlorination at the early stage of the incubation (day 36). As dechlorination proceeded, microcosms amended with 1,2,3,4-TeCB plus bioaugmentation with *D. ethenogenes* strain 195 were most active while dechlorination in other microcosms lagged. At day 126, the dechlorination in all active enrichments proceeded to similar levels. Clearly, the amendment with 1,2,3,4-TeCB and bioaugmentation with *D. ethenogenes* strain 195 shortened the lag period prior to onset of dechlorination. We theorize that indigenous dechlorinators eventually started to mediate the dechlorination in the non-bioaugmented treatments. In the late stage of the River Kymijoki sediment enrichments, dechlorination proceeded to more or less the same level in all treatments. This might due to the abundant supply of halogenated co-contaminants and indigenous dechlorinators in the sediments, which compensated for the lack of haloprimers and bioaugmentation. The bioaugmentation provided known dechlorinators of 1,2,3,4-TeCDD and the haloprimers might act to either induce the functional enzymes involved in the dechlorination or provide a growth substrate for dechlorinators to increase their population. Results here confirmed the positive effect of some haloprimers and bioaugmentation on the dechlorination of 1,2,3,4-TeCDD. However, the magnitude of enhancement was influenced by the characteristics of the sediments as well. The sediments contaminated with halogenated compounds (e. g. River Kymijoki sediments) appear to have an indigenous microbial community acclimated to the already present organohalides, thus the amendments with haloprimers and bioaugmentation might not be as necessary as to the sediments without the presence of these contaminants.

For the River Kymijoki microcosm set II, Ahn et al. (2007 Manuscript in preparation) carried out molecular analyses to observe the bacterial community changes brought about by different biostimulants. These analyses included terminal restriction fragment length polymorphism (TRFLP) analysis of 16S rRNA genes, denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA genes and quantitative, real-time polymerase chain reaction analysis (q-PCR) of total 16S rRNA genes from bacteria and the 16S rRNA and tceA genes of *D. ethenogenes* strain 195. These procedures were described in depth by Ahn et al. (2007 Manuscript in preparation). TRFLP analysis showed the presence of a TRF corresponding to *D. ethenogenes* strain 195. Interestingly this TRF was nearly undetectable in live and CP-amended sediments with or without bioaugmentation with *D. ethenogenes* strain 195. This TRF was only observed in TeCB-amended sediments with or without bioaugmentation with *D. ethenogenes* strain 195, after 4 months. This result agreed with the observed dominant 1,3-DCDD formation in the dechlorination pathway when strain 195 was added. Overall, the TRFLP patterns were quite similar among treatments, which is not surprising since dehalorespiring bacteria might not be a major community member in these enrichments.

DGGE analyses of bioaugmented enrichments showed one band which corresponded to *D. ethenogenes* strain 195. Sequences from two bands were 100% identical to the sequences of either *D. ethenogenes* strain 195 or *Dehalococcoides* sp. strain CBDB1. In non-bioaugmented enrichments amended with TeCB there was a strong band with associated sequences 100% (406 bp/406 bp) identical to a number of *Dehalococcoides* strains, namely CBDB1, JN18_A96, JN18_A30, H10, *Dehalococcoides* sp. BAV1, and FL2. The clear presence of this sequence in the non-bioaugmented enrichments suggests

that *Dehalococcoides* spp. are abundant in the River Kymijoki sediments and may be responsible for the dechlorination of 1,2,3,4-TeCDD.

Ahn et al. (2007 Manuscript in preparation) also quantified the dechlorinating bacterial populations by q-PCR. Primers designed to target the 16S rRNA and TCE reductive dehalogenase genes of *D. ethenogenes* strain 195 were used to measure the change in the population over the incubation period. Total 16S rRNA gene copies ranged from 0.1 ± 0.03 to 3.9 ± 1.8 × 10^6 (copies / ml) in all active enrichments. In the bioaugmented enrichments (with TeCB or CP as co-substrates), *Dehalococcoides* spp. 16S rRNA gene copies ranged from 3.8 ± 0.4 to 5.6 ± 2.5 × 10^4 (copies / ml), initially. In contrast, in non-bioaugmented cultures, *Dehalococcoides* 16S rRNA gene copies were less than 0.7 ± 0.1 × 10^4 (copies / ml). *Dehalococcoides* 16S rRNA gene copies were thus one order of magnitude higher in bioaugmented enrichments than in non-bioaugmented enrichments at time 0. Interestingly, in non-bioaugmented TeCB-amended cultures, *Dehalococcoides* 16S rRNA gene copies had increased to 1.2 ± 0.1 × 10^4 (copies / ml) after 4 months, which was similar to the gene copies detected in bioaugmented enrichments. These results correspond to 1,2,3,4-TeCDD dechlorination activity observed at the end of incubation. Initial dechlorination rates were higher in bioaugmented enrichments, but the extent of dechlorination after 4 months were similar. The *tceA* gene copies were much higher in the bioaugmented enrichments (0.9 ± 0.1 to 7.1 ± 0.1 × 10^5 copies / ml) than in the non-bioaugmented enrichments (0.2 ± 0.001 to 2 ± 0.001 × 10^3 copies / ml) between 0 to 4 months of incubation.

Influence of 1,2,3,4-TeCB on the dechlorination pathway was observed. 1,3-DCDD was the major intermediate in the microcosms amended with 1,2,3,4-TeCB alone and in

those amended with 1,2,3,4-TeCB plus bioaugmentation. Other microcosms had relatively equal levels of 1,3-DCDD and 2,3-DCDD. The mixed culture containing strain 195 has been shown to dechlorinate 1,2,3,4-TeCDD through 1,3-DCDD to 2-MCDD (Fennell et al. 2004). TRFLP results showed that a peak corresponding to strain 195 was present only in the 1,2,3,4-TeCB amended enrichments, which might lead to the conclusion that the presence of 1,2,3,4-TeCB assured the dominance of strain 195 among the dechlorinators, thus producing its characteristic pathway. This implies that application of haloprimer might channel the dechlorination through a desirable pathway. It is very important to not only dechlorinate PCDD/Fs but also to assure that the metabolites are less toxic than the parent compounds. Dechlorination from a peri position from the highly chlorinated 2,3,7,8-substituted PCDD/F congeners might produce a more toxic product relative to the substrate, as discussed previously, while preferential lateral dechlorination leads to detoxification (Liu & Fennell 2007).

The 1,2,3,4-TeCB was also shown to enhance the dechlorination of 1,2,3,4,7,8-HxCDF relative to the amendment with PCE and no haloprimer treatment in the microcosms prepared with the mixed culture containing strain 195. Although both 1,2,3,4-TeCB and PCE are growth substrates for strain 195, PCE did not enhance the dechlorination of 1,2,3,4,7,8-HxCDF (Liu & Fennell 2007). These two results implied that 1,2,3,4-TeCB might play a role in inducing the dehalogenases needed for dechlorination of PCDD/Fs.

Another interesting result from this study was the delay of dechlorination observed in the River Kymijoki sediment amended with PCE. Similar results from the mixed culture study were shown as well. PCE provides energy for the growth of strain 195 and thus

should result in a larger population. However PCE dechlorination could also compete with PCDD/F dechlorination for the limited amount of dehalogenase(s) present. The overall effect of PCE as a haloprimer seemed neutral or even slightly negative. Combined with the results of 1,2,3,4-TeCB addition, it seems the role of haloprimers may be more than just providing energy for the growth of dechlorinators.

Chapter 7

Summary and Environmental Implications

1. Summary

This research addressed pathways, kinetics and carbon stable isotope fractionation that occurs during the reductive dechlorination of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) by a mixed culture containing *Dehalococcoides ethenogenes* strain 195. Biostimulation experiments that were also performed in microcosms of contaminated sediments suggest that some of the behaviors observed for the mixed culture with *D. ethenogenes* strain 195 may have analogs in native PCDD/F dechlorinating communities.

The pathway of dechlorination of a 2,3,7,8-substituted congener, 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) by the mixed culture containing *D. ethenogenes* strain 195 was explored in chapter 3. The mixed culture removed the first chlorine substituent from a lateral position, thus resulting in much less toxic non-2,3,7,8-substituted products. Thus, importantly, the dechlorination was also a detoxification process. The addition of a haloprimer, 1,2,3,4-tetrachlorobenzene, enhanced the dechlorination about three fold compared with the treatment receiving PCE as a haloprimer, and the treatment without haloprimer. The mixed culture containing *D. ethenogenes* strain 195 did not dechlorinate 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD). The extremely low aqueous solubility and high hydrophobicity of this

congener or the planar structure with eight bulky chlorine substituents might hinder OCDD dechlorination.

The study of dechlorination kinetics in chapter 4 with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) as a model compound showed that dechlorination proceeded at a rate related to the initial concentration of 1,2,3,4-TeCDD, which is a characteristic of first order reactions. The dechlorination progress data at four different initial concentrations of 1,2,3,4-TeCDD were regressed based on first order kinetics to produce pseudo-first order reaction constants. Dechlorination results were also fit with a Monod-type biokinetic model, with the estimation of two parameters: $k_{PCDD/F}$, the maximum specific substrate utilization rate (μmol/cell·day); and $K_{S(PCDD/F)}$, the half-velocity coefficient for PCDD/F transformation (μmol/L). The estimated $K_{S(PCDD/F)}$, 290 μmol/L (nominal concentration assuming uniform distribution of the PCDF in solid and liquid phases), was much higher than the nominal concentrations of 1,2,3,4-TeCDD employed in this experiment. This may explain the fact that the data appear to fit pseudo-first order kinetics.

In Chapter 5, the carbon stable isotope fractionation during the dechlorination of 1,2,3,4-TeCDD was investigated. 1,2,3,4-TeCDD was dechlorinated to 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) and further to 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD). Compound specific isotope analysis revealed that the intermediate, 1,2,4-TrCDD was enriched in ^{13}C compared with the substrate, 1,2,3,4-TeCDD. The final product, 1,3-DCDD was depleted in ^{13}C relative to both the intermediate and substrate. The data were analyzed using the Rayleigh equation and the resulting enrichment factors were very small, on the order of magnitude of -1.0‰. These low values might have

resulted from the dilution effect of the 12 carbon atoms in one molecule of 1,2,3,4-TeCDD. When corrected for the dilution effect of the carbons, the intrinsic enrichment factors were of the same magnitude as those we observed for tetrachloroethene (PCE) dechlorination by the same culture. The enrichment of ^{13}C in 1,2,4-TrCDD relative to 1,2,3,4-TeCDD was probably a result of the further dechlorination of 1,2,4-TrCDD to 1,3-DCDD, where 1,3-DCDD was depleted in ^{13}C, and 1,2,4-TrCDD was enriched in ^{13}C. This suggests a greater magnitude of isotope fractionation for dechlorination of 1,2,4-TrCDD to 1,3-DCDD than for 1,2,3,4-TeCDD to 1,2,4-TrCDD.

A variety of amendments were applied to microcosms prepared with freshwater sediments to examine their influence on PCDD/F dechlorination. Results are shown in chapter 6. The amendments included addition of electron donors and haloprimers, and bioaugmentation of microcosms with the mixed culture containing *D. ethenogenes* strain 195, a dechlorinator with a known capability to dechlorinate PCDD/Fs. Haloprimers were also amended to the mixed culture containing *D. ethenogenes* strain 195 together with the selected PCDD/F congeners. Results revealed that amendment with a suitable haloprimer, such as 1,2,3,4-tetrachlorobenzene, enhanced the dechlorination of 1,2,3,4-TeCDD in both sediment microcosms and in the mixed culture. Bioaugmentation was helpful for boosting dechlorination extent and altering the dechlorination pathway during the early stages of stimulation in the River Kymijoki sediments.

2. Environmental Implications

Results from this study have important environmental implications for the bioremediation of PCDD/F contaminated matrices.

Reductive dechlorination could be a useful process to remediate PCDD/F contaminated sediments where highly chlorinated CDD/Fs are transformed to lightly chlorinated CDD/Fs, rendering them available for further aerobic biotransformation. While dechlorination may be useful as a remedial process, the possible formation of toxic products is of great concern. We have demonstrated detoxification during the dechlorination of an environmentally relevant 2,3,7,8-substituted PCDF congener by a mixed culture containing *D. ethenogenes* strain 195. Strain 195 also exhibited a consistent pattern in dechlorinating 1,2,3,4-TeCDD and 1,2,3,4,7,8-HxCDF by removing the first chlorine from a lateral position. It is not yet known if *D. ethenogenes* strain 195 grows using the PCDD/Fs as substrates and it is not known if this specific organism will be directly useful for environmental bioremediation applications. However the findings of this work suggest that the application of *D. ethenogenes* strain 195 or related dechlorinating bacteria might lead to detoxification of 2,3,7,8-substituted PCDD/Fs in environmental remediation. The identification and study of more PCDD/F-dechlorinating bacteria is needed. Furthermore, a better understanding of the reductive dehalogenases involved in the dechlorination of PCDD/Fs and the role 1,2,3,4-TeCB played in apparently enhancing the dechlorination process is critical.

The kinetic study examined the dechlorination of 1,2,3,4-TeCDD, determined pseudo-first order reaction rate constants, and estimated Monod-type dechlorination

kinetic parameters. The reaction rates of 1,2,3,4,7,8-HxCDF under different conditions were calculated as well. The results highlight different governing factors on the dechlorination of PCDD/F congeners. An important observation was that the dechlorination rate was related to the initial nominal PCDD/F concentration, which might explain the great variability of dechlorination rates reported in the literature (Ballerstedt et al. 1997; Albrecht et al. 1999; Fu et al. 1999). More importantly, the results revealed that the dechlorination of PCDDs might not be limited by their aqueous solubility, which could have important implications in the bioremediation of PCDD/F contaminated matrices. The calculated dechlorination rates could also help with the estimation of the half lives of PCDD/Fs in the environment under the influence of microbial dechlorinating activity. The half-velocity coefficient for 1,2,3,4-TeCDD dechlorination by strain 195 was estimated to be 290 μmol/L (nominal concentration assuming uniform distribution of the PCDF in solid and liquid phases). The half-velocity coefficient (K_s) describes the substrate concentration that produces a substrate utilization rate that is half the maximum specific substrate utilization rate. The K_s is to some extent a lumped parameter since an intrinsic value of the substrate concentration at the enzyme is not measured, but rather a bulk solution or matrix substrate concentration is measured, as is reported here. The K_s value may then incorporate phenomena such as mass transfer limitations and thus this value is influenced greatly by the physical and biological system under study. It is difficult to relate the estimated PCDD/F K_s to a specific concentration of PCDD/F that the PCDD/F dehalogenase enzyme interacts with. Our system contained a specific sediment, glass surface and microbial content. Thus, comparing this value to other studies is difficult. Rittmann and McCarty (2001) state that typical K_s values for easy to

degrade, soluble compounds is < 1 mg/L while that for difficult to degrade compounds with significant mass transfer limitations might be in excess of 100 mg/L. Our "bulk" value (290 μmol/L, 93 mg/L) fits this paradigm. Gaining a better understanding of how phase location and mass transfer limitations effect PCDD/F dechlorination should be an on-going effort.

Documenting reductive dechlorination of PCDDs *in situ* has proven to be quite difficult. Confirmation of pathways of dechlorination and increases in concentrations of dechlorination daughter products may be difficult to demonstrate because of the large numbers of congeners that may be present, and because regulatory requirements result in quantification of only the highly toxic 2,3,7,8-substituted congeners in most environmental samples. Observation of carbon isotope fractionation resulting from reductive dechlorination of PCDDs could provide an additional method for analysis of this process in the environment. The characteristic changes of isotope compositions of PCDDs related to reductive dechlorination could be obtained in the laboratory and applied to the field by extrapolation. The unique isotope pattern illustrated here might be used as an evidence for biotransformation processes. The carbon stable isotope analysis might be used as a monitoring tool for dechlorination of PCDD/Fs *in situ* both qualitatively and quantitatively.

The study on the various amendments applied to enhance the dechlorination of PCDD/Fs produced useful information. Experiments performed in contaminated sediments indicate that some of the behaviors observed with strain 195 may have analogs in the native PCDD/F dechlorinating communities. This was especially true of the nearly uniformly enhancing effect of 1,2,3,4-TeCB on dechlorination of PCDD/Fs. This

haloprimer enhanced PCDD dechlorination in this study and PCDD/F dechlorination in an earlier study (Ahn et al. 2005). Furthermore, rates of 1,2,3,4,7,8-HxCDF dechlorination were enhanced nearly three fold when strain 195 was co-amended with 1,2,3,4-TeCB. Conversely PCE seemed to have a neutral or slightly negative impact on PCDD/F dechlorination. One practical method for delivery of relatively large amounts of exogenous (bioaugmented) PCB and PCDD/F dechlorinators to sediments is to grow them on alternate halogenated electron acceptors such as PCE or 1,2,3,4-TeCB. Understanding the effect of choice of alternate growth substrate on the activation and field readiness of these organisms is needed. Enzyme or gene expression studies are needed to fully delineate the true impact of the haloprimer. The application of molecular techniques, such as quantitative real time PCR has for the first time provided inside information regarding the microbial community in the black box of PCDD-dechlorinating microcosms (Ahn et al. 2007). Gaining an understanding of how PCDD/F dechlorinators respond to stimulation and whether bioaugmented dechlorinators survive *in situ* are critical to eventual rational application of bioremediation to PCDD/F contaminated sediments. Certainly caution is needed when applying results from laboratory experiments to sediments with different contamination profiles and different physical, chemical and biological characteristics.

The integration of the knowledge from this study have enhanced the understanding of the dechlorination processes of PCDD/F congeners, in regard to pathway and kinetics by one of the known PCDD/F dechlorinating bacterial strains. Enhancement of dechlorination was achieved by several amendments, which could have further application in the bioremediation of PCDD/F contaminated sediments. The exploration of

carbon isotope fractionation of 1,2,3,4-TeCDD during dechlorination revealed the possibility of using this analytical tool as a monitoring approach for the *in situ* bioremediation process.

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Curriculum Vitae

FANG LIU

EDUCATION

Oct. 2007 Ph.D., Environmental Sciences, Rutgers, the State University of New Jersey

Dissertation "Microbial Dechlorination of Polychlorinated Dibenzo-*p*-Dioxins and Dibenzofurans (PCDD/Fs): Pathways, Kinetics and Environmental Implications" directed by Professor Donna E. Fennell.

Jul. 1999 B.S., Environmental Engineering, Tsinghua University, Beijing, China

RESEARCH EXPERIENCE

2003-Present Graduate assistant, Rutgers University, New Brunswick, NJ

Conducted research work towards a Ph.D. in Environmental Sciences. My dissertation work focused on

- Examining the spectrum of PCDD/F dechlorination by *Dehalococcoides ethenogenes* strain 195 and delineating *Dehalococcodes ethenogenes* strain 195 dechlorination pathways and environmental relevance
- Testing strategies for enhancing PCDD/F dechlorination in sediments
- Quantifying the kinetics of PCDD/F dechlorination by *Dehalococcodes ethenogenes* strain 195
- Exploring the feasibility of using compound specific isotope analysis to monitor PCDD/F dechlorination

Project funded by SERDP-Strategic Environmental Research and Development Program, Grant CU-1208 and ER-1492.

Jul-Oct 2005 Research fellow, Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany

Collaborated with Dr. Hans H. Richnow's group in the Department of Isotope Biogeochemistry, UFZ to investigate the isotope fractionation of PCDD/Fs during reductive dechlorination.

Project funded by a European Union Marie Curie Early Stage Training Fellowship (contract number MEST-CT-2004-8332).

TEACHING EXPERIENCE

2002-2003 and 2005-2006

Teaching assistant for Bioenvironmental Engineering Unit Process I & II laboratory, Rutgers University, New Brunswick, NJ

GRANTS, FELLOWSHIPS AND AWARDS

Sept 2005	Best poster award, The International Summer School, Genoa, Italy
Jul. 2005	European Union Marie Curie Early Stage Training Fellowship (€5000), Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany
Jun. 2005	Corporate Activities Program Student Travel Grants, American Society for Microbiology 105th General Meeting, Atlanta, GA
2004- 2005	Graduate Student Grants-In-Aid ($5000), New Jersey Water Resources Research Institute
May 2004	Graduate Student Poster Competition 1st Prize, New Jersey Water Environment Association 89th Annual Conference and Exposition, Atlantic City, NJ
2001- 2002	Cook College Excellence Fellowship ($20,000), Cook College, Rutgers University

PUBLICATIONS

- **Liu, F**.; Fennell, D. E. Microbial Dechlorinatation and Detoxification of 1,2,3,4,7,8-Hexachlorodibenzofuran by a Mixed Culture Containing *Dehalococcoides ethenogenes* Strain 195. In Press in *Environmental Science and Technology*.
- **Liu, F**.; Nijenhuis, I.; Richnow, H. H.; Fennell, D. E. Carbon Stable Isotope Fractionation of Chloroethenes and Polychlorinated Dibenzo-*p*-Dioxins by a Mixed Culture Containing *Dehalococcoides ethenogenes* Strain 195. To be submitted.
- **Liu, F**.; Fennell, D. E. Kinetics of Reductive Dechlorination of Polychlorinated Dibenzo-*p*-dioxins and Dibenzofurans by a *Dehalococcoides*- Containing Culture. In preparation.
- Ahn, Y.-B.; **Liu, F**.; Fennell, D. E.; Häggblom, M. M. Biostimulation and Bioaugmentation to Enhance Dechlorination of Polychlorinated Dibenzo-*p*-dioxins and Dibenzofurans in Sediment of River Kymijoki, Finland. In preparation.
- Zheng, S.; Yang, M.; Lv, W.; **Liu, F**. Study on Sludge Expansion During Treatment of Salad Oil Manufacturing Wastewater by Yeast. *Environ. Technol.* 2001, *22*, 533-542.

- Zheng, S.; Yang, M.; Park, Y. H.; **Liu, F**. Washout of a yeast population during continuous treatment of salad-oil-manufacturing wastewater. *Bioresource Technol.* 2003, *86*, 235-237.

PRESENTATIONS

- **Liu, F**.; Nijenhuis, I.; Richnow, H. H. Fennell, D. E. Microbial Dechlorination of Polychlorinated Dibenzo-*p*-dioxins by *Dehalococcoides sp.* and the Use of Carbon Stable Isotope Fractionation as a Possible Monitoring Tool. The ESEB/ISEB/JSEB International Symposium on Environmental Biotechnology Conference, July 2006, Leipzig, Germany (*Platform*).
- **Liu, F**.; Nijenhuis, I.; Richnow, H. H. Fennell, D. E. Investigation of Carbon Stable Isotope Fractionation of 1,2,3,4-Tetrachlorodibenzo-*p*-Dioxin During Reductive Dechlorination by *Dehalococcoides* sp. The American Society for Microbiology 106th General Meeting, May 2006, Orlando, FL (*Poster*).
- **Liu, F**.; Fennell, D. E. 2005. Microbial Dechlorination of 1,2,3,4,7,8-Hexachlorodibenzofuran by a *Dehalococcoides*-Containing Culture. The 21th Annual International Conference on Soils, Sediments and Water, October 2005, Amherst, MA (*Poster*).
- **Liu, F**.; Son, E-K.; Fennell, D. E. 2005. Dechlorination and Detoxification of 1,2,3,4,7,8-Hexachlorodibenzofuran by a *Dehalococcoides*-Containing Mixed Culture. The International Summer School "Biomonitoring, Bioavailability and Microbial Transformation of Pollutants in Sediments and Approaches to Stimulate Their Biodegradation". September 2005, Genoa, Italy (*Poster*).
- **Liu, F**.; Fennell, D. E. 2005. Dechlorination of Polychlorinated Dibenzo-*p*-Dioxins and Dibenzofurans by a *Dehalococcoides*-Containing Culture. The American Society for Microbiology 105th General Meeting, June 2005, Atlanta, GA (*Poster*).
- **Liu, F**.; Son, E.-K.; Fennell, D. E. 2005. Dechlorination of Polychlorinated Dioxins and Furans in a *Dehalococcoides*-Containing Culture. The Eighth International Symposium on *In Situ* and On-Site Bioremediation, June 2005, Baltimore, MD (*Platform*).
- **Liu, F**; Ahn, Y.-B.; Häggblom, M. M.; Fennell, D. E. 2004. Investigation of Microbiological Dechlorination of Dioxins in Dioxin-contaminated Sediments. The New Jersey Water Environment Association 89th Annual Conference, May, 2004, Atlantic City, NJ (*Poster*).
- **Liu, F**.; Ahn, Y.-B.; Häggblom, M. M.; Fennell, D. E. 2004. Investigation of Bacterially-Mediated Dechlorination in Polychlorinated Dibenzo-*p*-Dioxin Contaminated Sediments. The American Society for Microbiology 104th General Meeting, May, 2004, New Orleans, LA (*Poster*).
- **Liu, F**.; Ahn, Y.-B.; Häggblom, M. M.; Fennell, D. E. 2003. Dechlorination of Polychlorinated Dioxins in Cultures and Sediments: Results and Biokinetic Modeling. The 226th American Chemical Society National Meeting, September, 2003, New York, NY (*Poster*).